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Telephone: +86-10-85381892
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com
http://www.wjgnet.com

SUBSCRIPTION AND AUTHOR REPRINTS
Jing Wang
The WJG Press, Apartment 1066 Yishou Garden, 58 North Langxinzhuang Road, PO Box 2345, Beijing 100023, China
Telephone: +86-10-85381892
Fax: +86-10-85381893
E-mail: j.wang@wjgnet.com
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EDITORIAL

Social learning contributions to the etiology and treatment of functional abdominal pain and inflammatory bowel disease in children and adults

Rona L Levy, Shelby L Langer, William E Whitehead

Rona L Levy, Shelby L Langer, School of Social Work, University of Washington, United States
William E Whitehead, Center for Functional GI and Motility Disorders, University of North Carolina at Chapel Hill, United State
Supported in part by NIH grants R01 HD36069-06 awarded to Dr. Levy and R24 067674 awarded to Dr. Whitehead
Correspondence to: Rona L Levy, School of Social Work, University of Washington, Box 354900, 4101 15th Avenue NE, Seattle, WA 98105, United States. rlevy@u.washington.edu
Telephone: +1-206-5435917 Fax: +1-206-5431228
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Abstract

This paper reviews empirical work on cognitive and social learning contributions to the etiology and treatment of illness behavior associated with functional abdominal pain and inflammatory bowel disease. A particular emphasis is placed on randomized controlled trials, the majority of which are multi-modal in orientation, incorporating elements of cognitive behavioral therapy, social learning, and relaxation. Based on this review, we offer methodological and clinical suggestions: (1) Research investigations should include adequate sample sizes, long-term follow-up assessments, and a credible, active control group. (2) Standard gastrointestinal practice should include, when appropriate, learning opportunities for patients and family members, for example, instruction regarding the encouragement of wellness behavior.

Key words: Functional abdominal pain; Cognitive behavioral therapy; Social learning; Irritable bowel syndrome; Inflammatory bowel disease; Illness behavior

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SOCIAL LEARNING CONCEPTUALIZATION OF ILLNESS BEHAVIOR

How people react to health conditions, both acute and chronic, has sometimes been described as "illness behavior"[1]. Inappropriate illness behavior has several forms. In one extreme, an individual may fail to notice sensations indicative of a serious condition requiring attention or refuse to let even serious symptoms interrupt his or her normal routine. The other extreme may involve misinterpreting normal somatic sensations as symptoms of disease and seeking unnecessary medical treatment for minor complaints. Both ends of the continuum are of concern to health care providers: Denial may lead individuals to postpone diagnostic investigations, or not engage in activities, which would improve well-being. Preoccupation with illness, on the other hand, creates a costly burden on the individual and the health care system[2]. In the case of children, inappropriate parental reactions to children's expressions of discomfort may also contribute to a worsening of the disease experience and possibly even worsening of the disease itself[3]. Our research focuses on increasing appropriate and decreasing inappropriate illness behaviors in response to somatic sensations.

Social learning theory provides a strong conceptual framework for understanding much of the development and maintenance of illness behavior. In brief, behavior and thoughts exist in a particular form because of an individual's learning history. Thus, if we could know someone's complete learning history, including every experience (which of course, given the complexity of human experience, is impossible), an individual's thoughts and behavior would be recognized to be totally rational. While the theory can get quite complex, a core principle is the following: We internalize rules, or beliefs, which guide us to seek rewards and avoid negative consequences. Some patients may not have had the opportunity to learn beliefs which would be most beneficial to them. Other patients may operate under rules, which may have had some rational basis in the past, but have now changed.

From this perspective, illness behaviors such as effective coping strategies or somatic complaints may be learned and maintained in large part because they produce social rewards[4-7]. According to this model, a patient's illness behaviors may result in increased positive consequences for the patient, such as expressions of support, caring, or concern, or decreased negative events such as fewer demands for participation in taxing household activities or chores. This model does not imply that either direction of response indicates the symptoms of illness are not present or "real", nor does it imply a

conscious attempt to manipulate such contingencies.

Much of the empirical research examining this model has been conducted with patients suffering from chronic pain, where solicitous responses have been shown to be related to increased pain, activity, and disability[8-12]. While direct causal relationships are difficult to establish in such research, this theoretical model may account in part for the perpetuation of dysfunction and the excess disability seen in many patients with chronic pain, and possibly other chronic conditions, where the level of dysfunction appears to exceed that expected from observed pathophysiology.

CHILDHOOD SOCIAL LEARNING EFFECTS ON ADULT ILLNESS BEHAVIOR

Miller[13] and Whitehead[14,15] suggested that such learning might occur during childhood if parents rewarded somatic complaints and/or modeled illness behavior. Moreover, the learning model predicts a relationship between the specific somatic symptoms, which were rewarded during childhood, and the symptoms reported as an adult. Turkat[16] found evidence for the effect of modeling in diabetic patients, patients with chronic headaches[17] and in a small group of healthy subjects[18].

Several studies have interviewed adults about their experiences as children, and found higher reported rates of reinforcement and modeling for illness behavior during childhood among adults with Irritable Bowel Syndrome (IBS, a condition characterized by abdominal pain or discomfort and altered bowel habits occurring in the absence of underlying disease pathology) compared to asymptomatic control groups[15,19-21].

CHILDHOOD SOCIAL LEARNING EFFECTS ON CHILDHOOD ILLNESS BEHAVIOR

Our research provides evidence to support the effects of social learning on childhood illness behavior in general, and gastrointestinal symptoms in particular. Utilizing a health maintenance organization (HMO) automated database, we found that the children of adult IBS patients made 25% more health care visits per year than the children of control parents. Visits for gastrointestinal complaints occurred more often in the children of IBS parents, but GI-related visits made up only 22% of the excess health care visits[22].

In a subsequent prospective study[23], we also tested whether children whose mothers sought medical care for IBS during the two years prior to enrollment would show more illness behavior than control children. We compared the case children (i.e., children of women with IBS) to the control children (i.e., children of mothers without current symptoms or history of IBS), with respect to the number and types of GI symptoms reported by the child. We interviewed 296 case children from 208 families, and 335 control children. Measures of illness behavior were numbers of symptoms (reported by children) and school absences and medical clinic visits in the previous 3 mo reported by their mothers. We found that case children reported more bothersome gastrointestinal symptoms than control children, more frequent stomachaches, more school absences for GI symptoms, and more physician visits for gastrointestinal symptoms. These differences remained significant after adjusting for potential mediators.

To test the hypothesis that the findings in these studies could be explained by a genetic predisposition to illness, we examined data from a twin registry containing 6060 twin pairs. Regression analysis showed that the presence of IBS in the respondent's parents made a larger contribution to the risk of having IBS than did the presence of IBS in one's twin[24], and a study with a United Kingdom twin database provided confirmation of the influence of social learning over heredity in the development of IBS[25].

Further support for social learning comes from our research into the relationship between specific parental responses and children's behavior. We found that higher levels of parental solicitousness in response to their children's illness behaviors were related to key outcomes, specifically, higher levels of children's symptoms and disability as measured by school days missed[23]. Predictors of maternal solicitousness, in turn, included several maternal characteristics: non-Caucasian race, lower educational status, not being married or partnered, and perceiving the child's condition as more severe[26].

Other researchers have also explored the relationship between children's pain and parental influences[27,28]. Parents of children seen in a pediatric rheumatology clinic reported high levels of pain[29]. Furthermore, children's levels of pain were related to parents' seeking treatment for pain and parents' pain-related interference with activities. More recently, a study of parent-child interactions during exercise tasks in children with fibromyalgia or with juvenile rheumatoid arthritis as well as healthy controls, showed that parental discouragement of coping in response to child pain or task-related complaints was associated with less time on task for all children[30].

The effects of the social environment on illness have been heavily researched in some gastrointestinal disorders in children. Stone and Barbero[31] and Oster[32] noted that children with recurrent abdominal pain where no physiological basis can be found are significantly more likely than children without this condition to have parents who complain of abdominal pain. Hill and Blendis[33] reported that these children also come from large families in which somatic complaints are more likely to have been rewarded by attention. Our data support this[34].

Walker and her colleagues provide further support for the importance of social learning processes in the development of illness behavior. In studies of patients referred to a pediatric gastroenterology clinic for evaluation of abdominal pain, Walker's group found that these families were characterized by a higher frequency of non-specific somatic symptoms[35], gastrointestinal disorders, and other health problems[36] than was observed in the families of well patients. Furthermore, there was a significant positive association between severity of somatic symptoms in recurrent abdominal pain patients and similar symptoms in their parents[37]. This significant association between child and parent symptoms held for both maternal and paternal symptoms, suggesting that modeling by either parent may play a role in the development of

Table 1 Controlled trials of CBT in adults with irritable bowel syndrome

Authors	Design	Sample size	Outcome
Corney *et al*[62] (1991)	CBT *vs* SMT. FU at 4 & 9 mo	42	CBT = SMT
Greene *et al*[64] (1994)	CBT *vs* SMT. FU at 3 mo	20	CBT > SMT
Payne *et al*[65] (1995)	CBT *vs* Self-help support group *vs* SMT. FU at 3 mo	34	CBT > Self-help > SMT
Drossman *et al*[68] (2003)	CBT *vs* education, and desipramine *vs* placebo (parallel studies). No FU	431	CBT > education; desipramine: placebo
Boyce *et al*[61] (2003)	CBT *vs* relaxation training *vs* SMT. FU at 12 mo	105	CBT = Relaxation training = SMT
Blanchard *et al*[66] (2007)	Group CBT *vs* Self-help support group *vs* SMT. FU at 3 mo	210	CBT = Support group > SMT
Kennedy *et al*[63] (2006)	Multicenter primary care intervention by nurses. Open label. CBT + mebeverine *vs* mebeverine alone.	149	CBT + mebeverine > Mebeverine alone at 3 & 6 mo FU

SMT: standard medical care with symptom self-monitoring; FU = follow up; Mebeverine: anticholinergic for inhibition of smooth muscle contractions.

the functional complaints characteristic of children with chronic abdominal pain. Additional support for the effects of modeling came in a subsequent study[38] in which higher levels of paternal somatic symptoms were associated with continuation of the children's somatic symptoms one year following a clinic evaluation for recurrent abdominal pain. Maternal somatic complaints also predicted symptom continuation, but only for boys in families with high levels of negative life events. Finally, in a laboratory study, Walker recently found higher levels of pain complaints in children whose parents were trained to give attention to symptom talk, and the effect of this attention was more pronounced in pain patients than well children[39].

COGNITIVE-BEHAVIORAL INTERVENTIONS FOR CHRONIC SOMATIC PAIN IN ADULTS

Cognitive-behavioral interventions for chronic pain have used a multimodal approach, which addresses symptom-related cognitions, physiological arousal, and activity restructuring. Compas *et al*[40] reviewed the empirical support for these treatments for chronic pain. They concluded that, based on studies meeting criteria for randomized controlled treatment trials, operant-behavioral therapy and cognitive behavioral therapy qualified as efficacious treatments in decreasing disability, physical and psychological dysfunction, and level of pain. When compared with wait-list, no-treatment, or standard medical care control groups, operant behavioral interventions produced improvements in function[41-44], decreases in pain reports[42,43], and decreases in medication use[42]. In controlled studies of patients with chronic pain syndrome and low back pain, CBT resulted in improved activity levels and psychological functioning[42,45-47] and decreased pain reports[42,45,47].

More recent reviews of the literature provide additional evidence that CBT reduces pain, distress, and improves function in patients with chronic pain[48-50], and that intensive multidisciplinary biopsychosocial rehabilitation improves function and reduces pain in patients with chronic pain[51]. In a review of treatments for abdominal pain, Blanchard[52] concluded that the empirical literature also supports the positive effects of CBT in patients with IBS. More time-limited applications of CBT have also proven efficacious. In a randomized controlled trial[53], a four session CBT intervention resulted in significantly improved pain and functioning in patients with chronic temporomandibular joint disorder pain compared to controls, and these gains were maintained at 6 and 12 mo follow-up. Evidence is also accumulating that cognitive-behavioral interventions result in improved outcomes in children and adolescents with chronic pain[54,55], including pain due to specific medical conditions such as sickle cell disease[56].

A common feature of these treatment programs has been to incorporate the concepts of social learning theory into the interventions by training the spouse, significant other, or family member to increase positive responses for coping strategies, activity and well behaviors, and to reduce maladaptive solicitous responses to pain behaviors. Involvement of the partner in behavioral treatments for pain has long been recommended[6] and is frequently incorporated into multidisciplinary pain treatment programs[57,58]. Several controlled studies have documented that partner involvement in cognitive-behavioral treatment resulted in significant improvement in psychological and physical functioning in patients with chronic arthritic pain[59,60].

COGNITIVE-BEHAVIORAL STUDIES ON THE TREATMENT OF FUNCTIONAL GASTROINTESTINAL DISEASES

Table 1 summarizes controlled trials of CBT in adults with IBS. The majority of studies which have compared CBT to standard medical care (SMT)[61-63] or SMT plus symptom self-monitoring while waiting to receive CBT treatment[64-66], have found CBT to be superior, although the trials by Corney *et al*[62] and Boyce *et al*[61] are exceptions. However, caution should be exercised in interpreting this as strong evidence for the efficacy of CBT, since there is reason to believe that randomizing patients to receive SMT who have previously failed to respond to SMT, or assigning them to monitor symptoms while waiting to receive treatment, creates a negative expectation for benefit and tends to exaggerate differences between groups[67].

Three adult studies compared CBT to an active control group: The Drossman study[68] showed CBT to be superior to an educational control when outcomes were evaluated at the end of treatment; however, no follow-up data have been reported. Two studies[65,66], both from the same group, compared CBT to a self-help support group, which has a high degree of credibility for IBS patients. One of these studies showed CBT to be superior to the support group[65], but the total sample size was relatively

Table 2 Pediatric intervention studies for children with recurrent abdominal pain

Authors	Sample	Intervention	Design	# sessions	Comparison sample	Outcome for intervention group relative to comparison
Finney *et al*[71] (1989)	16 children with RAP (age 6-13)	1-5 components, tailored to each child: self-monitoring, limited reinforcement of illness behavior, relaxation training, prescribed dietary fiber, required school attendance	Case control	M = 2.5 visits plus 1-6 phone calls	16 untreated children with RAP matched for gender (age 4-18)	-improvement or resolution of pain symptoms (parent-report) -decreased school absences -decreased health care utilization[1]
Robins *et al*[72] (2005)	69 children with RAP (age 6-16)	CBT family including pain management, relaxation, distraction, parental encouragement of wellness behavior	RCT	5	Standard care (29 of the total 69)	-decreased pain (child- and parent-report) -fewer school absences
Sanders *et al*[69] (1989)	16 children with RAP (age 6-12)	CBT including self-monitoring, social learning, relaxation	RCT wait-list control	8	Wait-list control (8 of the total 16)	-decreased pain (child-report and maternal observation) -more pain-free days (child-report) -fewer pain behaviors (teacher observation) -fewer behavioral problems (parent-report)
Sanders *et al*[73] (1994)	44 children with RAP (age 7-14)	CBT including contingency management and self-management	RCT	6	Standard care (4-6 sessions)	-more pain-free days (child-report) -fewer pain behaviors (parental observation) -less pain-related interference (child- and parent-report)
Scharff & Blanchard (1996)[70] cited in Blanchard (2001)[52]	10 children with RAP (age 8-13)	Random assignment to social learning or stress management/relaxation	crossover	4	---	-decreased pain intensity (child-report) -decreased pain frequency (parent-report)

[1]In this study, the comparison group was used only as a reference for health care utilization, not the other outcome variables.

small (n = 10 to 12 per group). A larger study[66] failed to show any difference between CBT and the support group, although this might have been explained by the different modes of delivering CBT (group *vs* individual sessions). Taken together, these 7 studies provide suggestive but not compelling evidence for the efficacy of CBT. Other published studies evaluated multimodal psychological treatments for IBS, but the outcomes for these trials have been mixed.

There has also been limited intervention literature using CBT to treat children with recurrent abdominal pain (Table 2). While overall results have been quite positive, the research is characterized by methodological weaknesses, including low sample size[69-71], inadequate standard medical care control groups which do not control for intervention time and attention[72,73], or both.

The pediatric studies reviewed in Table 2 suggest that a cognitive behavioral approach, which includes a social learning component for treatment of the FGIDs, may be efficacious. Additionally, Sanders *et al*[73] note that research is needed which evaluates the effects of a briefer, more cost-effective program which could easily be incorporated into routine practice. Finally, Sanders *et al*[73] conclude with a recommendation for research which investigates the role of other potential predictors of outcome, such as child and family characteristics.

COGNITIVE-BEHAVIORAL INTERVENTIONS FOR INFLAMMATORY BOWEL DISEASE

The research on cognitive behavioral interventions for IBD has focused primarily on teaching patients coping strategies and stress management skills. Much of the research has also been limited by small sample sizes and inadequate control groups. In an early randomized trial, Milne, Joachim, and Niedhardt[74] assigned 80 IBD patients to a relaxation training or control group. There was no control for therapist attention. Self-reports of stress and disease activity dropped for the treatment group, but not the control group. Garcia-Vega and Fernandez-Rodriguez[75] assigned a total of 45 patients to one of two stress management groups (therapist or self-directed) or usual medical care. Patients in both self-management groups showed reductions in some gastrointestinal symptoms and tiredness, while the conventional medical group did not. Schwarz and Blanchard[76] compared a multi-component CBT intervention (consisting of IBD education, relaxation, biofeedback, and cognitive coping) to a symptom monitoring control group. The number of subjects in each group was small (10 and 11 respectively), and findings were mixed, not clearly showing a superior effect for the CBT group. In an uncontrolled study, Mussell *et al*[77] tested a 12-session cognitive behavioral group therapy intervention on 28 patients which included education about IBD, training in cognitive coping strategies, and progressive muscle relaxation. A significant decrease in worries and concerns related to disease was found for ulcerative colitis, but not Crohn's patients. Depressive coping also decreased in women, but not men. Education, often a component of CBT programs, was not effective by itself in reducing anxiety or improving quality of life in a group-based patient education program for IBD patients[78]. In 2001, Maunder and Esplen[79] stated that there was no consensus about the most appropriate psychosocial interventions for people with IBD. Similarly, in a comprehensive review, Levenstein[80] also concluded that, to date, research on the psychological and behavioral approaches to IBD treatment were disappointing. Small sample sizes, uncontrolled studies, and variation in treatment modalities likely provide

a basis for these conclusions and demonstrate the need for further research in this area. This suggests the need for well-designed, randomized, controlled studies to address whether such interventions may be effective in improving function and adjustment in patients where there also is an organic etiology for symptoms.

TREATMENT AND RESEARCH RECOMMENDATIONS

Based on this review, it seems appropriate to recommend integrating a cognitive behavioral approach into gastroenterology practice with adults. This would include both cognitive and relaxation components. The cognitive component would begin with an assessment of the patient's ideas about his/her illness. It would then be appropriate to work with the patient to reduce the negative effects of symptoms, by challenging and changing beliefs and ways of viewing the world which have an adverse effect on these symptoms. This is done by first having the patient understand interactions between thoughts and feelings and recognize the role that illness beliefs and behavior play in the experience of symptoms. Relaxation training should also be incorporated, with the goal of teaching patients to reduce the physical effects of stress and anxiety.

Studies with children have been less extensive, but the preliminary results with children indicate that a reasonable current recommendation for children would be similar. However, with children, of course, parents should be involved in any intervention strategies. Their cognitions about and response to their children's symptoms should be assessed and addressed. They could also be considered to assist with relaxation strategies.

Future research on the effectiveness of CBT with children and adults should meet the following minimum standards: (1) Include an adequate sample size to insure that the study is both generalizable and that there is sufficient power to detect a clinically meaningful effect. The sample size should be chosen prior to study initiation. (2) Assess maintenance of treatment effects by including a follow-up assessment at least 6 mo after the conclusion of treatment. (3) Include a credible control group (not SMT or wait list control) and assess whether subjects find the active and control groups equally credible[81]. (4) Include IBS symptom reduction as one of the outcome measures. The primary outcome may be a global rating of satisfaction with treatment or adequate relief of symptoms, but this should be confirmed by showing that treatment is also associated with a clinically meaningful reduction in gastrointestinal symptoms.

REFERENCES

1 **Mechanic D**. Sex, illness, illness-behavior, and the use of health services. *Social Science and Medicine* 1978; **12**: 207-214
2 **Drossman DA**, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari E. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580
3 **Gerson MJ**, Schonholtz J, Grega CH, Barr DR. The importance of the family context in inflammatory bowel disease. *Mt Sinai J Med* 1998; **65**: 398-403
4 **Hiller W**, Fichter MM. High utilizers of medical care: a crucial subgroup among somatizing patients. *J Psychosom Res* 2004; **56**: 437-443
5 **Whitehead WE**, Fedoravicius AS, Blackwell B, Wooley S. A behavioral conceptualization of psychosomatic illness: Psychosomatic symptoms as learned responses. In: JR M, ed. Behavioral approaches in medicine: Application and analysis. New York: Plenum, 1979: 65-99
6 **Fordyce W**. Behavioral methods for chronic pain and illness. St. Louis: Mosby, 1976
7 **Wooley SC**, Blackwell B, Winget C. A learning theory model of chronic illness behavior: theory, treatment, and research. *Psychosom Med* 1978; **40**: 379-401
8 **Keefe FJ**, Abernethy AP, C Campbell L. Psychological approaches to understanding and treating disease-related pain. *Annu Rev Psychol* 2005; **56**: 601-630
9 **Flor H**, Kerns RD, Turk DC. The role of spouse reinforcement, perceived pain, and activity levels of chronic pain patients. *J Psychosom Res* 1987; **31**: 251-259
10 **Flor H**, Turk DC, Rudy TE. Relationship of pain impact and significant other reinforcement of pain behaviors: the mediating role of gender, marital status and marital satisfaction. *Pain* 1989; **38**: 45-50
11 **Kerns RD**, Southwick S, Giller EL, Haythornthwaite JA, Jacob MC, Rosenberg R. The relationship between reports of pain-related social interactions and expressions of pain and affective distress. *Behavior Therapy* 1991; **22**: 101-111
12 **Romano JM**, Turner JA, Jensen MP, Friedman LS, Bulcroft RA, Hops H, Wright SF. Chronic pain patient-spouse behavioral interactions predict patient disability. *Pain* 1995; **63**: 353-360
13 **Miller NE**. Effect of learning on gastrointestinal functions. *Clin Gastroenterol* 1977; **6**: 533-546
14 **Whitehead W**, Fedoravicius, AS, Blackwell, B, Wooley, S. A behavioral conceptualization of psychosomatic illness: Psychosomatic symptoms as learned responses. In: McNamara J, ed. Behavioral approaches in medicine: Application and analysis. New York: Plenum, 1979: 65-99
15 **Whitehead WE**, Winget C, Fedoravicius AS, Wooley S, Blackwell B. Learned illness behavior in patients with irritable bowel syndrome and peptic ulcer. *Dig Dis Sci* 1982; **27**: 202-208
16 **Turkat ID**. An investigation of parental modeling in the etiology of diabetic illness behavior. *Behav Res Ther* 1982; **20**: 547-552
17 **Turkat ID**, Kuczmierczyk AR, Adams HE. An investigation of the aetiology of chronic headache. The role of headache models. *Br J Psychiatry* 1984; **145**: 665-666
18 **Turkat ID**, Noskin DE. Vicarious and operant experiences in the etiology of illness behavior: a replication with healthy individuals. *Behav Res Ther* 1983; **21**: 169-172
19 **Whitehead WE**, Busch CM, Heller BR, Costa PT. Social learning influences on menstrual symptoms and illness behavior. *Health Psychol* 1986; **5**: 13-23
20 **Whitehead WE**, Crowell MD, Heller BR, Robinson JC, Schuster MM, Horn S. Modeling and reinforcement of the sick role during childhood predicts adult illness behavior. *Psychosom Med* 1994; **56**: 541-550
21 **Lowman BC**, Drossman DA, Cramer EM, McKee DC. Recollection of childhood events in adults with irritable bowel syndrome. *J Clin Gastroenterol* 1987; **9**: 324-330
22 **Levy RL**, Whitehead WE, Von Korff MR, Feld AD. Intergenerational transmission of gastrointestinal illness behavior. *Am J Gastroenterol* 2000; **95**: 451-456
23 **Levy RL**, Whitehead WE, Walker LS, Von Korff M, Feld AD, Garner M, Christie D. Increased somatic complaints and health-care utilization in children: effects of parent IBS status and parent response to gastrointestinal symptoms. *Am J Gastroenterol* 2004; **99**: 2442-2451
24 **Levy RL**, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology. *Gastroenterology* 2001; **121**: 799-804
25 **Mohammed I**, Cherkas LF, Riley SA, Spector TD, Trudgill NJ. Genetic influences in irritable bowel syndrome: a twin study.

Am J Gastroenterol 2005; **100**: 1340-1344
26 **Langer SL**, Walker LS, Romano JM, Whitehead WE, Feld L, Levy RL. Predictors of maternal responses to child abdominal pain. *Child Health Care* 2007; **36**; 63-81
27 **Chen E**, Joseph MH, Zeltzer LK. Behavioral and cognitive interventions in the treatment of pain in children. *Pediatr Clin North Am* 2000; **47**: 513-525
28 **Zeltzer LK**, Barr RG, McGrath PA, Schechter NL. Pediatric pain: interacting behavioral and physical factors. *Pediatrics* 1992; **90**: 816-821
29 **Schanberg LE**, Anthony KK, Gil KM, Lefebvre JC, Kredich DW, Macharoni LM. Family pain history predicts child health status in children with chronic rheumatic disease. *Pediatrics* 2001; **108**: E47
30 **Reid GJ**, McGrath PJ, Lang BA. Parent-child interactions among children with juvenile fibromyalgia, arthritis, and healthy controls. *Pain* 2005; **113**: 201-210
31 **Stone RT,** Barbero GJ. Recurrent abdominal pain in childhood. *Pediatrics* 1970; **45**: 732-738
32 **Oster J**. Recurrent abdominal pain, headache and limb pains in children and adolescents. *Pediatrics* 1972; **50**: 429-436
33 **Hill OW**, Blendis L. Physical and psychological evaluation of 'non-organic' abdominal pain. *Gut* 1967; **8**: 221-229
34 **Whitehead WE**, Levy RL, Walker LS, Von Korff M, Lonczak HS, Bradshaw BH, Feld AD. Children's gastrointestinal complaints and family size. *Gastroenterology* 2001; **120** Suppl 1: A640
35 **Walker LS**, Greene JW. Children with recurrent abdominal pain and their parents: more somatic complaints, anxiety, and depression than other patient families? *J Pediatr Psychol* 1989; **14**: 231-243
36 **Walker LS**, Garber J, Greene JW. Psychosocial correlates of recurrent childhood pain: a comparison of pediatric patients with recurrent abdominal pain, organic illness, and psychiatric disorders. *J Abnorm Psychol* 1993; **102**: 248-258
37 **Walker LS**, Garber J, Greene JW. Somatization symptoms in pediatric abdominal pain patients: relation to chronicity of abdominal pain and parent somatization. *J Abnorm Child Psychol* 1991; **19**: 379-394
38 **Walker LS**, Garber J, Greene JW. Somatic complaints in pediatric patients: a prospective study of the role of negative life events, child social and academic competence, and parental somatic symptoms. *J Consult Clin Psychol* 1994; **62**: 1213-1221
39 **Walker LS**, Williams SE, Smith CA, Garber J, Van Slyke DA, Lipani TA. Parent attention versus distraction: impact on symptom complaints by children with and without chronic functional abdominal pain. *Pain* 2006; **122**: 43-52
40 **Compas BE**, Haaga DA, Keefe FJ, Leitenberg H, Williams DA. Sampling of empirically supported psychological treatments from health psychology: smoking, chronic pain, cancer, and bulimia nervosa. *J Consult Clin Psychol* 1998; **66**: 89-112
41 **Linton SJ**, Götestam KG. A controlled study of the effects of applied relaxation and applied relaxation plus operant procedures in the regulation of chronic pain. *Br J Clin Psychol* 1984; **23**(Pt 4): 291-299
42 **Nicholas MK**, Wilson PH, Goyen J. Operant-behavioural and cognitive-behavioural treatment for chronic low back pain. *Behav Res Ther* 1991; **29**: 225-238
43 **Turner JA**, Clancy S. Comparison of operant behavioral and cognitive-behavioral group treatment for chronic low back pain. *J Consult Clin Psychol* 1988; **56**: 261-266
44 **Turner JA**, Clancy S, McQuade KJ, Cardenas DD. Effectiveness of behavioral therapy for chronic low back pain: a component analysis. *J Consult Clin Psychol* 1990; **58**: 573-579
45 **Philips HC**. The effects of behavioural treatment on chronic pain. *Behav Res Ther* 1987; **25**: 365-377
46 **Puder RS**. Age analysis of cognitive-behavioral group therapy for chronic pain outpatients. *Psychol Aging* 1988; **3**: 204-207
47 **Turner JA**. Comparison of group progressive-relaxation training and cognitive-behavioral group therapy for chronic low back pain. *J Consult Clin Psychol* 1982; **50**: 757-765
48 **Astin JA**, Beckner W, Soeken K, Hochberg MC, Berman B. Psychological interventions for rheumatoid arthritis: a meta-analysis of randomized controlled trials. *Arthritis Rheum* 2002; **47**: 291-302
49 **McCracken LM**, Turk DC. Behavioral and cognitive-behavioral treatment for chronic pain: outcome, predictors of outcome, and treatment process. *Spine(Phila Pa 1976)* 2002; **27**: 2564-2573
50 **Morley S**, Eccleston C, Williams A. Systematic review and meta-analysis of randomized controlled trials of cognitive behaviour therapy and behaviour therapy for chronic pain in adults, excluding headache. *Pain* 1999; **80**: 1-13
51 **Guzmán J**, Esmail R, Karjalainen K, Malmivaara A, Irvin E, Bombardier C. Multidisciplinary bio-psycho-social rehabilitation for chronic low back pain. *Cochrane Database Syst Rev* 2002: CD000963
52 **Blanchard EB**. Irritable Bowel Syndrome: Psychosocial assessment and treatment. Washington, D.C.: American Psychological Association, 2001
53 **Turner JA**, Mancl L, Aaron LA. Short- and long-term efficacy of brief cognitive-behavioral therapy for patients with chronic temporomandibular disorder pain: a randomized, controlled trial. *Pain* 2006; **121**: 181-194
54 **Eccleston C**, Morley S, Williams A, Yorke L, Mastroyannopoulou K. Systematic review of randomised controlled trials of psychological therapy for chronic pain in children and adolescents, with a subset meta-analysis of pain relief. *Pain* 2002; **99**: 157-165
55 **Weydert JA**, Ball TM, Davis MF. Systematic review of treatments for recurrent abdominal pain. *Pediatrics* 2003; **111**: e1-11
56 **Chen E**, Cole SW, Kato PM. A review of empirically supported psychosocial interventions for pain and adherence outcomes in sickle cell disease. *J Pediatr Psychol* 2004; **29**: 197-209
57 **Loeser J**, Egan, KJ. Managing the chronic pain patient: Theory and practice at the University of Washington Multidisciplinary Pain Center. New York: Raven Press, 1989
58 **Sanders S**. Operant conditioning with chronic pain: Back to basics. In: Gatchel R, Turk, DC, ed. Psychological approaches to pain management: A practitioner's handbook. New York: Gilford Press, 1996: 112-130
59 **Keefe FJ**, Caldwell DS, Baucom D, Salley A, Robinson E, Timmons K, Beaupre P, Weisberg J, Helms M. Spouse-assisted coping skills training in the management of osteoarthritic knee pain. *Arthritis Care Res* 1996; **9**: 279-291
60 **Radojevic V**, Nicassio, PM, Weisman, MH. Behavioral intervention with and without family support for rheumatoid arthritis. *Behavior Therapy* 1992; **23**: 13-30
61 **Boyce PM**, Talley NJ, Balaam B, Koloski NA, Truman G. A randomized controlled trial of cognitive behavior therapy, relaxation training, and routine clinical care for the irritable bowel syndrome. *Am J Gastroenterol* 2003; **98**: 2209-2218
62 **Corney RH**, Stanton R, Newell R, Clare A, Fairclough P. Behavioural psychotherapy in the treatment of irritable bowel syndrome. *J Psychosom Res* 1991; **35**: 461-469
63 **Kennedy TM**, Chalder T, McCrone P, Darnley S, Knapp M, Jones RH, Wessely S. Cognitive behavioural therapy in addition to antispasmodic therapy for irritable bowel syndrome in primary care: randomised controlled trial. *Health Technol Assess* 2006; **10**: iii-iv, ix-x, 1-67
64 **Greene B**, Blanchard EB. Cognitive therapy for irritable bowel syndrome. *J Consult Clin Psychol* 1994; **62**: 576-582
65 **Payne A**, Blanchard EB. A controlled comparison of cognitive therapy and self-help support groups in the treatment of irritable bowel syndrome. *J Consult Clin Psychol* 1995; **63**: 779-786
66 **Blanchard EB**, Lackner JM, Sanders K, Krasner S, Keefer L, Payne A, Gudleski GD, Katz L, Rowell D, Sykes M, Kuhn E, Gusmano R, Carosella AM, Firth R, Dulgar-Tulloch L. A controlled evaluation of group cognitive therapy in the treatment of irritable bowel syndrome. *Behav Res Ther* 2007; **45**: 633-648
67 **Whitehead WE**. Control groups appropriate for behavioral interventions. *Gastroenterology* 2004; **126**: S159-S163

68 **Drossman DA**, Toner BB, Whitehead WE, Diamant NE, Dalton CB, Duncan S, Emmott S, Proffitt V, Akman D, Frusciante K, Le T, Meyer K, Bradshaw B, Mikula K, Morris CB, Blackman CJ, Hu Y, Jia H, Li JZ, Koch GG, Bangdiwala SI. Cognitive-behavioral therapy versus education and desipramine versus placebo for moderate to severe functional bowel disorders. *Gastroenterology* 2003; **125**: 19-31

69 **Sanders MR**, Rebgetz M, Morrison M, Bor W, Gordon A, Dadds M, Shepherd R. Cognitive-behavioral treatment of recurrent nonspecific abdominal pain in children: an analysis of generalization, maintenance, and side effects. *J Consult Clin Psychol* 1989; **57**: 294-300

70 **Scharff L**, Blanchard, EB. Behavioral treatment of recurrent abdominal pain: The effects of parent training and self-control in the management of symptoms. Albany: Center for Stress and Anxiety, 1996

71 **Finney J**, Lemanek, KL, Cataldo, MF, Katz, HP, Fuqua RW. Pediatric psychology in primary health care: Brief targeted therapy for recurrent abdominal pain. *Behavior Therapy* 1989; **20**: 283-291

72 **Robins PM**, Smith SM, Glutting JJ, Bishop CT. A randomized controlled trial of a cognitive-behavioral family intervention for pediatric recurrent abdominal pain. *J Pediatr Psychol* 2005; **30**: 397-408

73 **Sanders MR**, Shepherd RW, Cleghorn G, Woolford H. The treatment of recurrent abdominal pain in children: a controlled comparison of cognitive-behavioral family intervention and standard pediatric care. *J Consult Clin Psychol* 1994; **62**: 306-314

74 **Milne B**, Joachim G, Niedhardt J. A stress management programme for inflammatory bowel disease patients. *J Adv Nurs* 1986; **11**: 561-567

75 **García-Vega E**, Fernandez-Rodriguez C. A stress management programme for Crohn's disease. *Behav Res Ther* 2004; **42**: 367-383

76 **Schwarz SP,** Blanchard EB. Evaluation of a psychological treatment for inflammatory bowel disease. *Behav Res Ther* 1991; **29**: 167-177

77 **Mussell M**, Böcker U, Nagel N, Olbrich R, Singer MV. Reducing psychological distress in patients with inflammatory bowel disease by cognitive-behavioural treatment: exploratory study of effectiveness. *Scand J Gastroenterol* 2003; **38**: 755-762

78 **Larsson K**, Sundberg Hjelm M, Karlbom U, Nordin K, Anderberg UM, Lööf L. A group-based patient education programme for high-anxiety patients with Crohn disease or ulcerative colitis. *Scand J Gastroenterol* 2003; **38**: 763-769

79 **Maunder RG**, Esplen MJ. Supportive-expressive group psychotherapy for persons with inflammatory bowel disease. *Can J Psychiatry* 2001; **46**: 622-626

80 **Levenstein S**. Psychosocial factors in peptic ulcer and inflammatory bowel disease. *J Consult Clin Psychol* 2002; **70**: 739-750

81 **Borkovec T**, Nau, SD. Credibility of analogue therapy rationales. *J Behav Ther Exper Psych* 1972; **3**: 257-260

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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EDITORIAL

Recent advances in hepatitis C virus research and understanding the biology of the virus

Francoise Lunel-Fabiani

Francoise Lunel-Fabiani, Laboratory of Virology, University Hospital of Angers, France and Laboratory HIFI, UPRES EA 3859, University of Angers, France
Correspondence to: Francoise Lunel-Fabiani, Professor, Laboratory of Virology, Department of infectious agents, University Hospital of Angers, 4 rue Larrey, 49933 Angers Cedex 9, France. frlunel-fabiani@chu-angers.fr
Telephone: +33-2-41354709 Fax: +33-2-41354164
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Since the identification of hepatitis C virus (HCV) genome in 1989[1], a lot of progresses have been done about the understanding of HCV biology, natural history and therapeutic options. HCV is a member of the Flaviviridae viral family. Its genome is a positive simple strand RNA molecule which shows significant genetic variability. The HCV nomenclature has been recently re-examined by an international group of scientific experts in the field of HCV genetic variability or involved in HCV data base[2]. HCV sequence variability and HCV classification has considerably evolved since the consensus paper in 1994[3] proposing the classification of HCV by phylogenetic methods into 6 genotypes. Each genetic group contains a variable number of closely related but distinct subtypes at the nucleotide level. Genotypes differ from each other by 31% to 33%, and subtypes by 20% to 25%. Since this time, several molecular epidemiology studies have revealed a much higher diversity especially in certain region of the world demonstrating that HCV was present a long time ago in human populations, and that recent routes of spread, like transfusions, nosocomial transmission, or Intra Venous Drug use have allowed a rapid spread of HCV subtypes. Recombination between genotypes[4,5] were also described making the classification of certain strains more difficult. Because genotype identification is clinically important in terms of response to current HCV treatments (Pegylated Interferon and Ribavirin), experts have examined the must reliable methods for HCV classification especially for phylogenetic analysis of the core, E1, NS5B genes and complete genome sequences[2]. These combined methods support the primary division of HCV into the 6 genetic groups termed genotypes. Variants of HCV above 6 are being renamed according to the genotype group. The new proposal of the scientists and experts now serve as a framework for access to the 3 Databases (which follows the current revised nomenclature and the revised criteria for HCV classification). Particularly due to the variability of HCV strains, HCV infection is characterized by an extremely high rate (60%-80%) of chronic carrier state development associated with viral multiplication. The understanding and modelisation of natural history is still debated but the chronic carrier state is associated with the development of liver fibrosis and the risk of hepatocellular carcinoma (HCC). Histopathological and clinical studies have individualized by assimilation with HIV infection low and rapid progressors. However, risk factors for liver fibrosis progression seem to be linked to many individual or environmental factors (age, sex, age at infection, route of transmission, alcohol consumption, *etc.*). One of the most recent progress in the assessment of liver fibrosis is the development of blood scores (Hepascore®, Fibrometer®, Fibrotest®, ...) or physical methods like elastometry (Fibroscan®) which are modifying the management of patients with HCV infection and may probably facilitate the follow up of fibrosis progression[6-10]. The understanding of the HCV biology has also considerably benefit from new *in vitro* and *in vivo* systems of viral replication like the replicon system and transgenic mice models. These new culture and animal models together with the growing knowledge of molecular biology of HCV have reinforced the importance of HCV variability and its potential role in the natural history of infection but also in the mechanisms of resistance to antiviral agents. In this issue of World Journal of Gastroenterology, experts have examined the main aspects of HCV infection trying to focus on these new findings or understanding about biological, clinical and therapeutic progresses.

REFERENCES

1 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362

2 **Simmonds P**, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973

3 **Simmonds P**, Smith DB, McOmish F, Yap PL, Kolberg J, Urdea MS, Holmes EC. Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *J Gen Virol* 1994; **75** (Pt 5): 1053-1061

4 **Kalinina O**, Norder H, Mukomolov S, Magnius LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002; **76**: 4034-4043

5 **Noppornpanth S**, Lien TX, Poovorawan Y, Smits SL, Osterhaus AD, Haagmans BL. Identification of a naturally occurring recombinant genotype 2/6 hepatitis C virus. *J Virol* 2006; **80**: 7569-7577

6 **Oberti F**, Valsesia E, Pilette C, Rousselet MC, Bedossa P, Aubé C, Gallois Y, Rifflet H, Maïga MY, Penneau-Fontbonne D, Calès P. Noninvasive diagnosis of hepatic fibrosis or cirrhosis. *Gastroenterology* 1997; **113**: 1609-1616

7 **Castéra L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Lédinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

8 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075

9 **Calès P**, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konaté A, Gallois Y, Ternisien C, Chevailler A, Lunel F. A novel panel of blood markers to assess the degree of liver fibrosis. *Hepatology* 2005; **42**: 1373-1381

10 **Halfon P**, Bacq Y, De Muret A, Penaranda G, Bourliere M, Ouzan D, Tran A, Botta D, Renou C, Bréchot MC, Degott C, Paradis V. Comparison of test performance profile for blood tests of liver fibrosis in chronic hepatitis C. *J Hepatol* 2007; **46**: 395-402

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www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Hepatitis C virus proteins

Jean Dubuisson

Jean Dubuisson, Institut de Biologie de Lille (UMR8161), CNRS, Université de Lille I & II and Institut Pasteur de Lille, Lille, France
Correspondence to: Jean Dubuisson, DVM, PhD, Hepatitis C Laboratory, CNRS-UMR8161, Institut de Biologie de Lille, 1 rue Calmette, BP447, 59021 Lille Cedex, France. jean.dubuisson@ibl.fr
Telephone: +33-3-20871160 Fax: +33-3-20871201
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Abstract

Hepatitis C virus (HCV) encodes a single polyprotein, which is processed by cellular and viral proteases to generate 10 polypeptides. The HCV genome also contains an overlapping +1 reading frame that may lead to the synthesis of an additional protein. Until recently, studies of HCV have been hampered by the lack of a productive cell culture system. Since the identification of HCV genome approximately 17 years ago, structural, biochemical and biological information on HCV proteins has mainly been obtained with proteins produced by heterologous expression systems. In addition, some functional studies have also been confirmed with replicon systems or with retroviral particles pseudotyped with HCV envelope glycoproteins. The data that have accumulated on HCV proteins begin to provide a framework for understanding the molecular mechanisms involved in the major steps of HCV life cycle. Moreover, the knowledge accumulated on HCV proteins is also leading to the development of antiviral drugs among which some are showing promising results in early-phase clinical trials. This review summarizes the current knowledge on the functions and biochemical features of HCV proteins.

Key words: Hepatitis C virus; Viral hepatitis; Viral proteins; Molecular virology

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INTRODUCTION

As for the other members of the *Flaviviridae* family the genome of Hepatitis C virus (HCV) encodes a single polyprotein. This 3010 amino acid polyprotein is processed by cellular and viral proteases to generate 10 polypeptides[1] (Figure 1). The nonstructural proteins are released from the polyprotein after cleavage by HCV proteases NS2-3 and NS3-4A, whereas the structural proteins are released by host endoplasmic reticulum (ER) signal peptidase(s)[2]. Further processing mediated by a signal peptide peptidase also occurs at the C-terminus of the capsid protein[3]. In addition to the large open reading frame encoding the polyprotein, the HCV genome contains an overlapping +1 reading frame that may lead to the synthesis of an additional protein[4]. Despite the difficulties in propagating the virus in cell culture, a large body of data has accumulated on HCV proteins since the identification of HCV genome 17 years ago. A detailed knowledge of the functions of HCV proteins is important for the development of new antiviral drugs. This review summarizes the current knowledge of the functions and biochemical features of HCV proteins. A brief summary of the functions of HCV proteins is presented in Table 1.

CORE PROTEIN

The core protein is an RNA-binding protein that is supposed to form the viral nucleocapsid. It is removed from the polyprotein by a host signal peptidase cleavage at the C-terminus, yielding the immature form of the protein[5], and the signal peptide present at the C-terminus of the core is processed further by a host signal peptide peptidase, yielding the mature form of the protein[3] (Figure 1). It has been shown that the mature form of core is a dimeric alpha-helical protein, which behaves as a membrane protein[6]. This protein can be separated into two domains: an N-terminal two-thirds hydrophilic domain (D1) and a C-terminal one-third hydrophobic domain (D2)[7]. The D1 domain includes numerous positively charged amino acids and has similar characteristics to the capsid proteins of related pestiviruses and flaviviruses[6,7]. The D2 domain is required for proper folding of domain D1 and is critical for the membrane characteristics of the core[6,8]. It is worth noting that this domain is absent in the pestiviruses and flaviviruses but is found in GB virus B[6,9].

Little is known about the mechanisms of HCV nucleocapsid assembly. *In vitro* nucleocapsid reconstitution experiments with core segments have thus far yielded irregular particles larger than those isolated from infected subjects[10]. Full-length core protein has also been shown to assemble into nucleocapsid-like particles upon de novo

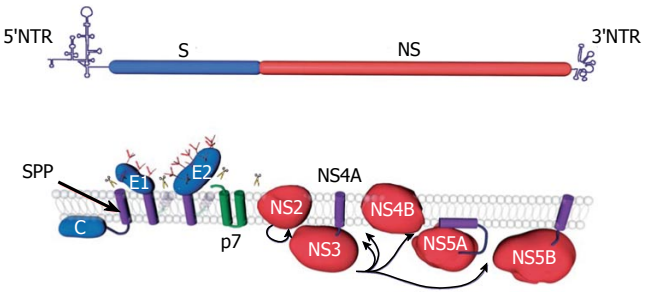

Figure 1 HCV genome organization (top) and polyprotein processing (bottom). HCV encodes a single polyprotein with the structural proteins (S) and the nonstructural proteins (NS) present in the N-terminal one-third and the C-terminal two-third of the polyprotein, respectively. The polyprotein processing and the location of the 10 proteins relative to the endoplasmic reticulum membrane are schematically represented. Scissors indicate cleavages by a host signal peptidase. Arrows indicate NS2-3 and NS3-4A cleavages. The intramembrane arrow indicates cleavage by a host signal peptide peptidase (SPP). The transmembrane domains of E1 and E2 are shown after signal-peptidase cleavage and reorientation of their C-terminus. In addition, the precleavage topology of the transmembrane domains of E1 and E2 is shown in light grey.

synthesis in cell-free systems made of rabbit reticulocyte lysate or wheat germ extracts[11]. It has also been suggested that the attachment of a core protein to a phospholipid layer is required as a template for proper assembly of the nucleocapsid[6]. Although, little is known on the assembly of the nucleocapsid, developing small molecules that block the signal peptide peptidase cleavage might be a way of inhibiting HCV assembly.

When expressed in the context of heterologous expression systems or HCV replicons, core is found both attached to the ER and at the surface of lipid droplets[7,12]. In some conditions, a minor proportion of the core protein has also been found to be located in the nucleus[13]. More recently, the core protein has also been found to colocalize with mitochondrial markers in Huh-7 cells containing a full-length HCV replicon[14]. However, in the context of an infectious virus, the core protein was only found in association with lipid droplets[15]. It has been reported that the traffic between rough ER membranes, the site of capsid protein synthesis, and lipid droplets is regulated by signal peptide peptidase cleavage in the C-terminal region of the core protein[3]. It is therefore likely that in the context of HCV-infected cells, transport of the C protein to the site of lipid droplet assembly is rapid due to rapid cleavage by the signal peptide peptidase.

The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions[7,16,17]. It has indeed been proposed to be involved in cell signaling, apoptosis, carcinogenesis and lipid metabolism. However, in most cases, it is unclear if these interactions occur in the course of a normal infection or are artifacts of ectopic expression or protein over-expression. Further studies with the recently developed cell culture system for HCV[18-20] should help clarify whether all the functions identified for HCV core protein can be observed in the context of infected cells.

E1 AND E2 GLYCOPROTEINS

HCV glycoproteins, E1 and E2, are released from the polyprotein by a host signal peptidase cleavage[12] (Figure 1). They are type-I transmembrane proteins with a large N-terminal ectodomain and a C-terminal transmembrane domain, and they assemble as noncovalent heterodimers[21]. The ectodomains of HCV envelope glycoproteins E1 and E2 are highly modified by N-linked glycans. Indeed, E1 and E2 possess up to 6 and 11 potential glycosylation sites, respectively, and most of them are well conserved[22,23]. It is worth noting that some glycans have been shown to play a role in HCV glycoprotein folding or in virus entry[24]. Because they are essential for virus entry, HCV envelope glycoproteins are a good target for the development of antiviral molecules that block HCV entry[25].

Table 1 Viral proteins and their functions in HCV life cycle

Protein	Molecular Mass[1]	Function
Core	21 kDa	RNA binding; nucleocapsid
E1	31-35 kDa	Envelope glycoprotein; associate with E2
E2	70 kDa	Envelope glycoprotein; receptor binding; associate with E1
p7	7 kDa	Ion channel
NS2	21 kDa	Component of NS2-3 proteinase
NS3	69 kDa	N-terminal proteinase domain; C-terminal NTPase/helicase domain
NS4A	6 kDa	NS3-4A proteinase cofactor
NS4B	27 kDa	Induces membrane alterations
NS5A	56-58 kDa	Phosphoprotein
NS5B	68 kDa	RNA-dependent RNA polymerase

[1]Estimated by SDS-PAGE.

Hypervariable regions (HVR) have been identified in the E2 envelope glycoprotein sequence[26]. The first 27 amino acids of the E2 ectodomain form HVR1. The apparent variability of this region seems to be driven by antibody selection of immune-escape variants. An HCV clone lacking HVR1 was found to be infectious but strongly attenuated in chimpanzees[27], supporting a functional role of this domain, likely in virus entry[28,29]. Despite the sequence variability of HVR1, the physico-chemical properties of the residues at each position and the conformation of HVR1 are highly conserved among the various genotypes[30]. In addition, HVR1 is a globally basic region and basic residues of HVR1 have been shown to play a role in modulating virus entry[29]. Another hypervariable region, HVR2, has also been described in E2[26], and this region has been proposed to modulate E2 receptor binding[31].

Although HCV glycoproteins can be detected at the plasma membrane when they are over-expressed[32-34], the E1E2 heterodimer is mainly retained in the ER[15,35]. The determinants for ER retention of HCV envelope glycoproteins have been mapped in the transmembrane domains of E1 and E2[36,37]. In addition to a membrane-proximal heptad repeat sequence in E2[38], these domains have also been shown to be essential for heterodimerization[39]. The transmembrane domains of HCV envelope glycoproteins are not canonical transmembrane domains[40], and dynamic changes have been shown to occur in these domains after cleavage by the signal peptidase[41]. Indeed, before cleavage by a host signal peptidase, the transmembrane domains of E1 and E2 adopt a hairpin structure, and after cleavage, the signal-like sequence is reoriented toward the cytosol,

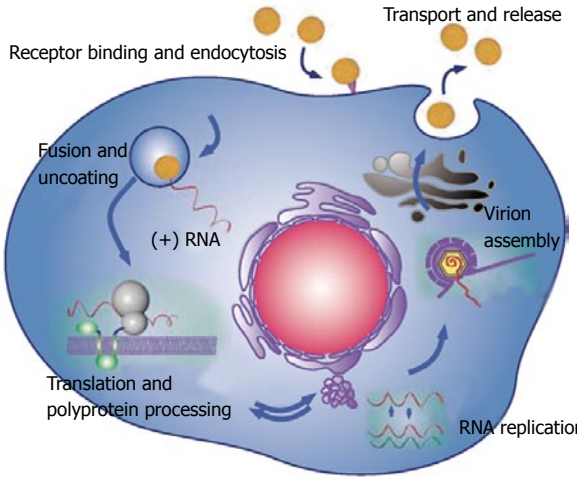

Figure 2 Schematic representation of the major steps of HCV life cycle. The virus binds to a receptor at the cell surface, which leads to endocytosis of the particle. Fusion between the viral envelope and an endosomal membrane leads to the release of HCV genome into the cytosol. HCV genome is a positive strand RNA, which is directly translated and all the viral proteins are simultaneously produced. Expression of HCV proteins induces intracellular membrane alterations (the membranous web), which is the site of RNA replication. The nonstructural proteins NS3 to NS5B assemble in association with cellular factors to form a replication complex, which is responsible for RNA replication. Accumulation of HCV genomic RNA and the structural proteins leads to the assembly of a nucleocapsid, which acquires its envelope within an intracellular compartment. The viral particle is then secreted by following the classical secretory spathway.

leading to a single transmembrane passage.

The two envelope glycoproteins, E1 and E2, play major roles at different steps of the HCV life cycle (Figure 2). They are essential for virus entry[42,43], and they participate in the assembly of infectious particles[19]. The E1 E2 heterodimer is the viral component present at the surface of HCV particles and it is therefore the obvious candidate ligand for cellular receptors. As a first approach to identify potential HCV receptor(s), a soluble form of HCV glycoprotein E2 has been used. This led to the identification of a series of putative receptors for HCV: CD81 tetraspanin[44], scavenger receptor class B type I (SR-BI)[45], heparan sulfate[46] and the mannose binding lectins DC-SIGN and L-SIGN[47-49]. An approach using virus-like particles produced in insect cells has also led to the identification of the asialoglycoprotein receptor as another candidate receptor for HCV[50]. In addition, because of the physical association of HCV with low- or very-low-density lipoproteins (LDL or VLDL) in serum, the LDL receptor has also been proposed as another candidate receptor for HCV[51,52]. Among these molecules, only CD81 and SR-BI have been shown to play a role in HCV entry[43]. However, co-expression of CD81 and SR-BI in non-hepatic cell lines does not lead to virus entry, indicating that other molecule(s) expressed only in hepatic cells, are necessary for HCV entry.

Interactions between viral envelope glycoproteins and potential receptors can have other consequences than a direct effect on virus entry. For instance, L-SIGN and DC-SIGN are not expressed on hepatocytes, and HCV interactions with these molecules may contribute to establishment or persistence of infection both by the capture and delivery of virus to the liver and by modulating dendritic cell functions as recently suggested[53,54]. It has also been shown that intracellular interaction between HCV envelope glycoproteins and CD81 can lead to secretion of exosomes containing E1 and E2 glycoproteins[55]. A soluble form of E2 is also able to bind CD81 at the surface of natural killer cells, and this interaction inhibits cytotoxicity and cytokine production by these cells[56,57]. Binding of a soluble form of E2 to CD81 can also provide a co-stimulatory signal for T cells[58,59], activate B lymphocytes[58] and up-regulate matrix metalloproteinase-2 in human hepatic stellate cells[60]. It remains however to be determined whether HCV glycoprotein expressed in the context of native particles will also have the same effects on cell functions.

Because they are exposed at the surface of the virion, the envelope proteins are targets of neutralizing antibodies. The recent development of retroviral particles pseudotyped by unmodified HCV E1 and E2 envelope glycoproteins (HCVpp)[32-34] has allowed to initiate studies on neutralizing antibodies. As determined with HCVpp, it seems that the majority of chronically infected patients have cross-reactive neutralizing antibodies[61,62]. In contrast, neutralizing antibodies have not been detected in several cases of acute resolving infection[61,62], and the detection of neutralizing antibodies in acutely infected individuals did not seem to be associated with viral clearance[61]. However, another study has shown in some patients a progressive emergence of a relatively strong neutralizing response in correlation with a decrease in viremia[63]. Further investigations on a large number of acutely infected patients will be necessary to determine the role of neutralizing antibodies in controlling HCV infections. Importantly, the majority of neutralizing anti-HCV monoclonal antibodies that have been described recognize E2[32,34,64-66]. In addition, some of the epitopes recognized by these antibodies have been mapped in the CD81 binding region of E2 and in the C-terminus of HVR1[34]. Studies with these neutralizing monoclonal antibodies will be essential to understand the mechanisms leading to HCV neutralization.

p7

The p7 polypeptide is located within the HCV polyprotein at the junction between the structural and nonstructural proteins[67,68]. It is released from the polyprotein by a host signal peptidase cleavage[12] (Figure 1). The p7 polypeptide is a small polytopic membrane protein composed of two transmembrane domains with both its N- and C-termini oriented toward the lumen of the ER[69]. The C-terminus of p7 contains a sequence for reinitiation of translocation, and when fused to a reporter protein, this sequence functions as a signal peptide[69,70]. The double membrane spanning topology of p7 with few residues accessible at one or the other side of the membrane suggests that p7 likely exerts its function(s) on membrane structures. When expressed by heterologous expression systems, p7 can be found in association with ER and/or mitochondrial membranes[69,71,72]. In addition, a small proportion of p7 can also be detected at the plasma membrane[69]. However, further investigations in the context of an infectious virus will be necessary to confirm these subcellular localizations. The

p7 polypeptide is not required for RNA replication, and it is uncertain whether it is a virion component. Interestingly, the p7 polypeptide has been shown to have an ion channel activity in artificial lipid membranes[72-75]. In addition, it has been shown to be essential for infectivity of HCV in chimpanzees[76]. These observations suggest that screening for small molecules that block the ion channel activity of p7 might be an approach to develop new anti-HCV molecules.

NS2

NS2 is an integral membrane protein that is not essential for the formation of the replication complex[77,78]. The function of NS2 in its mature form is unknown; however, before cleavage from the polyprotein, NS2 participates in a protease activity responsible for the cleavage at the NS2/NS3 junction[79] (Figure 1). The first 180 residues of NS3 are also required for this cleavage. In addition, the NS2-3 enzyme has been described as a cysteine proteinase[80]. The structure of NS2 reveals a dimeric cysteine protease with two composite active sites[81]. Surprisingly, for each active site, the catalytic histidine and glutamate residues are contributed by one monomer, and the nucleophilic cysteine by the other. The host-cell chaperone Hsp90 seems to be required to activate the NS2-3 proteinase[82]. Cleavage of the NS2 N-terminus from p7 is mediated by a signal peptidase within the ER[69,70]. When expressed alone, NS2 is found located in association with ER membranes[83]. NS2 contains several stretches of hydrophobic amino acids and is predicted to be a polytopic membrane protein[84,85]. The membrane topology of NS2 is unclear, but the presence of two internal signal-like sequences points to the existence of four transmembrane segments[85]. However, since the processing at the NS2/NS3 junction has to take place in the cytosolic space, the presence of the C-terminus of NS2 in the ER lumen suggests that a reorientation of this region would have to occur after cleavage between NS2 and NS3. Interestingly, crossover sites for natural or infectious artificial inter-genotypic HCV chimeras have been mapped in NS2[18,86,87]. These data suggest that in addition to its role in the processing at the NS2/NS3 cleavage site, NS2 is also involved in virus assembly and release. It remains however to be determined by which mechanism NS2 contributes to the latter process. Due to its involvement in NS2-3 protease activity, NS2 is an interesting target for the development of anti-HCV molecules.

NS2 has been shown to be a short-lived protein whose degradation by the proteasome is regulated in a phosphorylation-dependent manner through the protein kinase CK2[83]. In addition, it has been shown to interact with the liver-specific pro-apoptotic CIDE-B protein and to be an inhibitor of CIDE-B-induced apoptosis[88]. NS2 might also potentially affect cellular gene transcription[89]. However, all these properties need to be further investigated in the context of the newly developed cell culture system for HCV[18-20].

NS3 AND NS4A

NS3 is a multifunctional protein with an N-terminal serine-type protease domain and a C-terminal RNA helicase/NTPase domain. The NS3 protease domain has a typical chymotrypsin-like fold and is composed of two beta-barrel domains[90,91]. The protease activity of NS3 is enhanced by the NS4A cofactor. Indeed, NS4A contributes one beta-strand to the N-terminal protease domain and thereby allows its complete folding[90]. In addition, it induces a conformational change that leads to a repositioning of the catalytic triad. NS3 by itself has no transmembrane domain, but it associates non-covalently with the central domain of NS4A, which is a membrane protein. When co-expressed with NS4A, NS3 is found in association with ER or ER-like membranes whereas it is diffusely distributed in the cytoplasm and nucleus when expressed alone[92]. Deletion analyses have revealed that the hydrophobic N-terminal domain of NS4A is required for ER targeting of NS3. Interestingly, NS4A also stabilizes the protease against proteolytic degradation. The NS3-4A protease has an unusually shallow substrate-binding pocket and therefore requires rather long interaction surfaces with the substrate (reviewed in[1,93]). This made the design of efficient inhibitors of this protease challenging[94]. The NS3-4A protease is responsible for the polyprotein cleavage in the region downstream of NS3 (Figure 1), and this activity is essential for the generation of components of the viral RNA replication complex[95] (Figure 2). It is therefore not surprising that this protease has been the first target for the development of new anti-HCV molecules[94].

In addition to its role in the processing of the polyprotein, the NS3-4A protease activity is also involved in blocking the ability of the host cell to mount an innate antiviral response[96]. The NS3-4A has indeed been shown to interfere with double-stranded RNA signaling pathways. It disrupts the cellular RNA helicase retinoic acid-inducible gene I (RIG- I) pathway through proteolysis of a newly discovered essential adaptor protein of interferon regulatory factor-3 (IRF-3) activation[97]. Due to its recent simultaneous discovery by four different groups, this adaptor protein has received four different names: IPS-1, Cardif, VISA and MAVS[98]. NS3-4A cleavage of MAVS/IPS-1/VISA/Cardif results in its dissociation from the mitochondrial membrane and disruption of signaling to the antiviral immune response[99]. NS3-4A also cleaves the TRIF (also called TICAM-1) adaptor protein to ablate Toll-like receptor-3 (TLR-3) signaling of IRF-3 activation by extracellular double-stranded RNA[100]. However, this pathway has a minimal role in triggering the interferon antiviral response[101].

The C terminus of NS3 encodes a DexH/D-box RNA helicase[102]. Enzymes of this superfamily are capable of unwinding RNA-RNA duplexes in an ATP-dependent manner. The crystal structure of the HCV helicase shows a Y-shaped molecule composed of 3 nearly equally sized subdomains[103,104]. Although monomeric NS3 can bind RNA with high affinity, RNA unwinding requires an NS3 dimer[105]. Kinetic analyses indicate that this enzyme undergoes highly coordinated cycles of fast double-stranded RNA unwinding[105-107]. More recently, it has been reported that the cyclic movement of NS3 helicase is coordinated by ATP in discrete steps of 11 base pairs, and that actual unwinding occurs in rapid smaller sub-steps of 2 to 5 base pairs, also triggered by ATP binding, indicating that NS3 might move like an inchworm[108]. The NS3 helicase

activity can be modulated by interactions between the serine protease and helicase domains. Indeed the kinetics of duplex RNA unwinding is slower for the isolated helicase domain as compared with the full-length NS3 protein[109]. In addition, the presence of NS4A enhances productive RNA binding of a full-length NS3-4A complex[107]. The function of the NS3 helicase in the HCV life cycle is not known. It may be involved in initiation of RNA replication by unwinding stable stem-loop structures at the termini of positive and/or negative strand of HCV RNA. It may also contribute to the process of the replicase complex by removing stable RNA secondary structures and/or by displacing bound proteins that might interfere with RNA synthesis. Finally, it may also be required for dissociation of the replicative form. Due to its enzymatic activity, the helicase domain of NS3 is another potential target for the development of anti-HCV molecules.

The NS3 protein has been reported to interact with several cellular proteins[17], and it has been proposed to be involved in carcinogenesis[110]. However, the relevance of these interactions needs to be confirmed in the context of the recently developed cell culture system for HCV[18-20].

NS4B

The NS4B protein is a highly hydrophobic nonstructural protein, which is predicted to contain four transmembrane domains[111,112]. It has recently been shown that NS4B is palmitoylated in the C-terminal region of the protein[113]. The N- and C-termini of NS4B are localized in the cytosol; however, a fraction of the N-terminus can also be found in the ER lumen[112]. A putative amphipathic helix in the N-terminus of NS4B has been proposed to mediate membrane association[114]. The NS4B protein is detected in association with ER membranes[111,112,115]. In addition, NS4B also induces intracellular membrane alterations, suggesting that one of its functions is to induce the formation of membranous structures supporting RNA replication[116]. However, the structure of NS4B-induced membranes appears to be slightly distinct from the membranous web observed when all the HCV proteins are expressed, suggesting that other component(s) contribute to these membrane alterations. A nucleotide binding motif has been found in NS4B[117]. This structural motif binds and hydrolyzes GTP. Interestingly, mutation of this nucleotide binding motif affects HCV RNA replication[117]. The potential presence of NS4B domains on both sides of the ER membrane suggests that this protein plays a role in crosstalk between the ER lumen and the cytosol. Although a function can be attributed to this protein, it remains challenging to develop a high-throughput screening for small molecules targeting NS4B.

NS5A

NS5A is a membrane-associated protein containing a unique amphipathic alpha-helix at its N-terminus, which serves as an in-plane membrane anchor[118,119] (Figure 1). Like most HCV proteins, NS5A is detected in association with ER or ER-derived membranes[118]. Besides its membrane anchor sequence, NS5A contains three distinct domains that are separated by low complexity sequences (LCs) I and II [120]. Recently, the x-ray crystal structure of domain I was solved[121]. It is composed of a basic N-terminal subdomain IA and a predominantly acidic C-terminal subdomain IB. In subdomain IA a zinc ion is coordinated by a unique motif of 4 fully conserved cysteine residues, which are absolutely essential for RNA replication[120,121]. In subdomain IB an unusual disulfide bond linking 2 cysteine residues near the C-terminal subdomain border was found. However, this disulfide bond does not seem to be essential for HCV RNA replication. Domain I forms homodimers *via* contacts near the N-terminal end of the molecules. This dimerization results in the formation of a basic groove facing the cytosol at the surface of the membrane. This 'claw like' structure is believed to provide an RNA binding site that might be involved in regulated genome targeting within the replication complex[121]. In line with this observation, NS5A has been shown to bind to HCV RNA and more specifically to the 3'-ends of HCV plus and minus strand RNAs, with a preference for the polypyrimidine tract in the 3' non-translated region of positive strand RNA[122]. Therefore, the structure of domain I of NS5A provides a framework for the rational design of small antiviral molecules. The other two domains of NS5A are less characterized. Domain II has been proposed to be involved in inhibition of the interferon-induced double stranded RNA activated protein kinase PKR[123], and domain III is a less conserved region, which can tolerate insertions or partial deletions[124,125].

NS5A is a protein which is essential for genome replication[126,127]. Indeed, mutations that enhance RNA replication in cell culture map to the NS5A-coding sequence. In addition, NS5A has been shown to interact with NS5B, and this interaction is essential for maintenance of subgenomic replicons in Huh-7 cells[128,129]. NS5A is expressed as a basally phosphorylated and a hyperphosphorylated forms[93]. The functional relevance of the different phosphorylated forms is unknown. However, mutations that reduce NS5A hyperphosphorylation can lead to a dramatic enhancement of HCV genomic replication[124,130]. Furthermore, treatment of cells carrying non-adapted replicons with an inhibitor of the cellular kinase(s) responsible for NS5A hyperphosphorylation leads to an increase in HCV genomic replication[131]. In addition to its role in HCV genomic replication, NS5A has initially attracted considerable interest because of its potential role in modulating the interferon response[132]. NS5A has also been shown to interact with components of numerous cellular signaling pathways[17,133,134]. Among the potential cellular partners identified for NS5A, human vesicle-associated membrane protein-associated protein A (hVAP-A) is of particular interest because it is regulated by NS5A phos phorylation[130,135]. Indeed, NS5A hyperphosphorylation disrupts interaction with hVAP-A and negatively regulates viral RNA replication. VAP-A is a protein found on ER and Golgi membranes, which is involved in intracellular vesicle trafficking. It remains however to be determined why NS5A hijacks hVAP-A at some step of its life cycle. Another potentially important host cell factor interacting with NS5A is the geranylgeranylated protein FBL-2[136]. In

line with this observation, it has been shown that inhibition of geranylgeranylation in cells abolishes HCV RNA replication[137].

NS5B

NS5B is a membrane-associated protein containing a C-terminal transmembrane domain[138], which is essential for RNA replication in cell culture[139] (Figure 1). Like most HCV proteins, NS5B is detected in association with ER or ER-derived membranes[140]. NS5B is an RNA-dependent RNA polymerase, which is the catalytic component of the HCV RNA replication machinery. This enzyme synthesizes RNA using an RNA template. NS5B can initiate RNA synthesis de novo, at least *in vitro*, and it is assumed that de novo initiation is also operating *in vivo*[93]. The crystal structure of the NS5B catalytic domain shows a structural fold comparable with other polymerases with palm, finger, and thumb subdomains[141,142]. The palm domain contains the active site of the enzyme, whereas the fingers and the thumb modulate the interaction with the RNA chain. One structural peculiarity of the enzyme is the fully encircled active site, which is due to multiple interactions between the finger and thumb subdomains creating a tunnel in which a single-stranded RNA molecule is directly guided to the active site. NTPs enter the active site *via* another positively charged tunnel. Binding of the RNA template and initiation of RNA synthesis are supposed to be regulated by a highly flexible beta-hairpin loop located in the thumb domain and pointing toward the active site[126]. As observed for other viral polymerases, NS5B is an interesting and promising target for the development of new antiviral molecules targeting HCV[94].

The RNA-dependent RNA polymerase activity appears to be modulated by interaction with some other viral proteins (NS3 and NS5A)[93]. It has been shown that cyclophilin B, a peptidyl-prolyl cis-trans-isomerase, interacts with the C-terminal region of NS5B and appears to stimulate its RNA binding activity[143]. In addition, cyclosporin A, an inhibitor of cyclophilin B, inhibits HCV replication in cell culture[144]. However, how cyclophilin B activates replication remains to be determined. Furthermore, cyclophilin B does not seem to stimulate the RNA binding activity of NS5B in all genotypes[145]. NS5B has also been shown to interact with other cellular proteins[146-148].

ALTERNATIVE READING FRAME PROTEIN

In addition to the large open reading frame encoding the polyprotein, HCV genome contains an overlapping +1 reading frame that overlaps the sequence of the core protein[4]. This alternative reading frame (ARF) lacks an in-frame AUG start codon, suggesting that its expression involves unusual translation-level events. *In vitro* studies indicate that ribosomal frameshifting may the process leading to translation of the ARF. Frameshifting yields chimeric proteins that have segments encoded in the core sequence covalently attached to amino acids encoded in the ARF. Based on experiments with reporter gene constructs, the frameshift efficiency is in the range of 1% to 2%. The development of an immune response against the ARF protein in HCV infected patients indicates that this protein is expressed during natural HCV infections and stimulates specific immune responses[149]. The role of ARF protein in the HCV life cycle and/or pathogenesis is not yet known. However, the ARF protein is not required for HCV RNA replication. One cannot exclude that the ARF protein may be responsible for some of the effects attributed to the core protein. Indeed, most studies seeking to define the function of the core protein have used sequences likely to contain a combination of the core protein and ARF protein. Due to the lack of knowledge of its function, the ARF protein is not currently considered as a target for the development of new antiviral molecules.

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REFERENCES

1 **Penin F**, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5-19
2 **Reed KE**, Rice CM. Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. *Curr Top Microbiol Immunol* 2000; **242**: 55-84
3 **McLauchlan J**, Lemberg MK, Hope G, Martoglio B. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* 2002; **21**: 3980-3988
4 **Branch AD**, Stump DD, Gutierrez JA, Eng F, Walewski JL. The hepatitis C virus alternate reading frame (ARF) and its family of novel products: the alternate reading frame protein/F-protein, the double-frameshift protein, and others. *Semin Liver Dis* 2005; **25**: 105-117
5 **Santolini** E, Migliaccio G, La Monica N. Biosynthesis and biochemical properties of the hepatitis C virus core protein. *J Virol* 1994; **68**: 3631-3641
6 **Boulant S**, Vanbelle C, Ebel C, Penin F, Lavergne JP. Hepatitis C virus core protein is a dimeric alpha-helical protein exhibiting membrane protein features. *J Virol* 2005; **79**: 11353-11365
7 **McLauchlan J**. Properties of the hepatitis C virus core protein: a structural protein that modulates cellular processes. *J Viral Hepat* 2000; **7**: 2-14
8 **Boulant S**, Montserret R, Hope RG, Ratinier M, Targett-Adams P, Lavergne JP, Penin F, McLauchlan J. Structural determinants that target the hepatitis C virus core protein to lipid droplets. *J Biol Chem* 2006; **281**: 22236-22247
9 **Hope RG**, Murphy DJ, McLauchlan J. The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. *J Biol Chem* 2002; **277**: 4261-4270
10 **Kunkel M**, Lorinczi M, Rijnbrand R, Lemon SM, Watowich SJ. Self-assembly of nucleocapsid-like particles from recombinant hepatitis C virus core protein. *J Virol* 2001; **75**: 2119-2129
11 **Klein KC**, Polyak SJ, Lingappa JR. Unique features of hepatitis C virus capsid formation revealed by de novo cell-free assembly. *J Virol* 2004; **78**: 9257-9269
12 **Dubuisson J**, Penin F, Moradpour D. Interaction of hepatitis C virus proteins with host cell membranes and lipids. *Trends Cell Biol* 2002; **12**: 517-523
13 **Yasui K**, Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M. The native form and maturation process of hepatitis C virus core protein. *J Virol* 1998; **72**: 6048-6055
14 **Schwer B**, Ren S, Pietschmann T, Kartenbeck J, Kaehlcke K,

Bartenschlager R, Yen TS, Ott M. Targeting of hepatitis C virus core protein to mitochondria through a novel C-terminal localization motif. *J Virol* 2004; **78**: 7958-7968

15 **Rouillé Y**, Helle F, Delgrange D, Roingeard P, Voisset C, Blanchard E, Belouzard S, McKeating J, Patel AH, Maertens G, Wakita T, Wychowski C, Dubuisson J. Subcellular localization of hepatitis C virus structural proteins in a cell culture system that efficiently replicates the virus. *J Virol* 2006; **80**: 2832-2841

16 **Ray RB**, Ray R. Hepatitis C virus core protein: intriguing properties and functional relevance. *FEMS Microbiol Lett* 2001; **202**: 149-156

17 **Tellinghuisen TL**, Rice CM. Interaction between hepatitis C virus proteins and host cell factors. *Curr Opin Microbiol* 2002; **5**: 419-427

18 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626

19 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796

20 **Zhong J**, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299

21 **Deleersnyder V**, Pillez A, Wychowski C, Blight K, Xu J, Hahn YS, Rice CM, Dubuisson J. Formation of native hepatitis C virus glycoprotein complexes. *J Virol* 1997; **71**: 697-704

22 **Goffard A**, Dubuisson J. Glycosylation of hepatitis C virus envelope proteins. *Biochimie* 2003; **85**: 295-301

23 **Zhang M**, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. *Glycobiology* 2004; **14**: 1229-1246

24 **Goffard A**, Callens N, Bartosch B, Wychowski C, Cosset FL, Montpellier C, Dubuisson J. Role of N-linked glycans in the functions of hepatitis C virus envelope glycoproteins. *J Virol* 2005; **79**: 8400-8409

25 **Helle F**, Wychowski C, Vu-Dac N, Gustafson KR, Voisset C, Dubuisson J. Cyanovirin-N inhibits hepatitis C virus entry by binding to envelope protein glycans. *J Biol Chem* 2006; **281**: 25177-25183

26 **Weiner AJ**, Brauer MJ, Rosenblatt J, Richman KH, Tung J, Crawford K, Bonino F, Saracco G, Choo QL, Houghton M. Variable and hypervariable domains are found in the regions of HCV corresponding to the flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. *Virology* 1991; **180**: 842-848

27 **Forns X**, Thimme R, Govindarajan S, Emerson SU, Purcell RH, Chisari FV, Bukh J. Hepatitis C virus lacking the hypervariable region 1 of the second envelope protein is infectious and causes acute resolving or persistent infection in chimpanzees. *Proc Natl Acad Sci USA* 2000; **97**: 13318-13323

28 **Bartosch B**, Vitelli A, Granier C, Goujon C, Dubuisson J, Pascale S, Scarselli E, Cortese R, Nicosia A, Cosset FL. Cell entry of hepatitis C virus requires a set of co-receptors that include the CD81 tetraspanin and the SR-B1 scavenger receptor. *J Biol Chem* 2003; **278**: 41624-41630

29 **Callens N**, Ciczora Y, Bartosch B, Vu-Dac N, Cosset FL, Pawlotsky JM, Penin F, Dubuisson J. Basic residues in hypervariable region 1 of hepatitis C virus envelope glycoprotein e2 contribute to virus entry. *J Virol* 2005; **79**: 15331-15341

30 **Penin F**, Combet C, Germanidis G, Frainais PO, Deléage G, Pawlotsky JM. Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J Virol* 2001; **75**: 5703-5710

31 **Roccasecca R**, Ansuini H, Vitelli A, Meola A, Scarselli E, Acali S, Pezzanera M, Ercole BB, McKeating J, Yagnik A, Lahm A, Tramontano A, Cortese R, Nicosia A. Binding of the hepatitis C virus E2 glycoprotein to CD81 is strain specific and is modulated by a complex interplay between hypervariable regions 1 and 2. *J Virol* 2003; **77**: 1856-1867

32 **Bartosch B**, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. *J Exp Med* 2003; **197**: 633-642

33 **Drummer HE**, Maerz A, Poumbourios P. Cell surface expression of functional hepatitis C virus E1 and E2 glycoproteins. *FEBS Lett* 2003; **546**: 385-390

34 **Hsu M**, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, Rice CM, McKeating JA. Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc Natl Acad Sci USA* 2003; **100**: 7271-7276

35 **Duvet S**, Cocquerel L, Pillez A, Cacan R, Verbert A, Moradpour D, Wychowski C, Dubuisson J. Hepatitis C virus glycoprotein complex localization in the endoplasmic reticulum involves a determinant for retention and not retrieval. *J Biol Chem* 1998; **273**: 32088-32095

36 **Cocquerel L**, Duvet S, Meunier JC, Pillez A, Cacan R, Wychowski C, Dubuisson J. The transmembrane domain of hepatitis C virus glycoprotein E1 is a signal for static retention in the endoplasmic reticulum. *J Virol* 1999; **73**: 2641-2649

37 **Cocquerel L**, Meunier JC, Pillez A, Wychowski C, Dubuisson J. A retention signal necessary and sufficient for endoplasmic reticulum localization maps to the transmembrane domain of hepatitis C virus glycoprotein E2. *J Virol* 1998; **72**: 2183-219**1**

38 **Drummer HE**, Poumbourios P. Hepatitis C virus glycoprotein E2 contains a membrane-proximal heptad repeat sequence that is essential for E1E2 glycoprotein heterodimerization and viral entry. *J Biol Chem* 2004; **279**: 30066-30072

39 **Op De Beeck A**, Montserret R, Duvet S, Cocquerel L, Cacan R, Barberot B, Le Maire M, Penin F, Dubuisson J. The transmembrane domains of hepatitis C virus envelope glycoproteins E1 and E2 play a major role in heterodimerization. *J Biol Chem* 2000; **275**: 31428-31437

40 **Cocquerel L**, Wychowski C, Minner F, Penin F, Dubuisson J. Charged residues in the transmembrane domains of hepatitis C virus glycoproteins play a major role in the processing, subcellular localization, and assembly of these envelope proteins. *J Virol* 2000; **74**: 3623-3633

41 **Cocquerel L**, Op de Beeck A, Lambot M, Roussel J, Delgrange D, Pillez A, Wychowski C, Penin F, Dubuisson J. Topological changes in the transmembrane domains of hepatitis C virus envelope glycoproteins. *EMBO J* 2002; **21**: 2893-2902

42 **Bartosch B**, Cosset FL. Cell entry of hepatitis C virus. *Virology* 2006; **348**: 1-12

43 **Cocquerel L**, Voisset C, Dubuisson J. Hepatitis C virus entry: potential receptors and their biological functions. *J Gen Virol* 2006; **87**: 1075-1084

44 **Pileri P**, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941

45 **Scarselli E**, Ansuini H, Cerino R, Roccasecca RM, Acali S, Filocamo G, Traboni C, Nicosia A, Cortese R, Vitelli A. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. *EMBO J* 2002; **21**: 5017-5025

46 **Barth H**, Schafer C, Adah MI, Zhang F, Linhardt RJ, Toyoda H, Kinoshita-Toyoda A, Toida T, Van Kuppevelt TH, Depla E, Von Weizsacker F, Blum HE, Baumert TF. Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate. *J Biol Chem* 2003; **278**: 41003-41012

47 **Lozach PY**, Lortat-Jacob H, de Lacroix de Lavalette A, Staropoli I, Foung S, Amara A, Houles C, Fieschi F, Schwartz O, Virelizier JL, Arenzana-Seisdedos F, Altmeyer R. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. *J Biol Chem* 2003; **278**: 20358-20366

48 **Pöhlmann S**, Zhang J, Baribaud F, Chen Z, Leslie GJ, Lin G, Granelli-Piperno A, Doms RW, Rice CM, McKeating JA. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. *J Virol* 2003; **77**: 4070-4080

49 **Gardner JP**, Durso RJ, Arrigale RR, Donovan GP, Maddon PJ, Dragic T, Olson WC. L-SIGN (CD 209L) is a liver-specific

capture receptor for hepatitis C virus. *Proc Natl Acad Sci USA* 2003; **100**: 4498-4503
50 **Saunier B**, Triyatni M, Ulianich L, Maruvada P, Yen P, Kohn LD. Role of the asialoglycoprotein receptor in binding and entry of hepatitis C virus structural proteins in cultured human hepatocytes. *J Virol* 2003; **77**: 546-559
51 **Agnello V**, Abel G, Elfahal M, Knight GB, Zhang QX. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci USA* 1999; **96**: 12766-12771
52 **Monazahian M**, Böhme I, Bonk S, Koch A, Scholz C, Grethe S, Thomssen R. Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. *J Med Virol* 1999; **57**: 223-229
53 **Cormier EG**, Durso RJ, Tsamis F, Boussemart L, Manix C, Olson WC, Gardner JP, Dragic T. L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus. *Proc Natl Acad Sci USA* 2004; **101**: 14067-14072
54 **Lozach PY**, Amara A, Bartosch B, Virelizier JL, Arenzana-Seisdedos F, Cosset FL, Altmeyer R. C-type lectins L-SIGN and DC-SIGN capture and transmit infectious hepatitis C virus pseudotype particles. *J Biol Chem* 2004; **279**: 32035-32045
55 **Masciopinto F**, Giovani C, Campagnoli S, Galli-Stampino L, Colombatto P, Brunetto M, Yen TS, Houghton M, Pileri P, Abrignani S. Association of hepatitis C virus envelope proteins with exosomes. *Eur J Immunol* 2004; **34**: 2834-2842
56 **Crotta S**, Stilla A, Wack A, D'Andrea A, Nuti S, D'Oro U, Mosca M, Filliponi F, Brunetto RM, Bonino F, Abrignani S, Valiante NM. Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *J Exp Med* 2002; **195**: 35-41
57 **Tseng CT**, Klimpel GR. Binding of the hepatitis C virus envelope protein E2 to CD81 inhibits natural killer cell functions. *J Exp Med* 2002; **195**: 43-49
58 **Wack A**, Soldaini E, Tseng C, Nuti S, Klimpel G, Abrignani S. Binding of the hepatitis C virus envelope protein E2 to CD81 provides a co-stimulatory signal for human T cells. *Eur J Immunol* 2001; **31**: 166-175
59 **Rosa D**, Saletti G, De Gregorio E, Zorat F, Comar C, D'Oro U, Nuti S, Houghton M, Barnaba V, Pozzato G, Abrignani S. Activation of naïve B lymphocytes via CD81, a pathogenetic mechanism for hepatitis C virus-associated B lymphocyte disorders. *Proc Natl Acad Sci USA* 2005; **102**: 18544-18549
60 **Mazzocca A**, Sciammetta SC, Carloni V, Cosmi L, Annunziato F, Harada T, Abrignani S, Pinzani M. Binding of hepatitis C virus envelope protein E2 to CD81 up-regulates matrix metalloproteinase-2 in human hepatic stellate cells. *J Biol Chem* 2005; **280**: 11329-11339
61 **Logvinoff C**, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci USA* 2004; **101**: 10149-10154
62 **Meunier JC**, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci USA* 2005; **102**: 4560-4565
63 **Lavillette D**, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J Virol* 2005; **79**: 6023-6034
64 **Keck ZY**, Op De Beeck A, Hadlock KG, Xia J, Li TK, Dubuisson J, Foung SK. Hepatitis C virus E2 has three immunogenic domains containing conformational epitopes with distinct properties and biological functions. *J Virol* 2004; **78**: 9224-9232
65 **Owsianka A**, Tarr AW, Juttla VS, Lavillette D, Bartosch B, Cosset FL, Ball JK, Patel AH. Monoclonal antibody AP33 defines a broadly neutralizing epitope on the hepatitis C virus E2 envelope glycoprotein. *J Virol* 2005; **79**: 11095-11104
66 **Schofield DJ**, Bartosch B, Shimizu YK, Allander T, Alter HJ, Emerson SU, Cosset FL, Purcell RH. Human monoclonal antibodies that react with the E2 glycoprotein of hepatitis C virus and possess neutralizing activity. *Hepatology* 2005; **42**: 1055-1062
67 **Lin C**, Lindenbach BD, Prágai BM, McCourt DW, Rice CM. Processing in the hepatitis C virus E2-NS2 region: identification of p7 and two distinct E2-specific products with different C termini. *J Virol* 1994; **68**: 5063-5073
68 **Mizushima H**, Hijikata M, Asabe S, Hirota M, Kimura K, Shimotohno K. Two hepatitis C virus glycoprotein E2 products with different C termini. *J Virol* 1994; **68**: 6215-6222
69 **Carrère-Kremer S**, Montpellier-Pala C, Cocquerel L, Wychowski C, Penin F, Dubuisson J. Subcellular localization and topology of the p7 polypeptide of hepatitis C virus. *J Virol* 2002; **76**: 3720-3730
70 **Carrère-Kremer S**, Montpellier C, Lorenzo L, Brulin B, Cocquerel L, Belouzard S, Penin F, Dubuisson J. Regulation of hepatitis C virus polyprotein processing by signal peptidase involves structural determinants at the p7 sequence junctions. *J Biol Chem* 2004; **279**: 41384-41392
71 **Griffin S**, Clarke D, McCormick C, Rowlands D, Harris M. Signal peptide cleavage and internal targeting signals direct the hepatitis C virus p7 protein to distinct intracellular membranes. *J Virol* 2005; **79**: 15525-15536
72 **Griffin SD**, Harvey R, Clarke DS, Barclay WS, Harris M, Rowlands DJ. A conserved basic loop in hepatitis C virus p7 protein is required for amantadine-sensitive ion channel activity in mammalian cells but is dispensable for localization to mitochondria. *J Gen Virol* 2004; **85**: 451-461
73 **Griffin SD**, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, Harris MP, Rowlands DJ. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. *FEBS Lett* 2003; **535**: 34-38
74 **Pavlović D**, Neville DC, Argaud O, Blumberg B, Dwek RA, Fischer WB, Zitzmann N. The hepatitis C virus p7 protein forms an ion channel that is inhibited by long-alkyl-chain iminosugar derivatives. *Proc Natl Acad Sci USA* 2003; **100**: 6104-6108
75 **Premkumar A**, Wilson L, Ewart GD, Gage PW. Cation-selective ion channels formed by p7 of hepatitis C virus are blocked by hexamethylene amiloride. *FEBS Lett* 2004; **557**: 99-103
76 **Sakai A**, Claire MS, Faulk K, Govindarajan S, Emerson SU, Purcell RH, Bukh J. The p7 polypeptide of hepatitis C virus is critical for infectivity and contains functionally important genotype-specific sequences. *Proc Natl Acad Sci USA* 2003; **100**: 11646-11651
77 **Blight KJ**, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972-1974
78 **Lohmann V**, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110-113
79 **Lindenbach BD**, Rice CM. Flaviviridae: The Viruses and Their Replication. In: Knipe DM, Howley PM, editors. Fields Virology. 4th ed. Philadelphia, Pa: Lippincott Williams & Wilkins, 2001: 991-1042
80 **Pallaoro M**, Lahm A, Biasiol G, Brunetti M, Nardella C, Orsatti L, Bonelli F, Orrù S, Narjes F, Steinkühler C. Characterization of the hepatitis C virus NS2/3 processing reaction by using a purified precursor protein. *J Virol* 2001; **75**: 9939-9946
81 **Lorenz IC**, Marcotrigiano J, Dentzer TG, Rice CM. Structure of the catalytic domain of the hepatitis C virus NS2-3 protease. *Nature* 2006; **442**: 831-835
82 **Waxman L**, Whitney M, Pollok BA, Kuo LC, Darke PL. Host cell factor requirement for hepatitis C virus enzyme maturation. *Proc Natl Acad Sci USA* 2001; **98**: 13931-13935
83 **Franck N**, Le Seyec J, Guguen-Guillouzo C, Erdtmann L. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. *J Virol* 2005; **79**: 2700-2708
84 **Santolini E**, Pacini L, Fipaldini C, Migliaccio G, Monica N. The NS2 protein of hepatitis C virus is a transmembrane polypeptide. *J Virol* 1995; **69**: 7461-7471
85 **Yamaga AK**, Ou JH. Membrane topology of the hepatitis C

virus NS2 protein. *J Biol Chem* 2002; **277**: 33228-33234
86 **Kalinina O**, Norder H, Mukomolov S, Magnius LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002; **76**: 4034-4043
87 **Pietschmann T**, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* 2006; **103**: 7408-7413
88 **Erdtmann L**, Franck N, Lerat H, Le Seyec J, Gilot D, Cannie I, Gripon P, Hibner U, Guguen-Guillouzo C. The hepatitis C virus NS2 protein is an inhibitor of CIDE-B-induced apoptosis. *J Biol Chem* 2003; **278**: 18256-18264
89 **Dumoulin FL**, von dem Bussche A, Li J, Khamzina L, Wands JR, Sauerbruch T, Spengler U. Hepatitis C virus NS2 protein inhibits gene expression from different cellular and viral promoters in hepatic and nonhepatic cell lines. *Virology* 2003; **305**: 260-266
90 **Kim JL**, Morgenstern KA, Lin C, Fox T, Dwyer MD, Landro JA, Chambers SP, Markland W, Lepre CA, O'Malley ET, Harbeson SL, Rice CM, Murcko MA, Caron PR, Thomson JA. Crystal structure of the hepatitis C virus NS3 protease domain complexed with a synthetic NS4A cofactor peptide. *Cell* 1996; **87**: 343-355
91 **Love RA**, Parge HE, Wickersham JA, Hostomsky Z, Habuka N, Moomaw EW, Adachi T, Hostomska Z. The crystal structure of hepatitis C virus NS3 proteinase reveals a trypsin-like fold and a structural zinc binding site. *Cell* 1996; **87**: 331-342
92 **Wölk B**, Sansonno D, Kräusslich HG, Dammacco F, Rice CM, Blum HE, Moradpour D. Subcellular localization, stability, and trans-cleavage competence of the hepatitis C virus NS3-NS4A complex expressed in tetracycline-regulated cell lines. *J Virol* 2000; **74**: 2293-2304
93 **Bartenschlager R**, Frese M, Pietschmann T. Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* 2004; **63**: 71-180
94 **De Francesco R**, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. *Nature* 2005; **436**: 953-960
95 **Lindenbach BD**, Rice CM. Unravelling hepatitis C virus replication from genome to function. *Nature* 2005; **436**: 933-938
96 **Foy E**, Li K, Wang C, Sumpter R, Ikeda M, Lemon SM, Gale M. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003; **300**: 1145-1148
97 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
98 **Sen GC**, Sarkar SN. Hitching RIG to action. *Nat Immunol* 2005; **6**: 1074-1076
99 **Li XD**, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci USA* 2005; **102**: 17717-17722
100 **Li K**, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci* USA **2005**; **102**: 2992-2997
101 **Lin R**, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, Wilkinson P, Julkunen I, Vitour D, Meurs E, Hiscott J. Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J Virol* 2006; **80**: 6072-6083
102 **Tai CL**, Chi WK, Chen DS, Hwang LH. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J Virol* 1996; **70**: 8477-8484
103 **Kim JL**, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C, Caron PR. Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: the crystal structure provides insights into the mode of unwinding. *Structure* 1998; **6**: 89-100
104 **Yao N**, Hesson T, Cable M, Hong Z, Kwong AD, Le HV, Weber PC. Structure of the hepatitis C virus RNA helicase domain. *Nat Struct Biol* 1997; **4**: 463-467
105 **Serebrov V**, Pyle AM. Periodic cycles of RNA unwinding and pausing by hepatitis C virus NS3 helicase. *Nature* 2004; **430**: 476-480
106 **Levin MK**, Gurjar M, Patel SS. A Brownian motor mechanism of translocation and strand separation by hepatitis C virus helicase. *Nat Struct Mol Biol* 2005; **12**: 429-435
107 **Pang PS**, Jankowsky E, Planet PJ, Pyle AM. The hepatitis C viral NS3 protein is a processive DNA helicase with cofactor enhanced RNA unwinding. *EMBO J* 2002; **21**: 1168-1176
108 **Dumont S**, Cheng W, Serebrov V, Beran RK, Tinoco I, Pyle AM, Bustamante C. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* 2006; **439**: 105-108
109 **Frick DN**, Rypma RS, Lam AM, Gu B. The nonstructural protein 3 protease/helicase requires an intact protease domain to unwind duplex RNA efficiently. *J Biol Chem* 2004; **279**: 1269-1280
110 **Deng L**, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, Sada K, Hotta H. NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner. *J Gen Virol* 2006; **87**: 1703-1713
111 **Hügle T**, Fehrmann F, Bieck E, Kohara M, Kräusslich HG, Rice CM, Blum HE, Moradpour D. The hepatitis C virus nonstructural protein 4B is an integral endoplasmic reticulum membrane protein. *Virology* 2001; **284**: 70-81
112 **Lundin M**, Monné M, Widell A, Von Heijne G, Persson MA. Topology of the membrane-associated hepatitis C virus protein NS4B. *J Virol* 2003; **77**: 5428-5438
113 **Yu GY**, Lee KJ, Gao L, Lai MM. Palmitoylation and polymerization of hepatitis C virus NS4B protein. *J Virol* 2006; **80**: 6013-6023
114 **Elazar M**, Liu P, Rice CM, Glenn JS. An N-terminal amphipathic helix in hepatitis C virus (HCV) NS4B mediates membrane association, correct localization of replication complex proteins, and HCV RNA replication. *J Virol* 2004; **78**: 11393-11400
115 **Gretton SN**, Taylor AI, McLauchlan J. Mobility of the hepatitis C virus NS4B protein on the endoplasmic reticulum membrane and membrane-associated foci. *J Gen Virol* 2005; **86**: 1415-1421
116 **Egger D**, Wölk B, Gosert R, Bianchi L, Blum HE, Moradpour D, Bienz K. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J Virol* 2002; **76**: 5974-5984
117 **Einav S**, Elazar M, Danieli T, Glenn JS. A nucleotide binding motif in hepatitis C virus (HCV) NS4B mediates HCV RNA replication. *J Virol* 2004; **78**: 11288-11295
118 **Brass V**, Bieck E, Montserret R, Wölk B, Hellings JA, Blum HE, Penin F, Moradpour D. An amino-terminal amphipathic alpha-helix mediates membrane association of the hepatitis C virus nonstructural protein 5A. *J Biol Chem* 2002; **277**: 8130-8139
119 **Penin F**, Brass V, Appel N, Ramboarina S, Montserret R, Ficheux D, Blum HE, Bartenschlager R, Moradpour D. Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. *J Biol Chem* 2004; **279**: 40835-40843
120 **Tellinghuisen TL**, Marcotrigiano J, Gorbalenya AE, Rice CM. The NS5A protein of hepatitis C virus is a zinc metalloprotein. *J Biol Chem* 2004; **279**: 48576-48587
121 **Tellinghuisen TL**, Marcotrigiano J, Rice CM. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 2005; **435**: 374-379
122 **Huang L**, Hwang J, Sharma SD, Hargittai MR, Chen Y, Arnold JJ, Raney KD, Cameron CE. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. *J Biol Chem* 2005; **280**: 36417-36428
123 **Gale M**, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase

regulation. *Mol Cell Biol* 1998; **18**: 5208-5218
124 **Appel N**, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. *J Virol* 2005; **79**: 3187-3194
125 **Moradpour D**, Evans MJ, Gosert R, Yuan Z, Blum HE, Goff SP, Lindenbach BD, Rice CM. Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. *J Virol* 2004; **78**: 7400-7409
126 **Appel N**, Schaller T, Penin F, Bartenschlager R. From structure to function: new insights into hepatitis C virus RNA replication. *J Biol Chem* 2006; **281**: 9833-9836
127 **Seeger C**. Salient molecular features of hepatitis C virus revealed. *Trends Microbiol* 2005; **13**: 528-534
128 **Shimakami T**, Hijikata M, Luo H, Ma YY, Kaneko S, Shimotohno K, Murakami S. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. *J Virol* 2004; **78**: 2738-2748
129 **Shirota Y**, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J Biol Chem* 2002; **277**: 11149-11155
130 **Evans MJ**, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; **101**: 13038-13043
131 **Neddermann P**, Quintavalle M, Di Pietro C, Clementi A, Cerretani M, Altamura S, Bartholomew L, De Francesco R. Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J Virol* 2004; **78**: 13306-13314
132 **Tan SL**, Katze MG. How hepatitis C virus counteracts the interferon response: the jury is still out on NS5A. *Virology* 2001; **284**: 1-12
133 **Reyes GR**. The nonstructural NS5A protein of hepatitis C virus: an expanding, multifunctional role in enhancing hepatitis C virus pathogenesis. *J Biomed Sci* 2002; **9**: 187-197
134 **Macdonald A**, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 2004; **85**: 2485-2502
135 **Tu H**, Gao L, Shi ST, Taylor DR, Yang T, Mircheff AK, Wen Y, Gorbalenya AE, Hwang SB, Lai MM. Hepatitis C virus RNA polymerase and NS5A complex with a SNARE-like protein. *Virology* 1999; **263**: 30-41
136 **Wang C**, Gale M, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J. Identification of FBL2 as a geranylgeranylated cellular protein required for hepatitis C virus RNA replication. *Mol Cell* 2005; **18**: 425-434
137 **Ye J**, Wang C, Sumpter R, Brown MS, Goldstein JL, Gale M. Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. *Proc Natl Acad Sci USA* 2003; **100**: 15865-15870
138 **Ivashkina N**, Wölk B, Lohmann V, Bartenschlager R, Blum HE, Penin F, Moradpour D. The hepatitis C virus RNA-dependent RNA polymerase membrane insertion sequence is a transmembrane segment. *J Virol* 2002; **76**: 13088-13093
139 **Moradpour D**, Brass V, Bieck E, Friebe P, Gosert R, Blum HE, Bartenschlager R, Penin F, Lohmann V. Membrane association of the RNA-dependent RNA polymerase is essential for hepatitis C virus RNA replication. *J Virol* 2004; **78**: 13278-13284
140 **Schmidt-Mende J**, Bieck E, Hugle T, Penin F, Rice CM, Blum HE, Moradpour D. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. *J Biol Chem* 2001; **276**: 44052-44063
141 **Bressanelli S**, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, De Francesco R, Rey FA. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci USA* 1999; **96**: 13034-13039
142 **Lesburg CA**, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 1999; **6**: 937-943
143 **Watashi K**, Ishii N, Hijikata M, Inoue D, Murata T, Miyanari Y, Shimotohno K. Cyclophilin B is a functional regulator of hepatitis C virus RNA polymerase. *Mol Cell* 2005; **19**: 111-122
144 **Watashi K**, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282-1288
145 **Ishii N**, Watashi K, Hishiki T, Goto K, Inoue D, Hijikata M, Wakita T, Kato N, Shimotohno K. Diverse effects of cyclosporine on hepatitis C virus strain replication. *J Virol* 2006; **80**: 4510-4520
146 **Choi SH**, Park KJ, Ahn BY, Jung G, Lai MM, Hwang SB. Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase. *Mol Cell Biol* 2006; **26**: 3048-3059
147 **Shimakami T**, Honda M, Kusakawa T, Murata T, Shimotohno K, Kaneko S, Murakami S. Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon. *J Virol* 2006; **80**: 3332-3340
148 **Munakata T**, Nakamura M, Liang Y, Li K, Lemon SM. Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C virus NS5B RNA-dependent RNA polymerase. *Proc Natl Acad Sci USA* 2005; **102**: 18159-18164
149 **Bain C**, Parroche P, Lavergne JP, Duverger B, Vieux C, Dubois V, Komurian-Pradel F, Trépo C, Gebuhrer L, Paranhos-Baccala G, Penin F, Inchauspé G. Memory T-cell-mediated immune responses specific to an alternative core protein in hepatitis C virus infection. *J Virol* 2004; **78**: 10460-10469

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Genetic diversity of the hepatitis C virus: Impact and issues in the antiviral therapy

H Le Guillou-Guillemette, S Vallet, C Gaudy-Graffin, C Payan, A Pivert, A Goudeau, F Lunel-Fabiani

H Le Guillou-Guillemette, F Lunel-Fabiani, A Pivert, Laboratory of Virology, University Hospital of Angers, France and Laboratory HIFI, UPRES EA 3859, University of Angers, France
S Vallet, C Payan, Department of Microbiology, EA 3882, CHU Morvan, University Hospital of Brest, France
C Gaudy-Graffin, A Goudeau, Université François Rabelais, INSERM ERI 19 et CHRU de Tours, Tours, France
Correspondence to: F Lunel-Fabiani, Professor, Laboratory of Virology, Department of infectious agents, University Hospital of Angers, 4 rue Larrey, 49933 Angers Cedex 9, France. frlunel-fabiani@chu-angers.fr
Telephone: +33-2-41354709 Fax: +33-2-41354164
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Abstract

The hepatitis C Virus (HCV) presents a high degree of genetic variability which is explained by the combination of a lack of proof reading by the RNA dependant RNA polymerase and a high level of viral replication. The resulting genetic polymorphism defines a classification in clades, genotypes, subtypes, isolates and quasispecies. This diversity is known to reflect the range of responses to Interferon therapy. The genotype is one of the predictive parameters currently used to define the antiviral treatment strategy and the chance of therapeutic success. Studies have also reported the potential impact of the viral genetic polymorphism in the outcome of antiviral therapy in patients infected by the same HCV genotype. Both structural and non structural genomic regions of HCV have been suggested to be involved in the Interferon pathway and the resistance to antiviral therapy. In this review, we first detail the viral basis of HCV diversity. Then, the HCV genetic regions that may be implicated in resistance to therapy are described, with a focus on the structural region encoded by the E2 gene and the non-structural genes NS3, NS5A and NS5B. Both mechanisms of the Interferon resistance and of the new antiviral drugs are described in this review.

Key words: Hepatitis C virus; Genetic diversity; Therapy resistance; E2; NS3; NS5A; NS5B

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INTRODUCTION

The role of genetic variability in the natural history by hepatitis C Virus (HCV) infection and in the primary resistance to treatment remains unclear. In particular, the mechanisms underlying the resistance of HCV to Interferon treatment are very different from those by which the human immunodeficiency virus (HIV) becomes resistant to antiretroviral drugs. In this review, we will describe the impact of HCV genetic polymorphism in the treatment response.

HCV VARIABILITY: ORIGIN AND CLASSIFICATION

HCV has a single-strand positive RNA genome and displays a high genetic diversity. This diversity results from defects in the repair activity of the RNA-dependent RNA polymerase (resulting in nucleotide substitutions) and from the absence of 5' to 3' exonuclease activity (lack of error correction)[1]. The mean frequency of nucleotide mutations varies from 1.4×10^{3} to 1.9×10^{3} substitutions per nucleotide and per year. This estimate is based on comparisons of the major sequences of complete genomes obtained after eight years of evolution in a chimpanzee and 13 years in a human[2,3].

Some of the mutations accumulating during replication are silent or synonymous. These mutations have no impact on the amino-acid sequence of the viral protein, but may affect the secondary structure of the genomic RNA. Other so-called non-synonymous mutations lead to changes in protein sequence and the emergence of variants. Other mutations lead to the production of defective viral particles and are therefore lethal. The regions of the genome corresponding to essential viral functions (domains involved in translation or replication), or displaying major structural constraints (non-coding 5' and 3' ends) are the best conserved; indeed, the non coding 5' region is the most highly conserved region of the genome, with more than 90% identity between the sequences of distantly re-

lated strains[4]. The region encoding the capsid is also well conserved, with 81% to 88% sequence identity between isolates[5]. The most variable region of the genome is that encoding the envelope glycoproteins, E1 and E2. The sequences of hypervariable regions (HVR1 and HVR2) of E2 in strains isolated from different patients may differ by more than 50%. The polypeptides encoded by these hypervariable regions are therefore very tolerant to amino-acid substitutions[6,7].

The classification of HCV, redefined and simplified in 1998, is based on the topology of the trees obtained by phylogenetic relationships between viral variants. HCV variants can be classified into six clades, and then into subtypes corresponding to subgroups of the most closely related viruses within a clade[8]. The virus genotype is indicated by an Arabic number (from 1 to 6), associated with a lower-case letter to indicate the subtype. This new nomenclature has led to the reclassification of virus genotypes 7, 8, 9 and 11 as genotype 6 viruses, and the reclassification of genotype 10 viruses as genotype 3 viruses. Recently, the status of HCV-genotype nomenclature has been re-examined in an attempt to resolve conflicting subtype and genotype designations. A complete list of currently recognized genotypes and subtypes was published[9]. The euHCVDB (http://hepatitis.ibcp.fr), DDJB (http://www.ddbj.nig.ac.jp) and Los Alamos (http://hcv.lanl.gov) databases currently include a large number of HCV sequences, making it possible to compare an isolate with a large number of reference sequences[10]. Although currently restricted to specialist laboratories, the gold standard method for HCV genotyping is sequencing of the NS5B region followed by phylogenetic analysis including comparison to reference sequences[11,12].

A chimeric virus generated by an intergenotypic homologous recombination event (genotype 2k/genotype 1b) in the *NS2* gene was first described in the St Petersburg area: this demonstrated the occurrence of recombination phenomena in HCV[13]. Another recombinant form (genotype 2i/genotype 6p) was recently described in Vietnam, with a point of recombination between NS2 and NS3[14]. Such recombination events have already been described for GBV-C and Dengue virus[15,16]. In HCV, homologous recombination may be favoured by the nature of HCV risk behaviour in which there may be frequent exposures, for example among Intra Venous Drug Users. In practice, genotyping is based solely on polymorphism in a single genomic region (5' NC or NS5B), so it is currently impossible to estimate the frequency of recombination events.

Studies of the global distribution of HCV genotypes and analyses of their phylogenetic relationships have provided insight into their emergence and diversification over the centuries. The worldwide distribution of genotype 2 began some 90 to 150 years ago; that of subtype 1b began 60 to 70 years ago and that of genotype 3 began about 40 years ago[17] Genotypes 4 and 6 emerged much earlier: 350 and 700 years ago, respectively[18]. The simultaneous presence of a large number of subtypes of a viral genotype in a limited geographic region is indicative of the long-standing endemic presence of the virus in the population studied. Genotype 2 is frequent in West Africa (59% to 100%, depending on the region studied), and many subtypes have been identified (2c to 2l)[19]. The same is true of genotype 4 in Central Africa (4b, 4c, 4e and 4m)[20]. Conversely, the limited number of subtypes in Europe (where genotype 1, 2, 3 are the more frequently found), North America and Japan is consistent with the more recent introduction of HCV into these populations. Another level of complexity is found within a given infected patient termed as the quasispecies population. Quasispecies are populations of different but closely related genomes and data have suggested that Interferon alpha exerts a selective pressure on HCV quasispecies[21,22]. Thus, these molecular polymorphisms are clinically relevant and are one of the major factors in determining the outcome of the Interferon therapy.

Since 1986, before the discovery of HCV, the efficacy of Interferon alpha has been demonstrated in the treatment of chronic non A non B hepatitis[23,24]. Interferon alpha is a member of the cytokine family produced by cells in response to viral infections or other stimuli by binding to their specific receptors on the surface of target cells, Interferons stimulate a cascade of intracellular signalling pathways that result in the suppression of numerous Interferon-Stimulated Genes (ISGs) whose products (proteins) can mediate the effects of Interferon. Among these, are the INF induced, double stranded (ds) RNA dependant protein kinase (PKR), the 2' 5' oligoadenylate synthetase and the Mx proteins, of which the antiviral activities have been well demonstrated[25]. Interferon also acts as a stimulus of immunity and modulates cell growth, differentiation and apoptosis[26].

In an effort to understand the role of different factors on the outcome of Interferon therapy, numerous studies have been performed to define predictors of response and both viral and host factors have been studied.

Among the viral factors examined, viral genotype seems to be the most important predicting factor; it appears to be a predictive parameter of the SVR as strong as well-established predictive parameters such as the pre-treatment viral load, the stage of fibrosis on liver, and the age and the sex of the patient. Trials clearly reported a high SVR rate of 76% to 84% in patients infected with HCV genotypes 2 or 3 whereas a weaker SVR of 42% to 52% was obtained in HCV genotype 1-infected patients[27-29]. The SVR is likely to be higher for genotype 2 than for genotype 3 where a shortened treatment period was experienced, but ongoing studies will determine the role of the initial viral load and a rapid virologic response[30,31]. Therapeutic outcomes are well known for these three genotypes because of their geographic distribution; genotype 1 represents more than 70% of the HCV infections in the Western world, genotypes 2 and 3 infect 10% to 20% of the other patients. Studies focused on the outcome of the antiviral therapy conducted in patients infected by HCV genotype 4 are mainly provided by Egyptian cohorts and results showed an intermediate SVR rate comprised between 55% to 69%[32,33]. Genotypes 5 and 6 are less studied because of their minor distribution, patients infected by these genotypes may achieve a SVR at a level between the SVRs of genotype 1 and genotypes 2-3[34].

Differences in the SVR rate observed among differ-

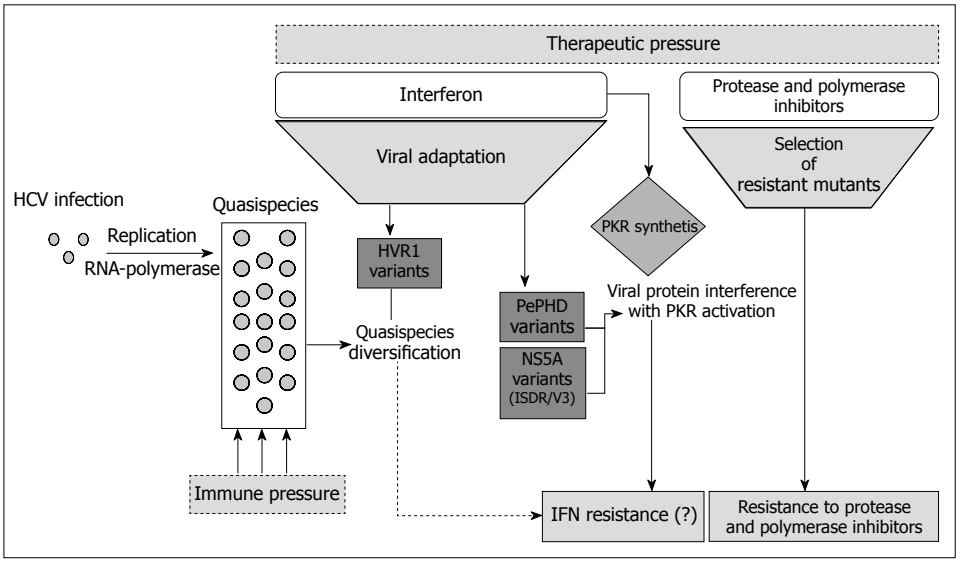

Figure 1 Mechanisms involved in HCV resistance to antiviral therapies.

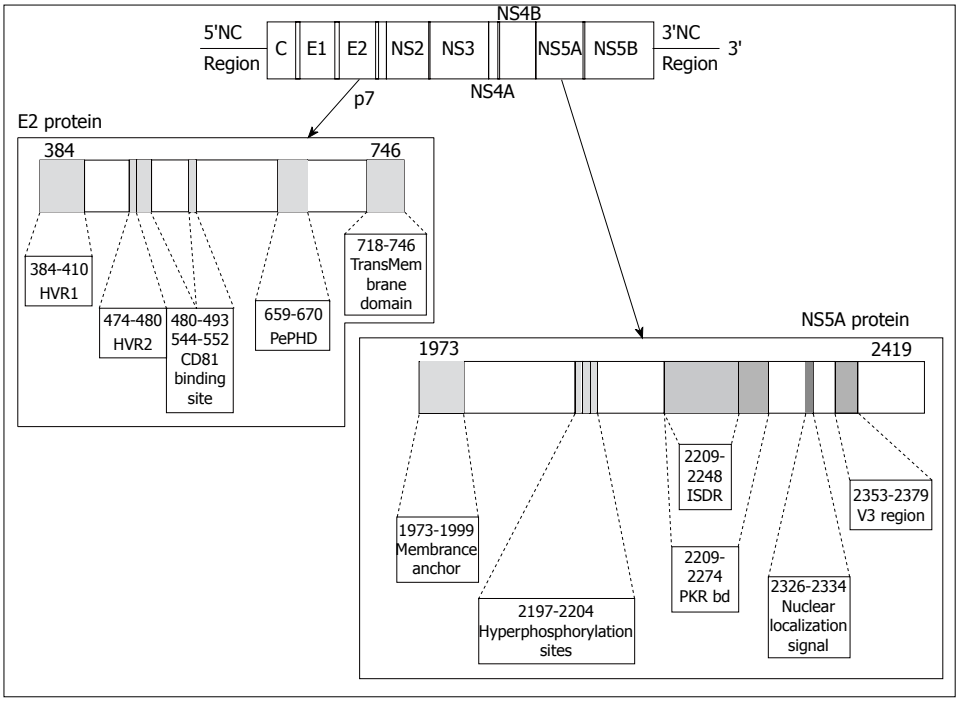

Figure 2 Linear representation of the E2 and NS5A proteins of the HCV, location of the main interesting domains of each protein. Each site of interest is represented by a grey zone on the protein and is linked to a box containing its name and amino acid positions.

ent HCV genotypes have highlighted a presumptive role of the viral genetic determinants and have suggested that some virus-encoded functions are involved in the response to Interferon. Although a very efficient *in vitro* or *in vivo* or animal models of HCV infection is not available, several studies have been undertaken to identify which viral gene may interfere with antiviral molecules. Both *in vivo* and *in vitro* studies demonstrated that the structural proteins E1 and E2, the non-structural proteins NS3, NS5A and NS5B may contribute to the resistance of the combined Interferon alpha and ribavirin therapy. Moreover, all these proteins share a genetic polymorphism involved in the resistance mechanisms as shown in Figure 1.

GENOMIC DOMAINS CONTRIBUTING TO RESISTANCE

As previously described by Pawlotsky in this issue of the journal, HCV presents a high genetic diversity. The intra-genotype analysis of this diversity along the viral genome shows different degrees of variation; regions such as the 5'UTR and the core are highly conserved, the non-structural regions 2, 3, 5b and the 3'UTR are relatively variable whereas the envelope regions E1 and E2 and the NS4 and the NS5A genes exhibit the highest sequence diversity.

Polymorphism and significance of amino acid substitutions within E2 regions in Interferon alpha resistance

E2 glycoprotein is a type I transmembrane protein of 70 kDa, with an N-terminal ectodomain and a C-terminal hydrophobic anchor. It assembles with E1 to form a heterodimer. It has been shown to interact with two potential HCV receptors, the human tetraspanin CD81 and the human scavenger receptor SR-BI, in experiments based on soluble E2 (sE2) binding (Figure 2). The role of E2 in the

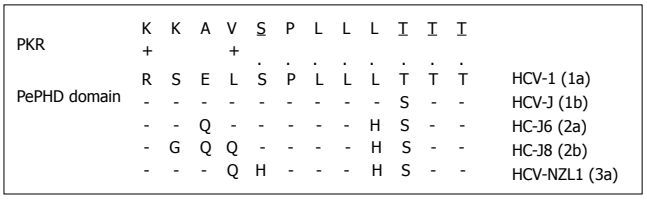

Figure 3 Sequence homology between HCV E2-PePHD domain and PKR. PePHD domains from various HCV genotypes (1a, 1b, 2a, 2b, 3a) are aligned and compared with the PKR sequence. Identical and similar amino acids to PKR are shown by (.) and (+), respectively. PKR auto-phosphorylation sites are underlined.

cell entry of HCV is extensively reviewed in this issue by Dubuisson *et al*.

One of the major ways by which cellular Interferon alpha inhibits viral replication involves the Interferon-alpha-inducible double-stranded RNA-activated Protein Kinase R (PKR). Indeed the Interferon alpha binds to the PKR and leads to its autophosphorylation which in turn initiates the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2α) by the PKR. This phosphorylation inhibits the RNA transcription. Indeed, eIF2α is necessary to initiate the translation by forming a complex with GTP and met-tRNA and then allowing binding to the 40S ribosomal subunit. The E2 glycoprotein, or more precisely, a 12-amino acid domain of this protein located between residues 659 and 670 and known as PePHD (PKR-eIF2α phosphorylation homology domain), is involved in PKR inhibition. Taylor *et al*[35] showed that PePHD was the main domain of E2 able to bind PKR *in vitro*. The PePHD motif sequence is very similar to that of the auto-phosphorylation sites of PKR and its target, eIF2α, and this similarity is more marked for genotype 1 viruses than for genotype 2 and 3 viruses (Figure 3). E2 glycoproteins of genotype 1 viruses (HCV-1 E2) behave *in vitro* as pseudosubstrates, inhibiting the kinase activity of PKR. In mammalian cells, the stimulation of translation by HCV-1 E2 is consistent with the hypothesis of PKR inhibition. This inhibitory activity has also been observed in a yeast model (*Saccharomyces cerevisiae* expressing HCV1 E2). The replacement of HCV-1 E2 by proteins identical to those of genotype 2 and 3 viruses abolishes this inhibitory effect. An interaction between genotype 1 strains and PKR, *via* PePHD, has therefore been proposed to account for the intrinsic resistance of the strains of this genotype to Interferon alpha[35].

The clinical relationship between aminoacid sequence of PePHD and the outcome of Interferon therapy has been a matter of controversy. A few studies have addressed polymorphism of the PePHD region in patients carrying strains of genotypes 1, 2 and 3. Abid *et al* studied a small number of patients infected with genotype 1b HCV. They initially showed that in some patients responding to treatment, the virus had a PePHD sequence identical to that of the HCV-J strain (RSELSPLLLSTT), calling Taylor's hypothesis into question[36]. For HCV 2a/b isolates, conflicting results about the association of PePHD mutations and treatment response have been published[37,38]. A number of studies focusing exclusively on the diversity of PePHD sequences in genotype 1b HCV before treatment have since shown strong conservation of this motif, regardless of the response subsequently obtained[39,40].

The genetic heterogeneity of the E2-HVR1 region can also be used to describe the composition of quasispecies precisely, provided a large number of clones are analysed. The preliminary studies in this area were based on analysis of single-strand polymorphisms or of a restricted number of clones (less than 10). A correlation between high levels of quasispecies complexity before Interferon alpha monotherapy and a lack of response to treatment was reported[41,42]. This led to the suggestion that genetic variability ensured a reservoir of potentially resistant strains. However, more recent studies with larger numbers of sequenced clones or based on SSCP enhanced sensitivity protocols have not confirmed the link between the genetic complexity of HCV and virological response[43]. These conflicting results illustrate the problems of methodological standardisation associated with studies of quasispecies.

Polymorphism of the HCV non-structural regions and impact on the treatment response.

The NS2, NS3 and NS4A/B proteins may not be implicated in the antiviral treatment resistance with the current combination pegylated Interferon alpha plus ribavirin. However, new therapies are in development targeting the protease encoded by NS3 and the polymerase encoded the NS5B. As already observed in the anti-HIV HAART treatment, the genetic polymorphism of NS3 and NS5B will be analysed in relation to therapy. HCV presents a high mutation rate and the antiviral pressure may favour the emergence of resistant strains. Mutation points have been already observed *in vitro* (replicon system).

Polymorphism of NS5A and Interferon resistance

The NS5A protein is the non structural HCV protein which is the protein most reported to be implicated in the Interferon resistance Many interactions between NS5A and different molecules from several intracellular pathways have been also demonstrated in cellular *in vitro* systems. An overview of the key roles of the genetic heterogeneity of this protein will be detailed in this paragraph.

Structure of the NS5A protein: NS5A is a phosphoprotein found in a basally phosphorylated form of 56kDa and a hyperphosphorylated form of 58 kDa. It varies in length, from 445 amino acids in genotype 4a, 447 amino acids in genotype 1b, 448 in genotypes 1a and 1c, 450 in genotype 5a, 451 in genotype 6a, 452 in genotype 3 to 452 amino acids in genotype 2a and b. The amino acid sequence varies depending on the genotype. The NS5A protein is a pleïotropic protein involved both in the viral replication and in many interactions with cellular signalling pathways. Although its function remains unclear, many domains of interest have been described in this protein (Figure 2).

Many interactions between NS5A and cellular signalling pathways have been reported and the interacting sequence has been identified for some of them. Although studies have been conducted *in vitro*, results strongly

suggest a potential involvement of the NS5A protein in the establishment of a chronic hepatitis and in the carcinogenesis and outcome of a liver tumour (for review, see[44]). In this review, we will focus only on the protein interaction of NS5A with the Interferon pathway.

Interaction with the cellular Interferon pathway: Phosphorylation of PKR triggered a global translastional repression comprising the viral replication. Gale *et al*[45] identified in the C-terminal NS5A a Protein Kinase R binding domain. They demonstrated *in vitro* that the NS5A protein is able to bind and inhibit the Interferon-alpha-inducible double-stranded RNA-activated PKR, the interaction occuring *via* the NS5A PKR binding domain. In that way, the NS5A protein has been suggested to balance the Interferon cellular antiviral pathway and to be involved in the resistance to the Interferon based-therapy.

The NS5A protein is also able to interact with the Interferon pathway in a PKR-independent manner. It has been demonstrated *in vitro* that NS5A induces the expression of the pro-inflammatory chemokine interleukin 8 (IL-8) at both the mRNA and protein levels. This chemokine is known to inhibit directly the Interferon alpha activity. The clinical relevance of these *in vitro* studies has been reported and it appears that the IL-8 serum levels were higher in infected patients than in healthy controls. A second study showed an increase of the pre-treatment IL-8 level in the non-responder than in the SVR[46,47].

Mutations in the NS5A protein and relationship with the Interferon resistance: The 40 first amino acids of the PKR binding domain present a high level of variability. It was termed the Interferon sensitivity determining region (ISDR) reported to play a key role in the Interferon therapy response[48,49]. Molecular analysis of this 40 amino acid region showed the potential role of mutations in the resistance to Interferon therapy. They demonstrated a correlation between the success of Interferon therapy and the variability of the ISDR domain in Japanese patients with HCV genotype 1b or 2 infection. Patients infected with viral strains whose ISDR harbours more than four mutations different from the HCV-J sequence (the Japanese prototype strain defined as the wild-type) were responders to Interferon therapy whereas patients infected with a wild-type or strain harbouring less than four mutations in ISDR were non-responders. Numerous studies investigating the correlation between the mutations in ISDR in genotype 1b HCV and the outcome of the Interferon-based therapy have been undertaken. Japanese results were concordant with the initial study whereas European and north-American groups did not describe such a correlation and debate remains controversial. A recent meta-analysis has been conducted on 1230 ISDR sequences from HCV 1b-infected patients by Pascu *et al*[50]. Sequences were provided from Japanese and European studies. Analyses were realised by logistic regression and clearly demonstrated an association between number of ISDR mutations and response to the treatment both in Japanese and European patients, irrespective from a geographical distribution. First, controversial results may be explained in part by the HCV European strains, most of the European HCV 1b strains present less than 3 mutations and it was difficult to identify a statistical difference between the Interferon therapy response among European infected patients. It is also important to note that treatment schedules were not the same in Europe and in Japan. A correlation between the variability in the ISDR region and a SVR has been demonstrated in the genotype 2a-infected patients. Conversely, investigations in the patients infected by genotypes 2b or 3a did not find any relation between the successful therapy and genetic variability of the NS5A gene[51,52]. Additionally, *in vitro* studies did not report any interaction between NS5A genotype 3a and PKR in viral resistant strains to the Interferon therapy[53].

The major implication of the variability in the resistance to antiviral agents has been recently pointed out, but few studies focused on the entire NS5A protein at the quasispecies level and its kinetics under therapy. Inshauspe *et al*[54] identified another domain localised in the C-terminal region of NS5A and termed it the V3 domain. It is 27 amino acids in length and it harbours a high variability level. Duverlie *et al*[55] demonstrated a relationship between the mutation level in V3 and the response to Interferon therapy. Resistant strains were highly conserved whereas sensitive strains were variable. Our group and others confirmed this correlation[56]. We followed HCV quasispecies diversity at baseline and under Interferon alpha-ribavirin combined treatment in the entire NS5A gene and in each region of interest (PKR-bd, ISDR and V3). As reported by Puig-Basoiti *et al*[57], the V3 domain showed a higher quasispecies diversity in responder patients than in non-responders in pre-treatment samples and these data confirmed the potential role of the genetic diversity in the success of the Interferon-based therapy.

NS3 protease as a target for specific antiviral therapy

Previous studies on the full-length genome sequence have led to the HCV NS3 domain as being classified as one of the less variable regions of the genome[58,59]. Nevertheless, it appears important to consider the diversity of HCV protease, because it is an attractive target for specific anti-HCV therapy.

Catalytic functions and the three-dimensional structure of HCV NS3 protease have been reviewed in this issue by Dubuisson *et al*. Some of the structural and functional constraints affecting the NS3 protease have been demonstrated by the definition of major domains, including the catalytic triad, the substrate binding pocket, the NS4A binding domain and the residues binding to the zinc ion[60].

Natural polymorphism of NS3 protease: The diversity of the HCV NS3 protease gene in clinical samples has been studied[61,62]. Although the protease NS3 is considered to be one of the less variable genes in the HCV genome, variability of both nucleotide and amino acid sequences exists. The proportion of synonymous substitutions affecting the region is significantly higher than the proportion of non synonymous ones suggesting that the NS3 protease mutations are the products of random genetic drift rather than of positive selection. NS3 protease structural and chemical integrity is required for it to process

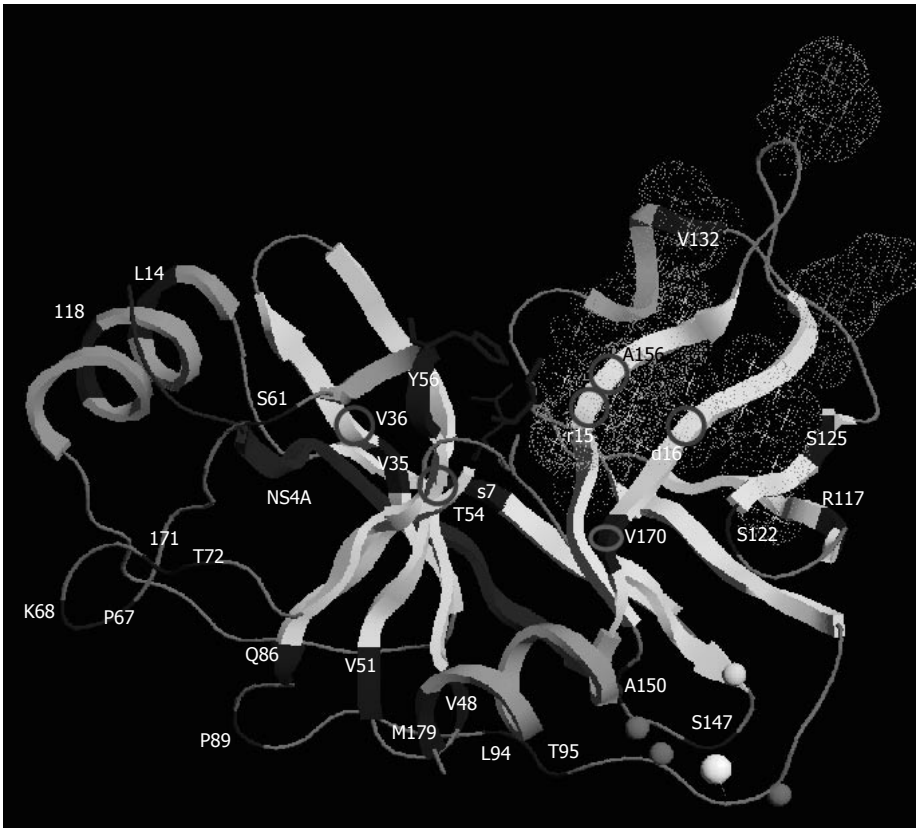

Figure 4 Three-dimensional structure of NS3 protease (PDB accession code 1NS3). Polymorphism and main residues implicated in resistance are shown. The protease is shown based on its secondary structure in light grey. Main polymorphic residues are shown in dark grey. The side chains of the residues forming the catalytic triad (H57, D81, and S139) are displayed in dark grey ball-and-stick representation. The NS4A cofactor is shown in dark grey and Zinc ion as a white ball. Zn2+ ligands (C97, C99 and C149) and H145 residue are modeled in light grey spheres. Stars correspond to the side chains of the residues forming the S1 to S6 substrate binding pockets (grey dots cloud) of the enzyme. Residues implicated in resistance to protease NS3 inhibitors are shown with circles. This figure was prepared using the RasTop programme version 2.0.3-VF.

the HCV polyprotein. The perpetuation of substitutions depends in large part on the extent to which the viral protease can tolerate them while remaining functional. The NS3 protease appears to be tolerant, particularly in its loop regions between helices and sheets (Figure 4). Sporadic mutations affecting the catalytic residues have already been observed from different patients. These catalytic-triad amino acid mutations probably lead to a loss of function, and therefore may be lethal. In that sense, they are not crucial for resistance to NS3 protease inhibitors[61].

The NS3 region is known to contain a large number of T-cell epitopes. T-cells directed against the NS3 domain seem to be of particular importance both during the acute and chronic phase of the disease as the CD4+ and CD8+ T-cell response to the NS3 protease correlates with clearance and control of the infection[63]. As reviewed by Dubuisson *et al*[64,65] in this issue, the NS3 serine protease influences the Interferon-dependent innate cellular host defence by inhibition of RIG-1 and TLR3 signalling. As use of Interferon alpha is the standard of care for HCV, the potential of protease inhibition to enhance Interferon therapy has attracted much interest.

NS3 Polymorphism and impact on response to antiviral specific treatment: The NS3 protease three-dimensional structure description has provided the necessary detailed insight to permit rational inhibitor design. Therapies based on such inhibitors are still at the development and testing stage[66,67]. The generation of such therapies based on the inhibition of site-specific proteolysis has been clearly illustrated in the development of effective inhibitors of human immunodeficiency virus type 1 (HIV-1)[68].

In vitro and *in vivo* phase I and II studies have shown that evaluated NS3 protease inhibitors (BILN 2061, VX-950 and SCH 503034) developed drug resistant mutations[69,70]. These new drugs meet the same problems of viral resistance that exist in the specific anti-viral treatment of HIV and HBV infected individuals. In untreated patients, less than five percent of protease variants present resistant mutations to inhibitors SCH6, SCH 503034 and VX-950[61,71,72]. The reduced fitness of the most resistant variant may explain that such variants are rarely found in naïve patients[69,70,73]. The replicative fitness of resistant viruses is a critical parameter of viral resistance and is an important factor to consider in achieving sustained virological response. It is underestimated due to low replicative resistant strains not being detected.

Both HCV protease inhibitors evaluated in humans, VX-950 and SCH-503034, have demonstrated a very strong correlation between their antiviral effect, their serum trough levels, and the development of resistance[67,74]. Changing in viral quasispecies with a selection of clones resistant to VX-950 has been observed in patients who experienced viral load breakthrough to VX-950 monotherapy[66]. Resistant variants may have poorer replicative fitness than wild-type viruses. However, VX-950-resistant clones isolated from patient plasma from the initial phase 1b study were thought to be associated with the lower doses of monotherapy, and selection for these clones was based on inadequate suppression of virus[70]. Four residues in the NS3 protease, when substituted, are known to be associated with resistance/reduced sensitivity to VX-950. R155K/T/S/M and A156T/V, located close to the VX-950 binding groove, a domain of NS3 protease, confer

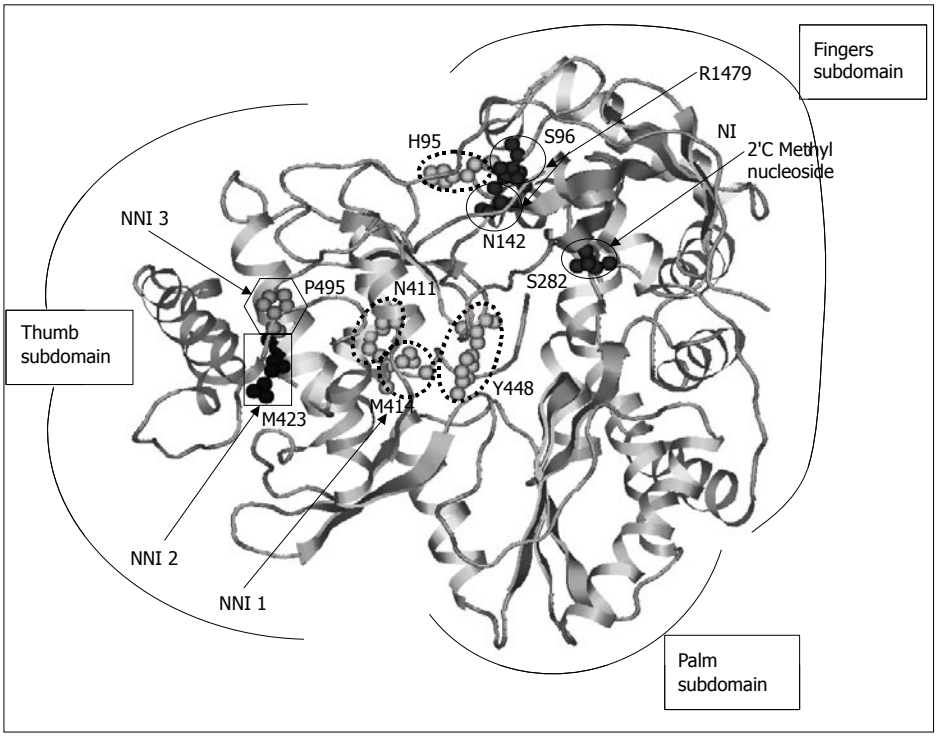

Figure 5 Three-dimensional structure of NS5B RNA-dependent RNA polymerase (PDB accession code 1QUV). The three subdomains of the polymerase are shown (Fingers, Thumb and Palm) as well as the main residues targeted by each class of polymerase inhibitors (NNI 1: Non nucleoside class 1, NNI 2: Non nucleoside class 2, NNI 3: Non nucleoside class 3, NI: Nucleoside Inhibitor). This figure was prepared using the RasTop programme version 2.0.3-VF.

moderate to high level resistance to VX-950; V36A/M and T54A located further from the binding groove confer low-level of resistance to VX-950 (Figure 4).

Three mutations, T54A, V170A and A156S mutations conferred low to moderate levels of resistance to SCH-503034 in a replicon model. Mutants with A156T substitution are highly resistant to this compound[73]. Combinations of VX-950 and SCH-503034 may be limited by the selection of cross-resistance mutations.

The polymorphic outside residues central to NS3 protease activity and substrate binding sites, may prove to be accessory substitutions of resistance mutations contributing to clinical resistance to HCV protease inhibitor therapy. This has been supposed for 3 separate second site mutations P89L, Q86R and G16R *in vitro* with the protease SCH6 inhibitor[75]. Moreover, resistance to future NS3 protease inhibitors could occur through mutations arising either in the protease gene itself or, as has been shown for HIV-1, in cleavage sites of the protein[68].

Resistance to specific HCV inhibitors will probably emerge in case of insufficient antiviral pressure. Viral load monitoring and checking for emergence of NS3 protease resistance mutations could contribute to surveillance of sustained viral response.

NS5B polymorphism and impact of the anti-polymerase therapy

Structure of the protein: The last gene of the HCV is the NS5B which encodes the HCV polymerase, which is an RNA-dependent RNA polymerase (RdRP). This 68 kDa protein plays a central role in HCV replication and in viral diversity because of the lack of a proof-reading activity. The NS5B protein belongs to the tail-anchored proteins as previously detailed by Dubuisson *et al*. The crystal structure of this enzyme has been fully determined. The three dimensional structure is similar to other polymerases; it shows a typical right-hand arrangement with fingers, palm and thumb domains. This organisation forms a cavity called a NTP tunnel in which the traffic of the nucleotides to the active site occurs[76,77] (Figure 5).

Genetic polymorphism and enzymatic activity: As all enzymatic proteins, the nucleic acid sequence is a fundamental determinant of the three-dimensional structure and it determines good enzymatic activity. It has been shown that the active site does not tolerate any mutations. Its amino acid sequence is highly conserved among all HCV genotypes and any mutation may inhibit viral replication. Several consensus sequence motifs have also been described along the NS5B polymerase; five of them are in the palm sub-domains (motifs A, B, C, D and E) and one is in the finger domain (motif F)[78,79]. Most of the single amino-acid substitutions introduced into the conserved motifs by directed mutagenesis resulted in decreased or abolished viral replication and enzymatic activity[80,81].

NS5B and viral genotype: Although the NS5B protein remains a relatively well conserved protein because of its enzymatic function, the NS5B domain, along with other domains of the HCV, supports a slight variability which provides a basis for distinguishing viral type and subtype. Indeed, the nucleic acid sequence is less conserved than the 5'NC but sufficient to define type and viral subtype. The relation between the structure of the NS5B and the response to the Interferon therapy has rarely been studied and most studies were focused on genotype 1b. Results were contradictory. Vuillermoz *et al*[82] did not find any mutation differences (point and rate mutations) between responders and non-responders to the HCV NS5B strain; conversely Kumagai *et al*[83] reported two polymorphisms in NS5B associated with an early viral clearance in the treatment of

HCV genotype 1b-infected patients.

New antiviral treatments targeting the HCV polymerase: The HCV polymerase represents an important target for the new inhibitors of HCV replication and numerous assays are currently in development using the *in vitro* subgenomic HCV replicon system. Thanks to the knowledge of the three dimensional structure of NS5B, screening of many chemical molecules has been undertaken. Three classes of inhibitors are now reported and include nucleoside analogues, non nucleoside analogues and pyrophosphate mimics. Drugs belonging to the nucleoside inhibitors are in competition with the substrate of the RdRP and inhibit polymerase elongation. The binding site is localised in the fingers subdomain of the polymerase. Molecules of this class have a 2'methylribose structure and some of them are now well characterised[84,85]. 2'-C-Methyl-Cytidine, 2'-C-Methyl-Adenosine and 2'-C-Methyl-Guanosine have been shown to be effective against genotype 1a and 1b in Con1 subgenomic replicons. A single mutation, S282T, confers a cross resistance to all the 2'-C-Methyl-Nucleoside, and to their prodrug as such the NM283 (prodrug of the 2'-C-Methyl-Cytidine known as the Valopicitabine)[86,87]. The R1479 (4'-Azido-Cytidine), which is another nucleoside inhibitor, is under development[88] and a recent study reported the mutation resistance points as S96T and S96T/N142T. This work showed no cross resistance between R1479 and the 2'C methyl nucleosides[89]. The NS5B genes of non genotype 1 (2a, 2b, 3, 4 and 6) have been studied in a genotype 1 replicon background; most of them reported a weak replication that enabled drug sensitivity assays. It is important to note that prototype sequences of genotype 4a and 6a encoded T282 and L495 which are implicated respectively in the *in vitro* resistance to the 2'-C-Methyl nucleoside inhibitors and to the benzimidazole non-nucleoside inhibitors[87]. NM283 (Idenix®) has demonstrated an anti-HCV activity in the trials of phases I and II a, it is now in the phase II b clinical development. Results of the phase I trial of R1626 (Roche®), another nucleoside inhibitor, were also encouraging. The first phase II development study is now starting[90].

Compounds belonging to the non-nucleoside inhibitors (NNI) act as allosteric inhibitors that may affect the initiation step of the polymerisation (inhibition of the conformational transition prior to the formation of an efficient pre-elongation complex, inhibition of RNA binding to NS5B or non-competitive inhibitors of NTP incorporation). Different molecules belong to this class and have distinct targets. Indeed, three binding pockets have been localised and are used to define each class of NNI (1 to 3). Two of them are located in the thumb domain. The benzothiazidines group (NNI 1) binds to one pocket located at the interface between the thumb and palm domains and centralised around Met414. *In vitro* studies have been carried out mainly with the genotype 1b subgenomic replicon and substitutions have been shown to decrease susceptibility (M414T, C451R, G558R, H95R)[91] or to confer resistance (H95Q, N411S, M414L/T, Y448H)[92] to benzothiazine drugs from different pharmaceutical companies. The second pocket, located at the base of the thumb domain, binds to the large group of molecules comprising thiophene 2-carboxylic acids, phenylalanine derivatives and substituted pyranones (NNI 2) and the binding central amino acid is Met423. *In vitro* assays reported differences in the inhibitory activity between the different genotypes tested. For example, Howe *et al*[93] showed that the susceptibility level to the HCV-371 (NNI 2) was equal among the genotype 1b isolates but varied with genotypes 1a, 3a and 4 (5 to 59 fold less susceptible than genotype 1b). The last pocket is located at the top of the thumb around Pro495 and is targeted by the benzimidazole 5-carboxamide inhibitors (NNI 3)[94]. As with the other nucleoside inhibitors, sensitivity has been studied *in vitro* in the subgenomic replicon; Tomei *et al*[95] have recently reported that HCV strains resistant to benzimidazole molecules harbored a substitution in Pro495. Interestingly, it has been shown that a chimaeric 1b:2b HCV replicon, harbouring a genotype 2b NS5B, was sensitive to the 2'-C-Methyl-Adenosine but completely insensitive to the NNI 1 (benzimidazole) and NNI 3 (thiophene)[96]. These results underlined that a genotype specificity may exist for each anti-polymerase inhibitor.

All of these results showed the importance of genetic diversity in the HCV therapeutic strategy. Preliminary results, both *in vitro* and *in vivo* trials, strongly suggest a relationship between genetic polymorphism and sensitivity to drug. Identification of the mutations set corresponding to each antiviral drug will be very helpful. It will represent a chance to anticipate treatment regimes at baseline based on the viral genotype, an approach currently only done for HIV therapy, many years after the beginning of HAART[97].

Finally, the genetic diversity of the HCV largely impacts in the global approach of this viral disease as in the treatment management as well as in the development of new HCV antiviral therapies.

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REFERENCES

1 **Steinhauer DA**, Domingo E, Holland JJ. Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase. *Gene* 1992; **122**: 281-288
2 **Ogata N**, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci USA* 1991; **88**: 3392-3396
3 **Okamoto H**, Kojima M, Okada S, Yoshizawa H, Iizuka H, Tanaka T, Muchmore EE, Peterson DA, Ito Y, Mishiro S. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. *Virology* 1992; **190**: 894-899
4 **Bukh J**, Purcell RH, Miller RH. Sequence analysis of the 5' noncoding region of hepatitis C virus. *Proc Natl Acad Sci USA* 1992; **89**: 4942-4946
5 **Simmonds P**, Smith DB, McOmish F, Yap PL, Kolberg J, Ur-

dea MS, Holmes EC. Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. *J Gen Virol* 1994; **75** (Pt 5): 1053-1061
6 **Smith DB**. Evolution of the hypervariable region of hepatitis C virus. *J Viral Hepat* 1999; **6** Suppl 1: 41-46
7 **Penin F**, Combet C, Germanidis G, Frainais PO, Deléage G, Pawlotsky JM. Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. *J Virol* 2001; **75**: 5703-5710
8 **Robertson B**, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, Gojobori T, Maertens G, Mizokami M, Nainan O, Netesov S, Nishioka K, Shin i T, Simmonds P, Smith D, Stuyver L, Weiner A. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. International Committee on Virus Taxonomy. *Arch Virol* 1998; **143**: 2493-2503
9 **Simmonds P**, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973
10 **Kuiken C**, Mizokami M, Deléage G, Yusim K, Penin F,Shin-I T, Charavay C, Tao N, Crisan D, Grando D, Dalwani A, Geourjon C, Agrawal A, Combet C. Hepatitis C databases, principles and utility to researchers. *Hepatology* 2006; **43**: 1157-1165
11 **Laperche S**, Lunel F, Izopet J, Alain S, Dény P, Duverlie G, Gaudy C, Pawlotsky JM, Plantier JC, Pozzetto B, Thibault V, Tosetti F, Lefrère JJ. Comparison of hepatitis C virus NS5b and 5' noncoding gene sequencing methods in a multicenter study. *J Clin Microbiol* 2005; **43**: 733-739
12 **Laperche S**, Saune K, Dény P, Duverlie G, Alain S, Chaix ML, Gaudy C, Lunel F, Pawlotsky JM, Payan C, Pozzetto B, Tamalet C, Thibault V, Vallet S, Bouchardeau F, Izopet J, Lefrère JJ. Unique NS5b hepatitis C virus gene sequence consensus database is essential for standardization of genotype determinations in multicenter epidemiological studies. *J Clin Microbiol* 2006; **44**: 614-616
13 **Kalinina O**, Norder H, Mukomolov S, Magnius LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002; **76**: 4034-4043
14 **Noppornpanth S**, Lien TX, Poovorawan Y, Smits SL, Osterhaus AD, Haagmans BL. Identification of a naturally occurring recombinant genotype 2/6 hepatitis C virus. *J Virol* 2006; **80**: 7569-7577
15 **Holmes EC**, Worobey M, Rambaut A. Phylogenetic evidence for recombination in dengue virus. *Mol Biol Evol* 1999; **16**: 405-409
16 **Worobey M**, Holmes EC. Homologous recombination in GB virus C/hepatitis G virus. *Mol Biol Evol* 2001; **18**: 254-261
17 **Simmonds P**. The origin and evolution of hepatitis viruses in humans. *J Gen Virol* 2001; **82**: 693-712
18 **Pybus OG**, Charleston MA, Gupta S, Rambaut A, Holmes EC, Harvey PH. The epidemic behavior of the hepatitis C virus. *Science* 2001; **292**: 2323-2325
19 **Jeannel D**, Fretz C, Traore Y, Kohdjo N, Bigot A, Pê Gamy E, Jourdan G, Kourouma K, Maertens G, Fumoux F, Fournel JJ, Stuyver L. Evidence for high genetic diversity and long-term endemicity of hepatitis C virus genotypes 1 and 2 in West Africa. *J Med Virol* 1998; **55**: 92-97
20 **Fretz C**, Jeannel D, Stuyver L, Hervé V, Lunel F, Boudifa A, Mathiot C, de Thé G, Fournel JJ. HCV infection in a rural population of the Central African Republic (CAR): evidence for three additional subtypes of genotype 4. *J Med Virol* 1995; **47**: 435-437
21 **Okada S**, Akahane Y, Suzuki H, Okamoto H, Mishiro S. The degree of variability in the amino terminal region of the E2/NS1 protein of hepatitis C virus correlates with responsiveness to interferon therapy in viremic patients. *Hepatology* 1992; **16**: 619-624
22 **Enomoto N**, Kurosaki M, Tanaka Y, Marumo F, Sato C. Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. *J Gen Virol* 1994; **75**(Pt 6): 1361-1369
23 **Kakumu S**, Arao M, Yoshioka K, Hayashi H, Kusakabe A, Hirofuji H, Kawabe M. Recombinant human alpha-interferon therapy for chronic non-A, non-B hepatitis: second report. *Am J Gastroenterol* 1990; **85**: 655-659
24 **Hoofnagle JH**, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, Waggoner JG, Park Y, Jones EA. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N Engl J Med* 1986; **315**: 1575-1578
25 **Knapp S**, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, Wright M, Chiaramonte M, Graves M, Thomas HC, Hill AV, Thursz MR. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1 and PKR. *Genes Immun* 2003; **4**: 411-419
26 **Conrad B**. Potential mechanisms of interferon-alpha induced autoimmunity. *Autoimmunity* 2003; **36**: 519-523
27 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
28 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
29 **Hadziyannis SJ**, Sette H, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
30 **Dalgard O**, Mangia A. Short-term therapy for patients with hepatitis C virus genotype 2 or 3 infection. *Drugs* 2006; **66**: 1807-1815
31 **von Wagner M**, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, Bergk A, Bernsmeier C, Häussinger D, Herrmann E, Zeuzem S. Peginterferon-alpha-2a (40KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. *Gastroenterology* 2005; **129**: 522-527
32 **El-Zayadi AR**, Attia M, Barakat EM, Badran HM, Hamdy H, El-Tawil A, El-Nakeeb A, Selim O, Saied A. Response of hepatitis C genotype-4 naïve patients to 24 weeks of Peg-interferon-alpha2b/ribavirin or induction-dose interferon-alpha2b/ribavirin/amantadine: a non-randomized controlled study. *Am J Gastroenterol* 2005; **100**: 2447-2452
33 **Kamal SM**, El Tawil AA, Nakano T, He Q, Rasenack J, Hakam SA, Saleh WA, Ismail A, Aziz AA, Madwar MA. Peginterferon {alpha}-2b and ribavirin therapy in chronic hepatitis C genotype 4: impact of treatment duration and viral kinetics on sustained virological response. *Gut* 2005; **54**: 858-866
34 **Nguyen MH**, Keeffe EB. Prevalence and treatment of hepatitis C virus genotypes 4, 5, and 6. *Clin Gastroenterol Hepatol* 2005; **3**: S97-S101
35 **Taylor DR**, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 1999; **285**: 107-110
36 **Abid K**, Quadri R, Negro F. Hepatitis C virus, the E2 envelope protein, and alpha-interferon resistance. *Science* 2000; **287**: 1555
37 **Saito T**, Ito T, Ishiko H, Yonaha M, Morikawa K, Miyokawa A, Mitamura K. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon therapy in Japanese patients infected with HCV genotypes 2a and 2b. *Am J Gastroenterol* 2003; **98**: 1377-1383
38 **Watanabe H**, Nagayama K, Enomoto N, Itakura J, Tanabe Y, Sato C, Izumi N, Watanabe M. Amino acid substitutions in PKR-eIF2 phosphorylation homology domain (PePHD) of hepatitis C virus E2 protein in genotype 2a/2b and 1b in Japan and interferon efficacy. *Hepatol Res* 2003; **26**: 268-274
39 **Gaudy C**, Lambelé M, Moreau A, Veillon P, Lunel F, Goudeau A. Mutations within the hepatitis C virus genotype 1b E2-

PePHD domain do not correlate with treatment outcome. *J Clin Microbiol* 2005; **43**: 750-754

40 **Polyak SJ**, Nousbaum JB, Larson AM, Cotler S, Carithers RL, Gretch DR. The protein kinase-interacting domain in the hepatitis C virus envelope glycoprotein-2 gene is highly conserved in genotype 1-infected patients treated with interferon. *J Infect Dis* 2000; **182**: 397-404

41 **Moribe T**, Hayashi N, Kanazawa Y, Mita E, Fusamoto H, Negi M, Kaneshige T, Igimi H, Kamada T, Uchida K. Hepatitis C viral complexity detected by single-strand conformation polymorphism and response to interferon therapy. *Gastroenterology* 1995; **108**: 789-795

42 **Toyoda H**, Kumada T, Nakano S, Takeda I, Sugiyama K, Osada T, Kiriyama S, Sone Y, Kinoshita M, Hadama T. Quasispecies nature of hepatitis C virus and response to alpha interferon: significance as a predictor of direct response to interferon. *J Hepatol* 1997; **26**: 6-13

43 **López-Labrador FX**, Ampurdanès S, Giménez-Barcons M, Guilera M, Costa J, Jiménez de Anta MT, Sánchez-Tapias JM, Rodés J, Sáiz JC. Relationship of the genomic complexity of hepatitis C virus with liver disease severity and response to interferon in patients with chronic HCV genotype 1b infection [correction of interferon]. *Hepatology* 1999; **29**: 897-903

44 **Macdonald A**, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. *J Gen Virol* 2004; **85**: 2485-2502

45 **Gale MJ**, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR, Katze MG. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 1997; **230**: 217-227

46 **Polyak SJ**, Khabar KS, Paschal DM, Ezelle HJ, Duverlie G, Barber GN, Levy DE, Mukaida N, Gretch DR. Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. *J Virol* 2001; **75**: 6095-6106

47 **Polyak SJ**, Khabar KS, Rezeiq M, Gretch DR. Elevated levels of interleukin-8 in serum are associated with hepatitis C virus infection and resistance to interferon therapy. *J Virol* 2001; **75**: 6209-6211

48 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Izumi N, Marumo F, Sato C. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. *J Clin Invest* 1995; **96**: 224-230

49 **Enomoto N**, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996; **334**: 77-81

50 **Pascu M**, Martus P, Höhne M, Wiedenmann B, Hopf U, Schreier E, Berg T. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. *Gut* 2004; **53**: 1345-1351

51 **Murakami T**, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. *Hepatology* 1999; **30**: 1045-1053

52 **Bagaglio S**, Bruno R, Lodrini S, De Mitri MS, Andreone P, Loggi E, Galli L, Lazzarin A, Morsica G. Genetic heterogeneity of hepatitis C virus (HCV) in clinical strains of HIV positive and HIV negative patients chronically infected with HCV genotype 3a. *J Biol Regul Homeost Agents* 2003; **17**: 153-161

53 **Castelain S**, Khorsi H, Roussel J, François C, Jaillon O, Capron D, Penin F, Wychowski C, Meurs E, Duverlie G. Variability of the nonstructural 5A protein of hepatitis C virus type 3a isolates and relation to interferon sensitivity. *J Infect Dis* 2002; **185**: 573-583

54 **Inchauspé G**, Zebedee S, Lee DH, Sugitani M, Nasoff M, Prince AM. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. *Proc Natl Acad Sci USA* 1991; **88**: 10292-10296

55 **Duverlie G**, Khorsi H, Castelain S, Jaillon O, Izopet J, Lunel F, Eb F, Penin F, Wychowski C. Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. *J Gen Virol* 1998; **79**(Pt 6): 1373-1381

56 **Veillon P**, Payan C, Le Guillou-Guillemette H, Gaudy C, Lunel F. Quasispecies evolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon-ribavirin therapy. *World J Gastroenterol* 2007;**13**:1195-1203

57 **Puig-Basagoiti F**, Forns X, Furcić I, Ampurdanés S, Giménez-Barcons M, Franco S, Sánchez-Tapias JM, Saiz JC. Dynamics of hepatitis C virus NS5A quasispecies during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. *J Gen Virol* 2005; **86**: 1067-1075

58 **Houghton M**. Hepatitis C virus. In: Howley DMKPM. Fields Virology. Philadelphia: Lippincott-Raven, 1996: 1035-1058

59 **Farci P**, Purcell RH. Clinical significance of hepatitis C virus genotypes and quasispecies. *Semin Liver Dis* 2000; **20**: 103-126

60 **Bartenschlager R**. The NS3/4A proteinase of the hepatitis C virus: unravelling structure and function of an unusual enzyme and a prime target for antiviral therapy. *J Viral Hepat* 1999; **6**: 165-181

61 **Vallet S**, Gouriou S, Nousbaum JB, Legrand-Quillien MC, Goudeau A, Picard B. Genetic heterogeneity of the NS3 protease gene in hepatitis C virus genotype 1 from untreated infected patients. *J Med Virol* 2005; **75**: 528-537

62 **Vallet S**, Gouriou S, Nkontchou G, Hotta H, Vilerio M, Legrand-Quillien MC, Beaugrand M, Trinchet JC, Nousbaum JB, Dény P, Gaudy C, Goudeau A, Picard B, Payan C. Is hepatitis C virus NS3 protease quasispecies heterogeneity predictive of progression from cirrhosis to hepatocellular carcinoma? *J Viral Hepat* 2007; **14**: 96-106

63 **Söderholm J**, Ahlén G, Kaul A, Frelin L, Alheim M, Barnfield C, Liljeström P, Weiland O, Milich DR, Bartenschlager R, Sällberg M. Relation between viral fitness and immune escape within the hepatitis C virus protease. *Gut* 2006; **55**: 266-274

64 **Gale M**. Effector genes of interferon action against hepatitis C virus. *Hepatology* 2003; **37**: 975-978

65 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172

66 **Kieffer T**, Sarrazin C, Bartels D, Hanzelka B, Müh U, Welker M, Wincheringer D, Lin C, Purdy S, Weegink C, Reesink H, Kwong A, Zeuzem S. Wild-type HCV NS3 protease re-emerges during follow-up after 14 days of dosing with VX-950 in patients with genotype 1 HCV. In: Colombo M. Journal of Hepatology. EASL 2006: Proceedings of the 41th Annual Meeting of the European Association for the Study of the Liver; 2006 Apr 26-30; Vienna, Austria. Shannon, Ireland: Elsevier, 2006: **44**: S7

67 **Zeuzem S**, Sarrazin C, Rouzier R, Tarral A, Brion N, Forestier N, Gupta S, Deckman D, Fellows K, Hussain M, Cutler D, Zhang J. Anti-viral activity of SCH503034, a HCV protease inhibitor, administrated as monotherapy in hepatitis C genotype-1 (HCV-1) patients refractory to pegylated interferon (peg-IFN-α). In: Blei AT. Hepatology. AASLD 2005: Proceedings of the 56th Annual Meeting of the American Association for the Study of Liver Diseases.; 11-15 November 2005; San Francisco, USA. Hoboken: John Wiley & Sons, 2005: **42**: 233A

68 **Clavel F**, Hance AJ. HIV drug resistance. *N Engl J Med* 2004; **350**: 1023-1035

69 **Lin C**, Lin K, Luong YP, Rao BG, Wei YY, Brennan DL, Fulghum JR, Hsiao HM, Ma S, Maxwell JP, Cottrell KM, Perni RB, Gates CA, Kwong AD. In vitro resistance studies of hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061: structural analysis indicates different resistance mechanisms. *J Biol Chem* 2004; **279**: 17508-17514

70 **Sarrazin C**, Kieffer T, Bartels D, Hanzelka B, Müh U, Welker M, Wincheringer D, Lin C, Grossman T, Purdy S, Weegink C, Reesink H, Zeuzem S, Kwong A. Characterization of viral variants in the HCV NS3 protease domain of genotype 1 patients that are selected during 14 days of dosing with VX-950. In: Blei AT. Hepatology. AASLD 2005: Proceedings of the 56th Annual Meeting of the American Association for the Study of

Liver Diseases; 2005 Nov 11-15; San Francisco, USA. Hoboken: John Wiley & Sons, 2005: **42**: 751A

71 **Franco S**, Clotet B, Martinez M. High genotypic and phenotypic resolution of HCV NS3/4 protease quasispecies: mutations associated with drug resistance in treatment-naive patients. In: Lange J RD. Antiviral Therapy. HIV Drug Resistance Worshop 2006: Proceedings of the 15th International HIV Drug resistance Workshop; 2006 Jun 13-17; Sitges, Spain. London: International Medical Press, 2006: **11**: S14

72 **Winters MA**, Welles SL, Holodniy M. Hepatitis C virus protease gene diversity in patients coinfected with human immunodeficiency virus. *J Virol* 2006; **80**: 4196-4199

73 **Tong X**, Chase R, Skelton A, Chen T, Wright-Minogue J, Malcolm BA. Identification and analysis of fitness of resistance mutations against the HCV protease inhibitor SCH 503034. *Antiviral Res* 2006; **70**: 28-38

74 **Reesink H**, Zeuzem S, Weegink C, Forestier N, van Vliet A, van de Wetering de Rooij J, McNair L, Purdy S, Chu H, Jansen P. Final results of a phase 1b, multiple-dose study of VX-950, a hepatitis C virus protease inhibitor. In: Blei AT. Hepatology. AASLD 2005: Proceedings of the 56th Annual Meeting of the American association for the Study of Liver Diseases; 2005 Nov 11-15; San Francisco, USA. Hoboken: John Wiley & Sons, 2005: **42**: 234A

75 **Yi M**, Tong X, Skelton A, Chase R, Chen T, Prongay A, Bogen SL, Saksena AK, Njoroge FG, Veselenak RL, Pyles RB, Bourne N, Malcolm BA, Lemon SM. Mutations conferring resistance to SCH6, a novel hepatitis C virus NS3/4A protease inhibitor. Reduced RNA replication fitness and partial rescue by second-site mutations. *J Biol Chem* 2006; **281**: 8205-8215

76 **Lesburg CA**, Cable MB, Ferrari E, Hong Z, Mannarino AF, Weber PC. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. *Nat Struct Biol* 1999; **6**: 937-943

77 **Bressanelli S**, Tomei L, Roussel A, Incitti I, Vitale RL, Mathieu M, De Francesco R, Rey FA. Crystal structure of the RNA-dependent RNA polymerase of hepatitis C virus. *Proc Natl Acad Sci USA* 1999; **96**: 13034-13039

78 **Lohmann V**, Körner F, Herian U, Bartenschlager R. Biochemical properties of hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. *J Virol* 1997; **71**: 8416-8428

79 **Lohmann V**, Roos A, Körner F, Koch JO, Bartenschlager R. Biochemical and kinetic analyses of NS5B RNA-dependent RNA polymerase of the hepatitis C virus. *Virology* 1998; **249**: 108-118

80 **Cheney IW**, Naim S, Lai VC, Dempsey S, Bellows D, Walker MP, Shim JH, Horscroft N, Hong Z, Zhong W. Mutations in NS5B polymerase of hepatitis C virus: impacts on in vitro enzymatic activity and viral RNA replication in the subgenomic replicon cell culture. *Virology* 2002; **297**: 298-306

81 **Labonté P**, Axelrod V, Agarwal A, Aulabaugh A, Amin A, Mak P. Modulation of hepatitis C virus RNA-dependent RNA polymerase activity by structure-based site-directed mutagenesis. *J Biol Chem* 2002; **277**: 38838-38846

82 **Vuillermoz I**, Khattab E, Sablon E, Ottevaere I, Durantel D, Vieux C, Trepo C, Zoulim F. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon-ribavirin therapy. *J Med Virol* 2004; **74**: 41-53

83 **Kumagai N**, Takahashi N, Kinoshita M, Tsunematsu S, Tsuchimoto K, Saito H, Ishii H. Polymorphisms of NS5B protein relates to early clearance of hepatitis C virus by interferon plus ribavirin: a pilot study. *J Viral Hepat* 2004; **11**: 225-235

84 **Carroll SS**, Tomassini JE, Bosserman M, Getty K, Stahlhut MW, Eldrup AB, Bhat B, Hall D, Simcoe AL, LaFemina R, Rutkowski CA, Wolanski B, Yang Z, Migliaccio G, De Francesco R, Kuo LC, MacCoss M, Olsen DB. Inhibition of hepatitis C virus RNA replication by 2'-modified nucleoside analogs. *J Biol Chem* 2003; **278**: 11979-11984

85 **Carroll SS**, Olsen DB. Nucleoside analog inhibitors of hepatitis C virus replication. *Infect Disord Drug Targets* 2006; **6**: 17-29

86 **Migliaccio G**, Tomassini JE, Carroll SS, Tomei L, Altamura S, Bhat B, Bartholomew L, Bosserman MR, Ceccacci A, Colwell LF, Cortese R, De Francesco R, Eldrup AB, Getty KL, Hou XS, LaFemina RL, Ludmerer SW, MacCoss M, McMasters DR, Stahlhut MW, Olsen DB, Hazuda DJ, Flores OA. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication *in vitro*. *J Biol Chem* 2003; **278**: 49164-49170

87 **Ludmerer SW**, Graham DJ, Boots E, Murray EM, Simcoe A, Markel EJ, Grobler JA, Flores OA, Olsen DB, Hazuda DJ, LaFemina RL. Replication fitness and NS5B drug sensitivity of diverse hepatitis C virus isolates characterized by using a transient replication assay. *Antimicrob Agents Chemother* 2005; **49**: 2059-2069

88 **Klumpp K**, Lévêque V, Le Pogam S, Ma H, Jiang WR, Kang H, Granycome C, Singer M, Laxton C, Hang JQ, Sarma K, Smith DB, Heindl D, Hobbs CJ, Merrett JH, Symons J, Cammack N, Martin JA, Devos R, Nájera I. The novel nucleoside analog R1479 (4'-azidocytidine) is a potent inhibitor of NS5B-dependent RNA synthesis and hepatitis C virus replication in cell culture. *J Biol Chem* 2006; **281**: 3793-3799

89 **Le Pogam S**, Jiang WR, Leveque V, Rajyaguru S, Ma H, Kang H, Jiang S, Singer M, Ali S, Klumpp K, Smith D, Symons J, Cammack N, Nájera I. In vitro selected Con1 subgenomic replicons resistant to 2′-C-methyl-cytidine or to R1479 show lack of cross resistance. *Virology* 2006; **351**: 349-359

90 **Roberts S**, Cooksley G, Shaw D, Berns H, Brandl M, Fettner S, Hill G, Ipe D, Klumpp K, Mannino M, O'Mara E, Tu Y, Washington C. Interim results of a multiple ascending dose study of R1626, a novel nucleoside analog targeting HCV polymerase in chronic HCV patients. In: Colombo M. Journal of Hepatology. EASL 2006: Proceedings of the 41th European Association for the Study of the Liver; 2006 Apr 26-30; Vienna, Austria. Shannon: Elsevier, 2006: **44**: S269

91 **Chan L**, Pereira O, Reddy TJ, Das SK, Poisson C, Courchesne M, Proulx M, Siddiqui A, Yannopoulos CG, Nguyen-Ba N, Roy C, Nasturica D, Moinet C, Bethell R, Hamel M, L'Heureux L, David M, Nicolas O, Courtemanche-Asselin P, Brunette S, Bilimoria D, Bédard J. Discovery of thiophene-2-carboxylic acids as potent inhibitors of HCV NS5B polymerase and HCV subgenomic RNA replication. Part 2: tertiary amides. *Bioorg Med Chem Lett* 2004; **14**: 797-800

92 **Mo H**, Lu L, Pilot-Matias T, Pithawalla R, Mondal R, Masse S, Dekhtyar T, Ng T, Koev G, Stoll V, Stewart KD, Pratt J, Donner P, Rockway T, Maring C, Molla A. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor *in vitro*. *Antimicrob Agents Chemother* 2005; **49**: 4305-4314

93 **Howe AY**, Bloom J, Baldick CJ, Benetatos CA, Cheng H, Christensen JS, Chunduru SK, Coburn GA, Feld B, Gopalsamy A, Gorczyca WP, Herrmann S, Johann S, Jiang X, Kimberland ML, Krisnamurthy G, Olson M, Orlowski M, Swanberg S, Thompson I, Thorn M, Del Vecchio A, Young DC, van Zeijl M, Ellingboe JW, Upeslacis J, Collett M, Mansour TS, O'Connell JF. Novel nonnucleoside inhibitor of hepatitis C virus RNA-dependent RNA polymerase. *Antimicrob Agents Chemother* 2004; **48**: 4813-4821

94 **Kukolj G**, McGibbon GA, McKercher G, Marquis M, Lefèbvre S, Thauvette L, Gauthier J, Goulet S, Poupart MA, Beaulieu PL. Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase. *J Biol Chem* 2005; **280**: 39260-39267

95 **Tomei L**, Altamura S, Paonessa G, De Francesco R, Migliaccio G. HCV antiviral resistance: the impact of in vitro studies on the development of antiviral agents targeting the viral NS5B polymerase. *Antivir Chem Chemother* 2005; **16**: 225-245

96 **Graham DJ**, Stahlhut M, Flores O, Olsen DB, Hazuda DJ, Lafemina RL, Ludmerer SW. A genotype 2b NS5B polymerase with novel substitutions supports replication of a chimeric HCV 1b:2b replicon containing a genotype 1b NS3-5A background. *Antiviral Res* 2006; **69**: 24-30

97 **De Francesco R**, Migliaccio G. Challenges and successes in developing new therapies for hepatitis C. *Nature* 2005; **436**: 953-960

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

New animal models for hepatitis C viral infection and pathogenesis studies

Dina Kremsdorf, Nicolas Brezillon

Dina Kremsdorf, Nicolas Brezillon, INSERM U812, Paris, France; Pasteur Institute, Paris, France; Université Paris Descartes, Faculté de Médecine René Descartes, CHU Necker, Paris 75015, France
Correspondence to: Dina Kremsdorf, INSERM U812, Université Paris Descartes, Faculté de Médecine René Descartes, CHU Necker, 156, rue de Vaugirard, Paris 75015, France. kremsdor@necker.fr
Telephone: +33-1-40615343 Fax: +33-1-40615581
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Abstract

Hepatitis C virus (HCV) is a major cause of chronic liver disease, cirrhosis and hepatocellular carcinoma (HCC). In man, the pathobiological changes associated with HCV infection have been attributed to both the immune system and direct viral cytopathic effects. Until now, the lack of simple culture systems to infect and propagate the virus has hampered progress in understanding the viral life cycle and pathogenesis of HCV infection, including the molecular mechanisms implicated in HCV-induced HCC. This clearly demonstrates the need to develop small animal models for the study of HCV-associated pathogenesis. This review describes and discusses the development of new HCV animal models to study viral infection and investigate the direct effects of viral protein expression on liver disease.

Key words: Hepatitis C virus; Viral infection; Transgenic mice; Pathogenesis

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INTRODUCTION

Despite the fact that infectious diseases account for at least one-third of all deaths worldwide, our capacity to study both their pathophysiology and host immune responses in man is often limited by the lack of simple laboratory models of infection. During the past decade, laboratory animals have been used as models of human diseases. In particular, transgenic mice models have been helpful to the understanding of the molecular basis of human diseases. The story of hepatitis C virus (HCV) started in the 1970s with the emergence of patients suffering from hepatitis syndrome which was not associated to hepatitis A or B infection (HAV or HBV). This new agent was named NANBH, for non-A non-B hepatitis[1]. In 1978, Alter *et al*[2] demonstrated that the inoculation of NANBH human sera in chimpanzees induced liver disease, and in 1989 Choo *et al*[3] identified a third viral agent for hepatitis by cloning a non-simian cDNA from the serum of a NANBH-infected chimpanzee. HCV infection leads to liver cirrhosis in up to 35% of cases and hepatocellular carcinoma develops in 2%-7% of cirrhotic patients per year. It is now accepted that the pathobiological changes induced by HCV infection are due to both the immune response and the direct viral cytopathic effects of the virus. As described by other authors in this issue, there is no prophylactic vaccine against HCV at present, and the therapeutic options are mainly limited by a lack of effective long-term treatment. Like other human hepatitis viruses, HCV needs fully functional human hepatocytes for its development. The discovery of anti-infectious agents and immune defense mechanisms has to date been severely restricted by the ethical and practical constraints of access to receptive cells. Due to the nearly strict human tropism of HCV, only man and higher primates such as chimpanzees have until recently been receptive to HCV infection and development. The purpose of this review is to focus on new HCV animal models independent of the chimpanzee, which will enable the study of viral infection and the direct effects of viral protein expression on liver disease.

NEW ANIMAL MODELS FOR HCV INFECTION

As summarized in Table 1, except for the chimpanzee, the only non-human primate permissive to HCV infection is the marmoset. Tupaia, a member of the Tree shrew genus is equally infected by HCV. The capacity for GBV-B, a hepatotrope virus of the flaviviridae family, to infect tamarins and marmosets has been used as a tool to better characterize HCV virus replication and to test HCV antiviral drugs[4-6]. However, none of these models is

Table 1 HCV infection trials in animals

Virus	Animal tested		Infection	Original reference
HCV	Non human primates	Chimpanzee	Yes	Alter *et al*[2] 1978
		Marmoset	Yes	Feinstone *et al*[71] 1981
		Cottontop tamarin	No	Garson *et al*[72] 1997
		Cynomoglus monkey	No	
		Rhesus monkey	No	
		Green monkey	No	
		Japanese monkey	No	Abe *et al*[73] 1993
		Doguera baboon	No	
		Chacma baboon	No	Sithebe *et al*[74] 2002
	Scandentia	Tupaia	Yes	Xie *et al*[75] 1998
	Rodents	uPA/SCID mice	Yes	Mercer *et al*[26] 2001
		Trimera mice	Yes	Galun *et al*[10] 1995
		Rat	Yes	Wu *et al*[8] 2005
		Woodchuck	No	Abe *et al*[73] 1993

Table 2 Characteristics of HCV infection in three rodent models

Rodent models	Humanization	Viremia	Duration of viral infection	Comments	Publications
uPA/SCID mice	Human hepatocyte transplantation	1×10^4 to 8×10^7 copies/mL	Up to 9 mo with maximum viremia as from one month	High viremia Variability of primary human hepatocytes Immunosuppressed mice	Mercer *et al*[26] 2001 Meuleman *et al*[22] 2005 Kneteman *et al*[29] 2006
Trimera mice	Xenograft of human liver tissue	Around 7×10^4 copies/mL	Around one month with peak viremia at 18 d	Low viremia Variability of human liver tissues Immunosuppressed mice	Ilan *et al*[12] 2002 Eren *et al*[11] 2006
Rat	Immunotolerization and transplantation of a human hepatoma cell line	1-2 × 10^4 copies/mL	Minimum 4 mo with peak viremia at 3 mo	Low viremia Transplantation of a hepatoma cell line Immunocompetent rat	Wu *et al*[8] 2005

perfect, particularly because of their inability to produce numerous animals in a short time (long gestation periods) and their high breeding costs. Rodents are certainly the most appropriate model for all biological studies. Their short gestation period (around 20 d for mice and rats), their small size and their low cost are particularly advantageous. Three interesting models for HCV infection: the immunotolerized rat model, the Trimera mouse model and the uPA/SCID model, are being developed (Table 2, Figure 1).

The rat model

Interestingly, Wu *et al* took account of the fact that the rat immune system does not develop until 15-17 d of gestation. They immunotolerized rat embryos to allow the transplantation and maintenance of a human hepatoma cell line (Huh7)[7] which could be infected with HCV[8] (Table 2, Figure 1C). Briefly, fetal rats are tolerized by an intraperitoneal injection of Huh7 cells into pregnant females at the 17th d of gestation. Twenty-four hours after birth, the rats are intrasplenically transplanted with the same hepatoma cell line which will represent around 6% of total hepatocytes after 14 d of development. Nearly 30% of the transplanted human hepatocytes are positive for HCV core protein after inoculation with HCV (genotype 1) positive human serum (Figure 1C). This new animal model is promising but validation remains necessary, for example by using this model to confirm the antiviral effects of drugs used for anti-HCV therapy in humans.

The Trimera mouse model

The Trimera mouse model involves the development of a chimeric mouse with a different source of tissue[9,10]. BNX (beige/nude/X-linked immunodeficient) mice are preconditioned by total body irradiation and reconstituted with SCID mouse bone marrow. These mice tolerate the transplantation of HCV-infected liver fragments from patients with HCV RNA-positive sera, as well as the transplantation of an *ex vivo* HCV-infected liver fragment[10-12]. The liver fragment is transplanted into the ear pinna or under the kidney capsule. In this way, the transplant can be maintained for several weeks and HCV messengers were detected in the serum for up to one month (Table 2, Figure 1B)[10-12]. The model has been validated as a tool for the testing of antiviral components. During a first set of experiments, an inhibitor of the internal ribosomal entry site and an anti-HCV monoclonal antibody were demonstrated to act as potential HCV

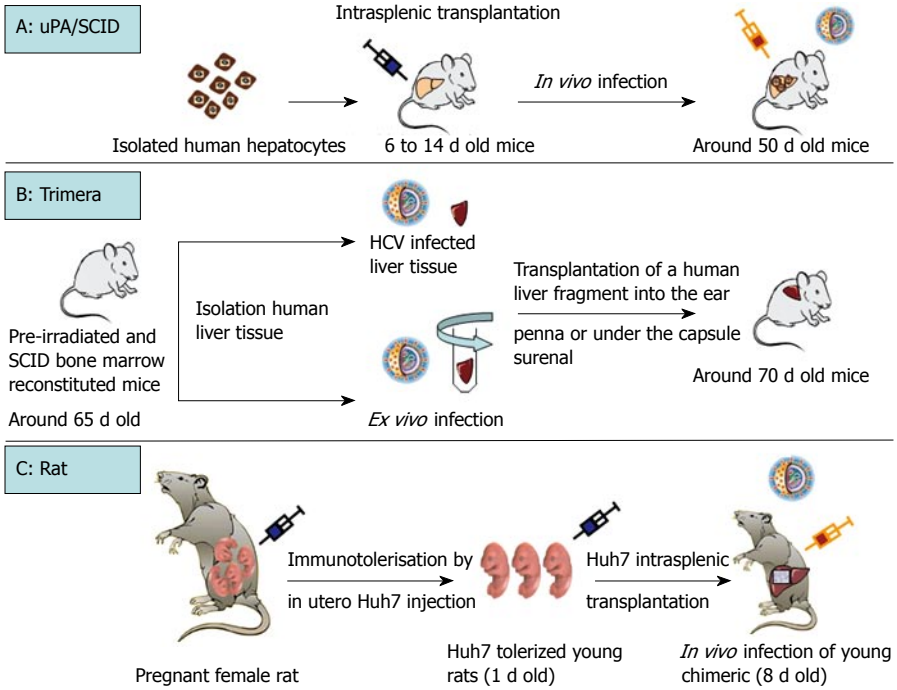

Figure 1 Transplantation protocols of three rodents model of HCV infection. **A**: uPA/SCID model: isolation of human hepatocyte, intrasplenic cell transplantation into homozygous mice and *in vivo* infection by a HCV positive human serum; **B**: Trimera model: irradiation and bone marrow reconstitution of mice, transplantation of intrinsically HCV infected human liver fragment or transplantation of an *ex vivo* pre-infected human liver fragment; **C**: Rat model: immunotolerisation of foetal rats by in utero injection of Huh7 cells, transplantation in new born rats with the same hepatoma cells and *in vivo* infection of young chimeric rats by a HCV positive human serum.

inhibitors[12]. Recently, the same team produced and characterized two monoclonal antibodies directed against HCV envelope protein E2. Following *in vitro* validation of the ability of these antibodies to immunoprecipitate HCV particles, they confirmed an inhibitory effect on HCV infection[11]. Indeed, in the HCV-Trimera model, both monoclonal E2 antibodies were shown to be capable of inhibiting the *ex vivo* HCV infection of human liver fragments (reduction of resultant viremia from 3×10^4 to 3×10^3 copies/mL). A reduction in viremia (3.1×10^4 to 5×10^3 copies/mL) was also demonstrated when these antibodies were used to treat HCV-trimera mice[11]. In conclusion, this mouse model appears to be well-suited to evaluating the inhibitory capacity of drugs, as had previously been shown with monoclonal antibodies directed against HBV[13,14]. Nevertheless, we should not forget that this approach involves the use of heterotopic and xenogenic grafts, so that we can never be entirely sure of the physiological relevance of observations.

The urokinase plasminogen activator protein (uPA) immunodeficient mouse model

Historically, urokinase plasminogen uPA transgenic mice were described in 1990 by Heckel *et al*[15]. The same team then demonstrated the ability of a small number of "normal" hepatocytes to repopulate ad-integrum the liver of transgenic uPA mice[16]. Indeed, the overexpression of uPA protein in hepatocytes is cytotoxic, giving rise to a continuous liver regeneration process. Under these conditions, hepatocytes which lose the transgene by somatic reversion, as well as healthy transplanted hepatocytes, have a strong survival advantage over resident cells[16,17]. Based on this advantage, uPA transgenic mice were back-crossed on an immunodeficient background (SCID or Rag2 mice) to obtain a mouse model which tolerated the xenotransplantation of human, woodchuck and tupaia hepatocytes[17-22]. Optimum liver repopulation requires the intrasplenic transplantation, within one or two weeks of birth, of high quality hepatocytes into mice which are homozygous for both the SCID trait and uPA transgene (Table 2, Figure 1A). The morphological and biochemical characterization of chimeric mice revealed satisfactory hepatic architecture and fusion of the mouse and human structures, indicating a physiological integration of transplanted cells[20,22]. The functionality of transplanted hepatocytes was attested by their susceptibility to infection with human hepatotropic pathogens such as *Plasmodium falciparum*[23], hepatitis B virus[22,24,25] and hepatitis C virus[22,26-28]. It was subsequently shown that this HCV-infected humanized mouse could be used to demonstrate the antiviral activity of two compounds which had already been shown to be effective during clinical trials: the administration in mice of IFNα2b and an anti protease agent (BILN-2061) significantly reduced HCV viremia[29]. Furthermore, it was shown that the antiviral effect was dependent on the viral genotype but appeared to be independent of the provenance of human hepatocytes. Interestingly, another team confirmed the antiviral effect of BILN-2061, but they also encountered cardiotoxic adverse effects with this compound (unpublished data, ISVHLD 2006 congress, Vanwolleghem *et al*). The uPA/SCID model is therefore appropriate for testing new antiviral molecules by evaluating their efficacy and toxicity.

It is clear that the immune response to viral infection plays a major role in the outcome of liver disease. By taking advantage of the absence of adaptive immune response in the chimeric uPA/SCID mouse model, Walters *et al*[30] were able to investigate the role of the innate antiviral immune response to HCV infection. The purpose of their study was to distinguish virus-induced gene expression changes from adaptive HCV-specific immune-mediated effects. Globally, in the uPA/SCID mouse model, HCV infection activates the transcription of interferon-stimulated genes which are in particular implicated in establishing the

innate immune response, and thus active in the inhibition of HCV replication. As previously shown in HCV-infected patients and HCV transgenic mice, these authors confirmed in the uPA/SCID mouse model the relationship between a severe HCV infection and lipid metabolism perturbation, suggesting that liver disease may not be mediated exclusively by an HCV-specific adaptive immune response. Thus, the innate immune response may play a fundamental role in limiting the viral HCV RNA copy number and can thus slow the progression of infection.

In summary, the recent development of small animal models for experimental HCV infection has opened new perspectives for the evaluation of novel therapeutic and/or prophylactic compounds against HCV. Indeed, these three rodent models are really promising, although relatively complicated to use, but they present the unquestionable advantage of being much less expensive and easier to maintain and breed than primates. The rat model may be the most accessible, notably because of the immunocompetent nature of the animals and the larger number of reproducible infected animals that could be obtained in theory using hepatocyte cell line transplantation. The two mice models are more physiologically relevant, in that they are based on the transplantation of human tissue or primary hepatocytes. The model most closely related physiologically to humans is certainly the uPA/SCID mouse. Indeed, even if this model is developed in an immunotolerant setting, humanized liver may contain around 75% of human hepatocytes as compared to just 6% of hepatoma cells in the rat (Table 2). Furthermore, viremia clearly lasts longer and at higher levels in uPA/SCID mice than in other models (Table 2). Under these conditions, the uPA/SCID mouse model appears to be the most relevant to building a bridge between *in vitro* research and clinical trials.

HCV TRANSGENIC MOUSE MODELS

An increasing body of evidence suggests a direct involvement of HCV in cellular metabolic disturbances. As described in this report, conditional expression of the HCV genome in transgenic mice has enabled study of the direct effect of HCV on hepatocytes, and investigation of the molecular pathways of HCV associated with liver injury. This has generated data crucial to our understanding of several aspects of HCV pathogenesis. As reported in Table 3, and despite some contradictory results, the development of HCV transgenic mouse models has enabled evaluation of the different, direct cytopathic effects of HCV protein and their correlation with the pathogenesis of chronic hepatitis C.

Hepatic steatosis and the derangement of lipid metabolism are characteristic of HCV protein expression

Recently, a correlation between HCV infection and both diabetes and insulin resistance was suggested, indicating that they might be metabolic diseases associated with viral infection[31,32]; this could be a critical factor in the pathogenesis of chronic hepatitis C. Transgenic mouse models have demonstrated a link between HCV core protein expression and elevated serum insulin levels, associated with a minor elevation of plasmatic glucose but without the development of diabetes[33,34]. Furthermore, the administration of insulin resulted in higher glycemia when compared to values in non-transgenic mice, indicating the presence of an insulin resistance phenotype in core transgenic mice[33,34]. This insulin resistance may be due to down-regulation in the expression of insulin receptor substrates 1 and 2 (IRS1 and 2), probably *via* an up-regulation of the suppressor of cytokine signaling 3 (SOCS3)[35]. One possible mechanism is that HCV core-induced SOCS3 promotes the proteosomal degradation of IRS1 and IRS2 through ubiquitination[35]. These results therefore provide direct experimental evidence for a role of HCV core protein in the development of insulin resistance mechanisms in HCV-infected patients.

Hepatic steatosis, which involves an accumulation of intracytoplasmic lipid droplets, is a common histological feature which is observed in more than 50% of chronic hepatitis C carriers[36]. Both host and viral factors have been demonstrated to play an important role in its development. By accelerating the development of fibrosis, steatosis may contribute to the progression of liver disease and the development of HCC. Consistent with the implication of viral protein in steatosis, it has been reported that the core protein of HCV targets microsomal triglyceride transfer protein activity, modifies hepatic VLDL assembly and secretion and increases the concentration of monosaturated fatty acids[37,38]. Furthermore, alcohol consumption in core transgenic mice has been shown to increase hepatic lipid peroxidation and hepatic TNF alpha and TGF beta expression[39]. This may participate in activating fibrogenesis and hence the development of HCC observed in HCV patients who abuse alcohol.

HCV and development of hepatocellular carcinoma

Epidemiological evidence favors a direct role for HCV in the development of hepatocellular carcinoma (HCC). Despite some contradictory results, transgenic mouse models have demonstrated the implication of core protein expression in HCC development. Moriya *et al*[40-42] showed that core protein expression in mice led to steatosis, oxidative stress and ultimately HCC in aging mice. RXR alpha is activated by cellular retinol binding protein II (CRBPII) in the liver of core-expressing transgenic mice[43]; suggesting that the modulation of RXR alpha-controlled gene expression *via* its interaction with core protein could contribute to liver pathogenesis. Alterations to other signaling pathways *via* activation of signal transducer and activator of transcription 3 (STAT3), activator protein-1 (AP-1), MAPK and the suppression of SOCS-1 expression may equally contribute to tumorigenesis in HCV core-expressing transgenic mice[44-47]. In another core transgenic line, the development of malignant lymphoma and hepatocellular adenoma has been observed[48]. In other reports, neither steatosis nor hepatic tumors were detected in core transgenic mice[49,50]. However, HCC was more frequently observed after diethylnitrosamine treatment or the repeated administration of carbon tetrachloride[49,51].

Table 3 Transgenic mice expressing HCV proteins

Viral protein	Original reference of the transgenic mouse	Mouse strain	Promoter	Comments	References
Core	Moriya *et al*[42] 1997	C57BL/6	HBV	Steatosis	Moriya *et al*[40] 1998
				HCC	Moriya *et al*[42] 1997
				Oxidative stress	Moriya *et al*[41] 2001
				Increased concentration of monounsaturated fatty acids	Moriya *et al*[37] 2001
				Inhibition of microsomal triglyceride transfer protein activity and VLDL secretion.	Perlemuter *et al*[38] 2002
				Alcohol and core protein increase lipid peroxidation	Perlemuter *et al*[39] 2003
				Alteration of intrahepatic cytokine expression and AP-1 activation	Tsutsumi *et al*[45] 2002
				Interaction with retinoid X receptor alpha	Tsutsumi *et al*[43] 2002
				Cooperation with ethanol activation of MAPK	Tsutsumi *et al*[44] 2003
				Insulin resistance	Shintani *et al*[34] 2004
					Koike *et al*[33] 2006
				Modulation of interferon pathway (Inhibition of SOCS-1 expression)	Miyoshi *et al*[47] 2005
				ER stress, apoptosis	Benali-Furet *et al*[78] 2005
Core	Honda *et al*[52] 2000	C57BL/6	HBV	Modulated sensitivity to Fas-mediated apoptosis	Honda *et al*[52] 2000
				Insulin resistance via down-regulation hepatic IRS1 and 2	Kawaguchi *et al*[35] 2004
				Constitutive activation of STAT3, implication in HCC	Yoshida *et al*[46] 2002
Core	Pasquinelli *et al*[50] 1997	C57BL/6	Major urinary protein	No liver disease	Pasquinelli *et al*[50] 1997
Core (Korean wt and mutants)	Wang *et al*[79] 2004	C57BL/6J	HBV	Cell dysplasia for S99Q core mutant	Wang *et al*[79] 2004
Core (in T cell)	Soguero *et al*[53] 2002	C57BL/6	CD2	Increased Fas-mediated apoptosis and liver infiltration of peripheral T cells	Soguero *et al*[53] 2002
Double Tg core X TCR	Cruise *et al*[55] 2005	C57BL/6 × DO11.10 (H-2d)		Increased Fas ligand expression of CD4+ T cells associated with liver inflammation	Cruise *et al*[55] 2005
				Role of CXCR3 ligands via Fas induction, during the inflammatory response	Cruise *et al*[54] 2006
Core	Ishikawa *et al*[48] 2003	C57BL/6N	serum amyloid	Malignant lymphoma and Hepatocellular adenoma in old mice	Ishikawa *et al*[48] 2003
Core	Kato *et al*[49] 2003	C57BL/6	serum amyloid P	Adenoma and HCC development in transgenic mice following repeated CCl_4 administrations.	Kato *et al*[49] 2003
Core Core-E1-E2	Kamegaya *et al*[51] 2005	FVB × C57BL/6	Albumin	After DEN treatment, core-E1-E2 mice develop tumors with a larger size than core mice (diminution of apoptotic index)	Kamegaya *et al*[51] 2005
Core-E1-E2-p7	Lerat *et al*[62] 2002	C57BL/6	Albumin	Steatosis	Lerat *et al*[62] 2002
				HCC (rare)	
				Sensitivity to oxidative stress	Okuda *et al*[80] 2002
Core-E1-E2-p7	Korenaga *et al*[81] 2005	C57BL/6J	Albumin	Increase in ROS production by mitochondrial electron transport complex I	Korenaga *et al*[81] 2005
Core-E1-E2	Kawamura *et al*[82] 1997	FVB	Albumin or major urinary protein	No liver disease	Kawamura *et al*[82] 1997
Core-E1-E2	Honda *et al*[56] 1999	C57BL/6	H2-Kd	Sensitivity to anti-fas administration	Honda *et al*[56] 1999
Core-E1-E2	Naas *et al*[57] 2005	C57BL/6	CMV	Steatosis. Acceleration of liver and lymphoid tumor development	Naas *et al*[57] 2005
Core-E1-E2-NS2	Wakita *et al*[59] 1998	Balb/C	Cre/Lox system (CAG promoter)	Hepatitis injury associated with HCV-specific CTL response	Wakita *et al*[59] 1998 Wakita *et al*[58] 2000
				Suppression of Fas-mediated cell death	Machida *et al*[83] 2001
				HCV-specific CD8+ CTLs specifically induce liver injury	Takaku *et al*[62] 2003
E1-E2	Koike *et al*[63] 1995	CD1	HBV	No liver disease Expression in salivary glands	Koike *et al*[63] 1995
				Exocrinopathy resembling Sjogren syndrome	Koike *et al*[84] 1997
E2	Pasquinelli *et al*[50] 1997	C57BL/6	Albumin	No liver disease	Pasquinelli *et al*[50] 1997
NS3-NS4A	Frelin *et al*[64] 2006	C57BL/6	Major urinary protein	No liver disease. Alteration of hepatic immune cell subsets. Reduced sensitivity to TNF alpha mediated liver disease.	Frelin *et al*[64] 2006

NS5	Majumder *et al*[65] 2002	FVB	ApoE	No liver disease	Majumder *et al*[65] 2002
				Protection against TNF alpha mediated liver disease (inhibition of NF-kappaB activation)	Majumder *et al*[66] 2003
Full polyprotein	Alonzi *et al*[60] 2004 Blindenbacher *et al*[67] 2003	C57BL/6	Alpha1 antitrypsin	Steatosis T cell infiltrate	Alonzi *et al*[60] 2004
				Inhibition of IFN alpha-induced signaling	Blindenbacher *et al*[67] 2003
				Increased expression of protein phosphatase 2A	Duong *et al*[68] 2004
Full polyprotein	Lerat *et al*[62] 2002	C57BL/6	Albumin	Hepatic steatosis, HCC	Lerat *et al*[62] 2002
				Impairment of intrahepatic immune response (absence of elimination of adenovirus-infected hepatocytes)	Disson *et al*[85] 2004
				Down-regulation of pro-apoptotic CIDE-B protein in adenovirus-infected transgenic mice	Erdtmann *et al*[86] 2003
				Iron overload induces increase the risk of HCC (mitochondrial injury)	Furutani *et al*[87] 2006

HCV-core protein in liver cells may affect the persistence of Fas-mediated liver cell injury[52]. Liver inflammation induced by HCV core-expression in CD4+ T cells is associated with high expression levels of the Fas ligand and CXCR3 chemokine induction[53-55]. In this context, liver inflammation is abolished by anti-Fas antibody treatment[54,55]. Similarly, transgenic mice expressing Core-E1-E2 present with hepatocyte necrosis associated with increased Fas-mediated injury[56,57]. Furthermore, the expression of core-E1-E2-NS2 viral proteins, as well as of the entire HCV polyprotein, may induce steatosis with lymphocyte cell infiltrate, mito-chondrial injury and sensitivity to oxidative stress and HCC in aging animals[58-62]. By contrast, E1-E2, E2, NS3-NS4A or NS5A transgenic animals have not been shown to exhibit any major histological changes to the liver[50,63-66].

HCV and interferon pathway modulation.

In vivo experiments in transgenic mice have confirmed that the expression of full HCV polyproteins and core protein inhibits interferon alpha signaling. The HCV core protein has been shown to induce an aberrant expression of SOCS1, which can suppress Jak-STAT signaling activation[47]. Similarly, STAT signaling was found to be strongly inhibited in the liver of HCV transgenic mice[67]. In this model, STAT phosphorylation by Jak was not reduced, but the binding of STAT transcription factors to the promoters of interferon-stimulated genes was inhibited[67]. HCV expression in the liver is associated with the inhibition of STAT function by concomitant induction of the expression of the protein inhibitor of activated STAT (PIAS)[68]. This may be mediated *via* an up-regulation of protein phosphatase 2A and by STAT demethylation[68,69]. In addition, NS5 protein activates STAT3 through interaction with Jak1[70].

In summary, and despite some discordant data, studies involving transgenic mice expressing one or a combination of HCV viral proteins have been essential to a clearer understanding of the mechanisms involved in HCV-induced pathogenesis. However, to be rigorous the interpretation of any data must take account of variations in the genetic background of mice, constitutive transgene expression and the absence of any immune response. Nevertheless, even if we consider the variability of morphological observations and the diversity of biochemical data, most reports strongly support a direct role for core protein in liver pathogenesis. Core protein may predispose subjects to HCC development through its contribution to the onset of steatosis, fibrosis and oxidative stress, particularly by acting on the expression of cell growth-related genes, the interferon pathway and lipid metabolism.

CONCLUSION

This review underlines the usefulness of rodent models in the field of HCV infection studies. Indeed, as pointed out, exponential information has been obtained using small animal models which are susceptible to HCV infection or allow HCV protein expression. The principal advance in this area is the establishment of small animal models which can support the entire life cycle of the virus. However, the contribution of different viral proteins to HCV-related pathogenesis is far from being clarified. Indeed, if we are to reach definite conclusions regarding the mechanisms linked to HCV pathogenesis, the next step must be to develop a rodent model which can harbor both a human immune system and human liver cells susceptible to HCV infection. This rodent model will constitute a new tool to allow the efficient screening of HCV vaccine candidates. Finally, without forgetting the indispensable role of the chimpanzee (which remains the optimum model to study the efficacy of future vaccines), new animal models such as transgenic mice and HCV infection-permissive rodents constitute extremely promising and complementary tools which will enable us to better understand and fight against HCV infection.

REFERENCES

1 **Feinstone SM**, Kapikian AZ, Purcell RH, Alter HJ, Holland PV. Transfusion-associated hepatitis not due to viral hepatitis type A or B. *N Engl J Med* 1975; **292**: 767-770
2 **Alter HJ**, Purcell RH, Holland PV, Popper H. Transmissible agent in non-A, non-B hepatitis. *Lancet* 1978; **1**: 459-463
3 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362
4 **Bright H**, Carroll AR, Watts PA, Fenton RJ. Development of a GB virus B marmoset model and its validation with a novel series of hepatitis C virus NS3 protease inhibitors. *J Virol* 2004;

78: 2062-2071
5 **Nam JH**, Faulk K, Engle RE, Govindarajan S, St Claire M, Bukh J. In vivo analysis of the 3' untranslated region of GB virus B after in vitro mutagenesis of an infectious cDNA clone: persistent infection in a transfected tamarin. *J Virol* 2004; **78**: 9389-9399
6 **Rijnbrand R**, Yang Y, Beales L, Bodola F, Goettge K, Cohen L, Lanford RE, Lemon SM, Martin A. A chimeric GB virus B with 5' nontranslated RNA sequence from hepatitis C virus causes hepatitis in tamarins. *Hepatology* 2005; **41**: 986-994
7 **Ouyang EC**, Wu CH, Walton C, Promrat K, Wu GY. Transplantation of human hepatocytes into tolerized genetically immunocompetent rats. *World J Gastroenterol* 2001; **7**: 324-330
8 **Wu GY**, Konishi M, Walton CM, Olive D, Hayashi K, Wu CH. A novel immunocompetent rat model of HCV infection and hepatitis. *Gastroenterology* 2005; **128**: 1416-1423
9 **Lubin I**, Faktorowich Y, Lapidot T, Gan Y, Eshhar Z, Gazit E, Levite M, Reisner Y. Engraftment and development of human T and B cells in mice after bone marrow transplantation. *Science* 1991; **252**: 427-431
10 **Galun E**, Burakova T, Ketzinel M, Lubin I, Shezen E, Kahana Y, Eid A, Ilan Y, Rivkind A, Pizov G. Hepatitis C virus viremia in SCID-->BNX mouse chimera. *J Infect Dis* 1995; **172**: 25-30
11 **Eren R**, Landstein D, Terkieltaub D, Nussbaum O, Zauberman A, Ben-Porath J, Gopher J, Buchnick R, Kovjazin R, Rosenthal-Galili Z, Aviel S, Ilan E, Shoshany Y, Neville L, Waisman T, Ben-Moshe O, Kischitsky A, Foung SK, Keck ZY, Pappo O, Eid A, Jurim O, Zamir G, Galun E, Dagan S. Preclinical evaluation of two neutralizing human monoclonal antibodies against hepatitis C virus (HCV): a potential treatment to prevent HCV reinfection in liver transplant patients. *J Virol* 2006; **80**: 2654-2664
12 **Ilan E**, Arazi J, Nussbaum O, Zauberman A, Eren R, Lubin I, Neville L, Ben-Moshe O, Kischitzky A, Litchi A, Margalit I, Gopher J, Mounir S, Cai W, Daudi N, Eid A, Jurim O, Czerniak A, Galun E, Dagan S. The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. *J Infect Dis* 2002; **185**: 153-161
13 **Eren R**, Ilan E, Nussbaum O, Lubin I, Terkieltaub D, Arazi Y, Ben-Moshe O, Kitchinzky A, Berr S, Gopher J, Zauberman A, Galun E, Shouval D, Daudi N, Eid A, Jurim O, Magnius LO, Hammas B, Reisner Y, Dagan S. Preclinical evaluation of two human anti-hepatitis B virus (HBV) monoclonal antibodies in the HBV-trimera mouse model and in HBV chronic carrier chimpanzees. *Hepatology* 2000; **32**: 588-596
14 **Galun E**, Eren R, Safadi R, Ashour Y, Terrault N, Keeffe EB, Matot E, Mizrachi S, Terkieltaub D, Zohar M, Lubin I, Gopher J, Shouval D, Dagan S. Clinical evaluation (phase I) of a combination of two human monoclonal antibodies to HBV: safety and antiviral properties. *Hepatology* 2002; **35**: 673-679
15 **Heckel JL**, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* 1990; **62**: 447-456
16 **Sandgren EP**, Palmiter RD, Heckel JL, Daugherty CC, Brinster RL, Degen JL. Complete hepatic regeneration after somatic deletion of an albumin-plasminogen activator transgene. *Cell* 1991; **66**: 245-256
17 **Rhim JA**, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. *Science* 1994; **263**: 1149-1152
18 **Petersen J**, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1998; **95**: 310-315
19 **Dandri M**, Burda MR, Gocht A, Török E, Pollok JM, Rogler CE, Will H, Petersen J. Woodchuck hepatocytes remain permissive for hepadnavirus infection and mouse liver repopulation after cryopreservation. *Hepatology* 2001; **34**: 824-833
20 **Tateno C**, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, Asahara T, Yokoi T, Furukawa T, Yoshizato K. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004; **165**: 901-912
21 **Dandri M**, Burda MR, Zuckerman DM, Wursthorn K, Matschl U, Pollok JM, Rogiers X, Gocht A, Köck J, Blum HE, von Weizsäcker F, Petersen J. Chronic infection with hepatitis B viruses and antiviral drug evaluation in uPA mice after liver repopulation with tupaia hepatocytes. *J Hepatol* 2005; **42**: 54-60
22 **Meuleman P**, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, Roskams T, Leroux-Roels G. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* 2005; **41**: 847-856
23 **Morosan S**, Hez-Deroubaix S, Lunel F, Renia L, Giannini C, Van Rooijen N, Battaglia S, Blanc C, Eling W, Sauerwein R, Hannoun L, Belghiti J, Brechot C, Kremsdorf D, Druilhe P. Liver-stage development of Plasmodium falciparum, in a humanized mouse model. *J Infect Dis* 2006; **193**: 996-1004
24 **Dandri M**, Burda MR, Török E, Pollok JM, Iwanska A, Sommer G, Rogiers X, Rogler CE, Gupta S, Will H, Greten H, Petersen J. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* 2001; **33**: 981-988
25 **Tsuge M**, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, Takahashi S, Iwao E, Fujimoto Y, Ochi H, Chayama K, Tateno C, Yoshizato K. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005; **42**: 1046-1054
26 **Mercer DF**, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, Addison WR, Fischer KP, Churchill TA, Lakey JR, Tyrrell DL, Kneteman NM. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001; **7**: 927-933
27 **Turrini P**, Sasso R, Germoni S, Marcucci I, Celluci A, Di Marco A, Marra E, Paonessa G, Eutropi A, Laufer R, Migliaccio G, Padron J. Development of humanized mice for the study of hepatitis C virus infection. *Transplant Proc* 2006; **38**: 1181-1184
28 **Walters KA**, Joyce MA, Thompson JC, Smith MW, Yeh MM, Proll S, Zhu LF, Gao TJ, Kneteman NM, Tyrrell DL, Katze MG. Host-specific response to HCV infection in the chimeric SCID-beige/Alb-uPA mouse model: role of the innate antiviral immune response. *PLoS Pathog* 2006; **2**: e59
29 **Kneteman NM**, Weiner AJ, O'Connell J, Collett M, Gao T, Aukerman L, Kovelsky R, Ni ZJ, Zhu Q, Hashash A, Kline J, Hsi B, Schiller D, Douglas D, Tyrrell DL, Mercer DF. Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* 2006; **43**: 1346-1353
30 **Walters KA**, Joyce MA, Thompson JC, Proll S, Wallace J, Smith MW, Furlong J, Tyrrell DL, Katze MG. Application of functional genomics to the chimeric mouse model of HCV infection: optimization of microarray protocols and genomics analysis. *Virol J* 2006; **3**: 37
31 **Koike K**. Hepatitis C as a metabolic disease: Implication for the pathogenesis of NASH. *Hepatol Res* 2005; **33**: 145-150
32 **Asselah T**, Rubbia-Brandt L, Marcellin P, Negro F. Steatosis in chronic hepatitis C: why does it really matter? *Gut* 2006; **55**: 123-130
33 **Koike K**. Hepatitis C virus infection can present with metabolic disease by inducing insulin resistance. *Intervirology* 2006; **49**: 51-57
34 **Shintani Y**, Fujie H, Miyoshi H, Tsutsumi T, Tsukamoto K, Kimura S, Moriya K, Koike K. Hepatitis C virus infection and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840-848
35 **Kawaguchi T**, Yoshida T, Harada M, Hisamoto T, Nagao Y, Ide T, Taniguchi E, Kumemura H, Hanada S, Maeyama M, Baba S, Koga H, Kumashiro R, Ueno T, Ogata H, Yoshimura A, Sata M. Hepatitis C virus down-regulates insulin receptor substrates 1 and 2 through up-regulation of suppressor of cytokine signaling 3. *Am J Pathol* 2004; **165**: 1499-1508
36 **Ramalho F**. Hepatitis C virus infection and liver steatosis. *Antiviral Res* 2003; **60**: 125-127
37 **Moriya K**, Todoroki T, Tsutsumi T, Fujie H, Shintani Y, Miyoshi H, Ishibashi K, Takayama T, Makuuchi M,

Watanabe K, Miyamura T, Kimura S, Koike K. Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biochem Biophys Res Commun* 2001; **281**: 1207-1212

38 **Perlemuter G**, Sabile A, Letteron P, Vona G, Topilco A, Chrétien Y, Koike K, Pessayre D, Chapman J, Barba G, Bréchot C. Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; **16**: 185-194

39 **Perlemuter G**, Lettéron P, Carnot F, Zavala F, Pessayre D, Nalpas B, Bréchot C. Alcohol and hepatitis C virus core protein additively increase lipid peroxidation and synergistically trigger hepatic cytokine expression in a transgenic mouse model. *J Hepatol* 2003; **39**: 1020-1027

40 **Moriya K**, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4**: 1065-1067

41 **Moriya K**, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Miyazawa T, Ishibashi K, Horie T, Imai K, Todoroki T, Kimura S, Koike K. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. *Cancer Res* 2001; **61**: 4365-4370

42 **Moriya K**, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K. Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; **78** (Pt 7): 1527-1531

43 **Tsutsumi T**, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, Fujie H, Matsuura Y, Koike K, Miyamura T. Interaction of hepatitis C virus core protein with retinoid X receptor alpha modulates its transcriptional activity. *Hepatology* 2002; **35**: 937-946

44 **Tsutsumi T**, Suzuki T, Moriya K, Shintani Y, Fujie H, Miyoshi H, Matsuura Y, Koike K, Miyamura T. Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003; **38**: 820-828

45 **Tsutsumi T**, Suzuki T, Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Matsuura Y, Kimura S, Koike K, Miyamura T. Alteration of intrahepatic cytokine expression and AP-1 activation in transgenic mice expressing hepatitis C virus core protein. *Virology* 2002; **304**: 415-424

46 **Yoshida T**, Hanada T, Tokuhisa T, Kosai K, Sata M, Kohara M, Yoshimura A. Activation of STAT3 by the hepatitis C virus core protein leads to cellular transformation. *J Exp Med* 2002; **196**: 641-653

47 **Miyoshi H**, Fujie H, Shintani Y, Tsutsumi T, Shinzawa S, Makuuchi M, Kokudo N, Matsuura Y, Suzuki T, Miyamura T, Moriya K, Koike K. Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression. *J Hepatol* 2005; **43**: 757-763

48 **Ishikawa T**, Shibuya K, Yasui K, Mitamura K, Ueda S. Expression of hepatitis C virus core protein associated with malignant lymphoma in transgenic mice. *Comp Immunol Microbiol Infect Dis* 2003; **26**: 115-124

49 **Kato T**, Miyamoto M, Date T, Yasui K, Taya C, Yonekawa H, Ohue C, Yagi S, Seki E, Hirano T, Fujimoto J, Shirai T, Wakita T. Repeated hepatocyte injury promotes hepatic tumorigenesis in hepatitis C virus transgenic mice. *Cancer Sci* 2003; **94**: 679-685

50 **Pasquinelli C**, Shoenberger JM, Chung J, Chang KM, Guidotti LG, Selby M, Berger K, Lesniewski R, Houghton M, Chisari FV. Hepatitis C virus core and E2 protein expression in transgenic mice. *Hepatology* 1997; **25**: 719-727

51 **Kamegaya Y**, Hiasa Y, Zukerberg L, Fowler N, Blackard JT, Lin W, Choe WH, Schmidt EV, Chung RT. Hepatitis C virus acts as a tumor accelerator by blocking apoptosis in a mouse model of hepatocarcinogenesis. *Hepatology* 2005; **41**: 660-667

52 **Honda A**, Hatano M, Kohara M, Arai Y, Hartatik T, Moriyama T, Imawari M, Koike K, Yokosuka O, Shimotohno K, Tokuhisa T. HCV-core protein accelerates recovery from the insensitivity of liver cells to Fas-mediated apoptosis induced by an injection of anti-Fas antibody in mice. *J Hepatol* 2000; **33**: 440-447

53 **Soguero C**, Joo M, Chianese-Bullock KA, Nguyen DT, Tung K, Hahn YS. Hepatitis C virus core protein leads to immune suppression and liver damage in a transgenic murine model. *J Virol* 2002; **76**: 9345-9354

54 **Cruise MW**, Lukens JR, Nguyen AP, Lassen MG, Waggoner SN, Hahn YS. Fas ligand is responsible for CXCR3 chemokine induction in CD4+ T cell-dependent liver damage. *J Immunol* 2006; **176**: 6235-6244

55 **Cruise MW**, Melief HM, Lukens J, Soguero C, Hahn YS. Increased Fas ligand expression of CD4+ T cells by HCV core induces T cell-dependent hepatic inflammation. *J Leukoc Biol* 2005; **78**: 412-425

56 **Honda A**, Arai Y, Hirota N, Sato T, Ikegaki J, Koizumi T, Hatano M, Kohara M, Moriyama T, Imawari M, Shimotohno K, Tokuhisa T. Hepatitis C virus structural proteins induce liver cell injury in transgenic mice. *J Med Virol* 1999; **59**: 281-289

57 **Naas T**, Ghorbani M, Alvarez-Maya I, Lapner M, Kothary R, De Repentigny Y, Gomes S, Babiuk L, Giulivi A, Soare C, Azizi A, Diaz-Mitoma F. Characterization of liver histopathology in a transgenic mouse model expressing genotype 1a hepatitis C virus core and envelope proteins 1 and 2. *J Gen Virol* 2005; **86**: 2185-2196

58 **Wakita T**, Katsume A, Kato J, Taya C, Yonekawa H, Kanegae Y, Saito I, Hayashi Y, Koike M, Miyamoto M, Hiasa Y, Kohara M. Possible role of cytotoxic T cells in acute liver injury in hepatitis C virus cDNA transgenic mice mediated by Cre/loxP system. *J Med Virol* 2000; **62**: 308-317

59 **Wakita T**, Taya C, Katsume A, Kato J, Yonekawa H, Kanegae Y, Saito I, Hayashi Y, Koike M, Kohara M. Efficient conditional transgene expression in hepatitis C virus cDNA transgenic mice mediated by the Cre/loxP system. *J Biol Chem* 1998; **273**: 9001-9006

60 **Alonzi T**, Agrati C, Costabile B, Cicchini C, Amicone L, Cavallari C, Rocca CD, Folgori A, Fipaldini C, Poccia F, Monica NL, Tripodi M. Steatosis and intrahepatic lymphocyte recruitment in hepatitis C virus transgenic mice. *J Gen Virol* 2004; **85**: 1509-1520

61 **Machida K**, Cheng KT, Sung VM, Lee KJ, Levine AM, Lai MM. Hepatitis C virus infection activates the immunologic (type II) isoform of nitric oxide synthase and thereby enhances DNA damage and mutations of cellular genes. *J Virol* 2004; **78**: 8835-8843

62 **Takaku S**, Nakagawa Y, Shimizu M, Norose Y, Maruyama I, Wakita T, Takano T, Kohara M, Takahashi H. Induction of hepatic injury by hepatitis C virus-specific CD8+ murine cytotoxic T lymphocytes in transgenic mice expressing the viral structural genes. *Biochem Biophys Res Commun* 2003; **301**: 330-337

63 **Koike K**, Moriya K, Ishibashi K, Matsuura Y, Suzuki T, Saito I, Iino S, Kurokawa K, Miyamura T. Expression of hepatitis C virus envelope proteins in transgenic mice. *J Gen Virol* 1995; **76** (Pt 12): 3031-3038

64 **Frelin L**, Brenndorfer ED, Ahlen G, Weiland M, Hultgren C, Alheim M, Glaumann H, Rozell B, Milich DR, Bode JG, Sallberg M. The hepatitis C virus and immune evasion: non-structural 3/4A transgenic mice are resistant to lethal tumour necrosis factor (alpha) mediated liver disease. *Gut* 2006; **55**: 1475-1483

65 **Frelin L**, Brenndörfer ED, Ahlén G, Weiland M, Hultgren C, Alheim M, Glaumann H, Rozell B, Milich DR, Bode JG, Sällberg M. Hepatitis C virus NS5A protein impairs TNF-mediated hepatic apoptosis, but not by an anti-FAS antibody, in transgenic mice. *Virology* 2002; **294**: 94-105

66 **Majumder M**, Steele R, Ghosh AK, Zhou XY, Thornburg L, Ray R, Phillips NJ, Ray RB. Expression of hepatitis C virus non-structural 5A protein in the liver of transgenic mice. *FEBS Lett* 2003; **555**: 528-532

67 **Blindenbacher A**, Duong FH, Hunziker L, Stutvoet ST, Wang X, Terracciano L, Moradpour D, Blum HE, Alonzi T, Tripodi M, La Monica N, Heim MH. Expression of hepatitis c virus proteins inhibits interferon alpha signaling in the liver of transgenic mice. *Gastroenterology* 2003; **124**: 1465-1475

68 **Duong FH**, Filipowicz M, Tripodi M, La Monica N, Heim MH.

Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastroenterology* 2004; **126**: 263-277

69 **Duong FH**, Christen V, Berke JM, Penna SH, Moradpour D, Heim MH. Upregulation of protein phosphatase 2Ac by hepatitis C virus modulates NS3 helicase activity through inhibition of protein arginine methyltransferase 1. *J Virol* 2005; **79**: 15342-15350

70 **Sarcar B**, Ghosh AK, Steele R, Ray R, Ray RB. Hepatitis C virus NS5A mediated STAT3 activation requires co-operation of Jak1 kinase. *Virology* 2004; **322**: 51-60

71 **Feinstone SM**, Alter HJ, Dienes HP, Shimizu Y, Popper H, Blackmore D, Sly D, London WT, Purcell RH. Non-A, non-B hepatitis in chimpanzees and marmosets. *J Infect Dis* 1981; **144**: 588-598

72 **Garson JA**, Whitby K, Watkins P, Morgan AJ. Lack of susceptibility of the cottontop tamarin to hepatitis C infection. *J Med Virol* 1997; **52**: 286-288

73 **Abe K**, Kurata T, Teramoto Y, Shiga J, Shikata T. Lack of susceptibility of various primates and woodchucks to hepatitis C virus. *J Med Primatol* 1993; **22**: 433-434

74 **Sithebe NP**, Kew MC, Mphahlele MJ, Paterson AC, Lecatsas G, Kramvis A, de Klerk W. Lack of susceptibility of Chacma baboons (Papio ursinus orientalis) to hepatitis C virus infection. *J Med Virol* 2002; **66**: 468-471

75 **Xie ZC**, Riezu-Boj JI, Lasarte JJ, Guillen J, Su JH, Civeira MP, Prieto J. Transmission of hepatitis C virus infection to tree shrews. *Virology* 1998; **244**: 513-520

76 **Simons JN**, Pilot-Matias TJ, Leary TP, Dawson GJ, Desai SM, Schlauder GG, Muerhoff AS, Erker JC, Buijk SL, Chalmers ML. Identification of two flavivirus-like genomes in the GB hepatitis agent. *Proc Natl Acad Sci USA* 1995; **92**: 3401-3405

77 **Lanford RE**, Chavez D, Notvall L, Brasky KM. Comparison of tamarins and marmosets as hosts for GBV-B infections and the effect of immunosuppression on duration of viremia. *Virology* 2003; **311**: 72-80

78 **Benali-Furet NL**, Chami M, Houel L, De Giorgi F, Vernejoul F, Lagorce D, Buscail L, Bartenschlager R, Ichas F, Rizzuto R, Paterlini-Bréchot P. Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. *Oncogene* 2005; **24**: 4921-4933

79 **Wang AG**, Moon HB, Lee YH, Yu SL, Kwon HJ, Han YH, Fang W, Lee TH, Jang KL, Kim SK, Yu DY, Lee DS. Transgenic expression of Korean type hepatitis C virus core protein and related mutants in mice. *Exp Mol Med* 2004; **36**: 588-593

80 **Okuda M**, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366-375

81 **Korenaga M**, Wang T, Li Y, Showalter LA, Chan T, Sun J, Weinman SA. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. *J Biol Chem* 2005; **280**: 37481-37488

82 **Kawamura T**, Furusaka A, Koziel MJ, Chung RT, Wang TC, Schmidt EV, Liang TJ. Transgenic expression of hepatitis C virus structural proteins in the mouse. *Hepatology* 1997; **25**: 1014-1021

83 **Machida K**, Tsukiyama-Kohara K, Seike E, Toné S, Shibasaki F, Shimizu M, Takahashi H, Hayashi Y, Funata N, Taya C, Yonekawa H, Kohara M. Inhibition of cytochrome c release in Fas-mediated signaling pathway in transgenic mice induced to express hepatitis C viral proteins. *J Biol Chem* 2001; **276**: 12140-12146

84 **Koike K**, Moriya K, Ishibashi K, Yotsuyanagi H, Shintani Y, Fujie H, Kurokawa K, Matsuura Y, Miyamura T. Sialadenitis histologically resembling Sjogren syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94**: 233-236

85 **Disson O**, Haouzi D, Desagher S, Loesch K, Hahne M, Kremer EJ, Jacquet C, Lemon SM, Hibner U, Lerat H. Impaired clearance of virus-infected hepatocytes in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2004; **126**: 859-872

86 **Erdtmann L**, Franck N, Lerat H, Le Seyec J, Gilot D, Cannie I, Gripon P, Hibner U, Guguen-Guillouzo C. The hepatitis C virus NS2 protein is an inhibitor of CIDE-B-induced apoptosis. *J Biol Chem* 2003; **278**: 18256-18264

87 **Furutani T**, Hino K, Okuda M, Gondo T, Nishina S, Kitase A, Korenaga M, Xiao SY, Weinman SA, Lemon SM, Sakaida I, Okita K. Hepatic iron overload induces hepatocellular carcinoma in transgenic mice expressing the hepatitis C virus polyprotein. *Gastroenterology* 2006; **130**: 2087-2098

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Epidemiology of hepatitis C virus infection

Miriam J Alter

Miriam J Alter, Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, Texas USA 77555, United States
Correspondence to: Miriam J Alter, PhD, University of Texas Medical Branch, Mail Route 0435, 301 University Boulevard, Galveston, Texas 77555, United States. mjalter@utmb.edu
Telephone: +1-409-7470229 Fax: +1-409-7470220
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Abstract

Globally, hepatitis C virus (HCV) has infected an estimated 130 million people, most of whom are chronically infected. HCV-infected people serve as a reservoir for transmission to others and are at risk for developing chronic liver disease, cirrhosis, and primary hepatocellular carcinoma (HCC). It has been estimated that HCV accounts for 27% of cirrhosis and 25% of HCC worldwide. HCV infection has likely been endemic in many populations for centuries. However, the wave of increased HCV-related morbidity and mortality that we are now facing is the result of an unprecedented increase in the spread of HCV during the 20th century. Two 20th century events appear to be responsible for this increase; the widespread availability of injectable therapies and the illicit use of injectable drugs.

Key words: Hepatitis C virus; Global epidemiology; Incidence; Prevalence; Transmission; Natural history

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PREVALENCE AND INCIDENCE

The estimated global prevalence of HCV infection is 2.2%, corresponding to about 130 000 000 HCV-positive persons worldwide (Figure 1)[1]. Because many countries lack data, this estimate is based on weighted averages for regions rather than individual countries. Region-specific estimates range from < 1.0% in Northern Europe to > 2.9% in Northern Africa. The lowest prevalence (0.01%-0.1%) has been reported from countries in the United Kingdom and Scandinavia; the highest prevalence (15%-20%) has been reported from Egypt[2,3]. An estimated 27% of cirrhosis and 25% of HCC worldwide occur in HCV-infected people[4].

There are both geographic and temporal differences in the patterns of HCV infection[5]. For example, vastly different countries, including the United States, Australia, Turkey, Spain, Italy, and Japan, belong to regions of the world with similar overall average prevalences of HCV infection (1.0%-1.9%), but have different patterns of age-specific prevalence (Figure 2A). In the United States, prevalence is highest among persons 30-49 years old, who account for two-thirds of all infections, and lower than average among persons less than 20 and greater than 50 years old[6,7]. This pattern indicates that most HCV transmission occurred in the last 20-40 years, and primarily among young adults, a pattern similar to that observed in Australia[8]. In the United States[9,10], Australia[11], and countries in western and northern Europe with similar HCV epidemiology[12,13], the greatest variations in prevalence occur among persons with different risk factors for infection.

In contrast, the age-specific prevalences of HCV infection increase steadily with age in Turkey, Spain, Italy, Japan, and China, (Figure 2A)[14-18]. In these countries, persons > 50 years old account for most infections, which suggests a cohort effect in which the risk for HCV infection was higher in the distant past, i.e., 40-60 years previously. In many countries with this pattern, the greatest variations in HCV prevalence occur geographically. In Italy, Japan and China, for example, there are hyperendemic areas of the country in which older persons have an HCV prevalence 20-fold greater than the average overall and 1.5-2-fold greater than the prevalence among older persons in other areas of the country[19-22].

The highest HCV prevalence in the world occurs in Egypt, where the prevalence of infection increases steadily with age, and high rates of infection are observed among persons in all age groups (Figure 2B)[4,23]. This pattern indicates an increased risk in the distant past followed by an ongoing high risk for acquiring HCV infection, although there are regional differences in average overall prevalence[4,24].

Determining the incidence of HCV infection (i.e., the rate of newly acquired infections) is difficult because most acute infections are asymptomatic, available assays do not distinguish acute from chronic or resolved infection, and most countries do not systematically collect data on cases of acute disease. Even in countries with well-established surveillance systems, acute disease reporting systems

Figure 1 Estimated HCV prevalence by region[1].

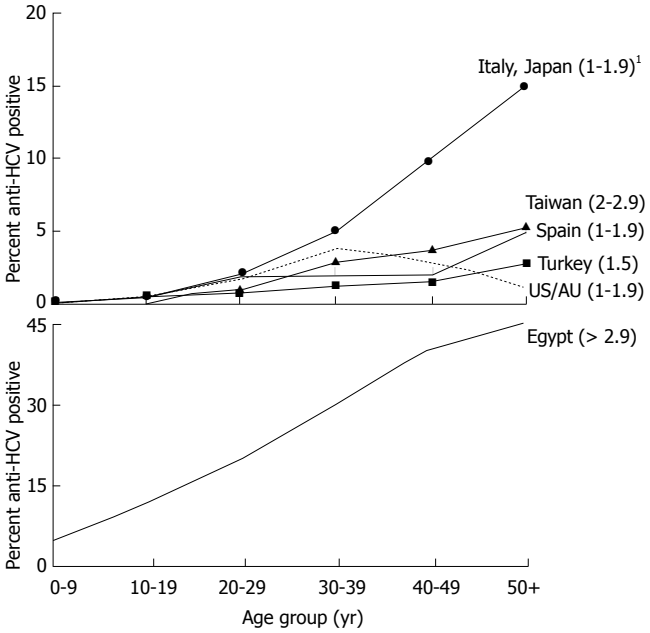

Figure 2 Age-Specific prevalence of antibody to hepatitis C virus by selected countries. [1]Numbers in parentheses indicate average regional prevalence in which country resides (see Figure 1).

underestimate the incidence of HCV infection[25-27]. For several countries, mathematical models have been used to infer trends in incidence, which rely on the assumption that current age-specific prevalence reflects the cumulative risk of acquiring infection.

In the United States, trends in HCV incidence were modeled using age-specific incidence from reported cases of acute disease[28] and age-specific prevalence from a cross-sectional national survey[6]. This model showed a large increase in the incidence of newly acquired HCV infections from the late 1960s to the early 1980s[29]. The estimated annual incidence was low (18 per 100 000) before 1965, increased steadily through 1980, and remained high (130 per 100 000) through 1989, corresponding to an average of 240 000 infections per year in the 1980s. Since 1989, the incidence of reported cases of hepatitis C has declined by more than 80%[30], consistent with the finding that the national seroprevalence of infection remained unchanged between 1988 and 2002[7]. The rate of new HCV infections also declined in Italy in the 1990s according to reported cases of acute disease[27]. In both the United States and Italy, most newly acquired infections are in young adults (30-35 years old)[30,31]. A model of HCV burden in France, which employed death rates from hepatocellular carcinoma in addition to cross-sectional seroprevalence studies to estimate past incidence, showed a trend similar to that of the United States with increasing incidence through the 1980s[32], whereas an alternative approach to modeling disease burden in Australia showed a steady increase in new HCV infections in that country from 1961-2001[8].

Several other countries have measured HCV infection incidence by determining the rate of seroconversions in HCV-negative cohorts followed over time. Cohort studies conducted in hyperendemic areas in Taiwan and Japan found incidence rates of HCV infection of 110/10 000 and 28-36/10 000 persons, respectively[15,22,33]. The mean age of persons with newly acquired HCV infection was 50 years in the Taiwan cohort and 40 and 60 years, respectively, in the two Japan cohorts. Cohort studies in Egypt found incidence rates of 0.8/1000 person-years in an area of Upper Egypt where the background prevalence was 9% and 6.8/1000 in the Nile Delta where the background prevalence was 24%[34]. Sixty-seven percent of the incident infections were in persons < 20 years old.

Because chronic liver disease may develop many years after infection, the past incidence is a major determinant of the future burden of HCV-associated complications[35]. In the United States and other countries where the emergence of HCV infection is a more recent event, the full magnitude of the burden of HCV-related chronic liver disease has yet to be realized as the duration of infection among most infected persons has not reached the point at which complications from chronic liver disease typically occur[29,32]. In countries where the emergence of HCV infection occurred in the distant past (such as Japan and Italy), the burden of HCV-related chronic disease already might have reached its highest magnitude, but changes in disease transmission patterns that result in younger persons acquiring infection could result in future increases in chronic disease as this cohort ages[36]. In Egypt, where

Table 1 Importance of different exposures to HCV transmission patterns in low, moderate and high prevalence areas worldwide

	The extent exposure contributes to HCV transmission by level of HCV prevalence		
Exposure	Low	Moderate	High
Injecting drug use	++++	++	+
Transfusions (unscreened)	+++	+++	+++
Unsafe therapeutic injections	+	++++	++++
Occupational	+	+	+
Perinatal	+	+	+
High-risk sex	++	+	+/-

there has been an ongoing high risk for decades, the high magnitude of the current burden of HCV-related chronic disease is predicted to continue into the future[37].

MODES OF TRANSMISSION

The most efficient transmission of HCV is through large or repeated direct percutaneous exposures to blood (e.g., transfusion or transplantation from infectious donors, injecting drug use)[10]. HCV is less efficiently transmitted by single small dose percutaneous exposures (e.g., accidental needlesticks)[10,38] or by mucosal exposures to blood or serum-derived fluids (e.g., birth to an infected mother, sex with an infected partner)[10,39,40].

There is also evidence that the environment can serve as a reservoir for infectious virus. HCV transmission by inapparent percutaneous exposures has been caused by cross-contamination from reused needles and syringes, multiple-use medication vials, infusion bags, and injecting-drug use paraphernalia[41,42]. These epidemiologic data implicating transmission from environmental sources of HCV are supported by an experimental study that demonstrated the infectivity of HCV in blood after exposure to drying and storage at room temperature[43]. Similar results have been reported for hepatitis B virus (HBV)[44]. However, the risk for transmission of HBV from such inapparent exposures appears to be greater, probably because of differences in characteristics between the two viruses, including the longer environmental survival of HBV[44] and concentrations in the blood of infected persons two- to four-logs higher[45].

However, in the recent cohort study in Egypt, the strongest predictor of incident HCV infection was having an anti-HCV–positive family member[34]. Among those that did, incidence was 5.8/1000 PY compared with 1.0/1000 PY ($P < 0.01$) among those with no positive family members. The highest incidence rate (14.1/1000 PY) was in children younger than 10 who were living in households with an anti-HCV–positive parent. The study did not determine the factors responsible for this association.

RISK FACTORS

Risk factors associated with acquiring infection as determined from cohort (prospective) and case control (retrospective) studies of persons with acute disease (or infection) have included transfusion of blood and blood products and transplantation of solid organs from infected donors, injecting drug use, unsafe therapeutic injections, occupational exposure to blood (primarily contaminated needle sticks), birth to an infected mother, sex with an infected partner, and sex with multiple partners[9,10]. Among these, transfusion from unscreened donors, injecting drug use, and unsafe therapeutic injections have been the most important, however, there are temporal and geographical differences in the extent to which these risk factors have contributed to HCV transmission (Table 1).

BLOOD TRANSFUSION AND IATROGENIC EXPOSURES

Transfusion-associated HCV infection was a worldwide risk before HCV testing became available. It has been virtually eliminated in those countries that implemented routine HCV testing of donors[46], but in others, receipt of blood transfusions remains an important source for infection. Some countries continue to use commercial donors to supplement their blood supplies, have not considered blood safety a priority, and lack the resources to implement donor screening[47].

Of even greater importance in the spread of HCV, are unsafe therapeutic injections performed by both professionals and non-professionals. It has been estimated that approximately 2 million HCV infections are acquired annually from contaminated health care injections, and may account for up to 40% of all HCV infections worldwide[48]. In many developing countries, supplies of sterile syringes may be inadequate or nonexistent, non-professionals often administer injections outside the medical setting, and injections are often given to deliver medications that could otherwise be delivered by the oral route. Reuse of glass syringes during the early campaign to treat schistosomiasis in Egypt appeared to be responsible for the largest outbreak of iatrogenic transmission of a bloodborne pathogen ever recorded[3]. In addition to unsafe injection practices, lack of attention to appropriate cleaning and disinfection of equipment used in hospital and dental settings also may be a source for HCV transmission.

ILLEGAL INJECTING DRUG USE

Injecting drug use has been the predominant mode of transmission during the past 40 years in the United States and Australia, and now accounts for most newly acquired infections in many other countries, including those in Western, Northern, and Southern Europe. Although cumulative infection rates among young injection drug users during the first 2-3 years of injecting have slowed in recent years, declining from 80% during the late 1980s to 30% during the late 1990s, incidence among new injectors remains high, ranging from 15% to > 30% annually[49]. Fewer sharing partners are necessary to sustain HCV transmission than are necessary for other bloodborne viruses[50], and indirect drug sharing and preparation practices, such as backloading and sharing cotton, cooker, and rinse water, have been associated with HCV transmission[51]. These results indicate that harm

reduction messages and access to drug treatment need to be expanded to prevent new HCV infections, particularly among young injectors.

OTHER EXPOSURES

In contrast, the contributions of occupational, perinatal, and high-risk sexual exposures have been relatively constant over time and with substantially less geographic variation (Table 1)[52]. The relatively low efficiency of these routes for transmission compared to those involving large or repeated percutaneous exposures may explain these differences.

Occupational transmission of HCV infection is largely confined to health care workers who have sustained contaminated needlestick injuries; average incidence of anti-HCV seroconversion from an HCV-positive source is 1.8%; transmission has been associated with hollow-bore needles and deep injuries[53]. Transmission rarely occurs from mucous membrane or non-intact skin exposures to blood[54], and no transmission to health care workers has been documented from intact skin exposures to blood. Furthermore, the prevalence of HCV infection among health care workers, including orthopedic, general, and oral surgeons, is no greater than the general population, averaging 1%-2%, and is 10 times lower than that for HBV infection. Even more rarely, HCV-infected health-care workers have transmitted to patients, and the risk was extremely low-averaging about 0.5%, even for those episodes involving surgeons.

The rate of perinatal transmission of HCV is 4% to 7% per pregnancy and occurs only when HCV RNA is detectable in maternal serum at delivery. Transmission may be related to higher levels (above 10^6 copies per mL), although data on the effect of virus concentration have been inconsistent[39]. Prolonged labor after membrane rupture and internal fetal monitoring have been associated with perinatal infection[39,55]. There has been no association with vaginal delivery, caesarian section or breastfeeding. Co-infection with human immunodeficiency virus (HIV) increases the rate of transmission 4- to 5-fold.

The extent to which HCV is transmitted by sexual activity and under what circumstances is one of the most controversial aspects of the epidemiology of hepatitis C. The results of different types of studies have been inconsistent. The strongest evidence for heterosexual activity as a risk factor for HCV infection came from case-control studies of persons with acute non-A, non-B hepatitis (now known as hepatitis C) in the United States during the 1970s and 1980s, which identified sex with an infected partner or with multiple partners as independently associated with acquiring disease[56,57]. Since then, 15%-20% of cases of acute hepatitis C have reported no other risk factor except one of these sexual exposures. In contrast, no association was found with male homosexual activity, and cross-sectional studies conducted since 1990 of men who have sex with men (MSM) and heterosexual persons in long term monogamous relationships with a partner with chronic HCV have found little evidence for sexual transmission of HCV[10,40]. One possible explanation for these apparent inconsistencies is that HCV is more likely to be transmitted by sexual intercourse when the infected partner is in the early phase of acute infection; virus concentration is high and there is no antibody to complex with antigen. The early case-control studies of acute disease were performed when the incidence of HCV infection was at its peak. During that time a high proportion of the general adult population had a history of multiple sex partners[6], which may have increased their probability of having sex with an infectious partner. A higher rate of sexual transmission of HCV during the acute phase of infection in combination with a high proportion of persons having unsafe sex with multiple partners could explain the disproportionate amount of the HCV-related disease burden accounted for by sexual activity relative to the low efficiency by which the virus is transmitted by this mode, as well as the rare episodes of infection among partners of persons with chronic HCV. A similar association with acute hepatitis C and multiple sex partners has been reported from Italy[58], and since 2000, there have been reports from several European countries of episodes of acute hepatitis C among HIV-infected MSM[59-62].

Because of the wide variety of human activities that involve the potential for percutaneous exposure to blood or blood-derived body fluids, there are numerous other biologically-plausible modes of transmission besides those with clearly-demonstrated epidemiologic associations with infection. These include cosmetic procedures (tattooing, body-piercing), intranasal drug use, and religious or cultural practices such as ritual scarification, circumcision, acupuncture, and cupping. In most regions of the world, there are insufficient data to determine whether these risk factors make any measurable contribution to overall HCV transmission. In those countries where adequate studies have been done, none of these activities have been consistently associated with HCV transmission[9,63].

SUMMARY

Thus, most of the HCV-related disease burden in developed countries has resulted from injection drug use, receipt of transfusions before donor screening, and high-risk sexual activity. In contrast, most of the disease burden in developing countries is related to receipt of unsafe therapeutic injections and contaminated blood. Characterizing the epidemiology of HCV infection in individual countries is crucial to developing and implementing effective preventive measures. In some, ensuring safe blood supplies and health-care related procedures are the highest priorities. In others, priorities need to focus on preventing injecting drug use, improving access to drug treatment, harm reduction counseling, and testing to identify HCV-infected persons for medical evaluation and management.

REFERENCES

1 **Global burden of disease (GBD) for hepatitis C.** *J Clin Pharmacol* 2004; **44**: 20-29

2 **Shepard CW**, Finelli L, Fiore AE, Bell BP. Epidemiology of hepatitis B and hepatitis B virus infection in United States children. *Pediatr Infect Dis J* 2005; **24**: 755-760

3 **Frank C**, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR, Magder LS, El Khoby T, Abdel-Wahab Y, Aly Ohn ES, Anwar W, Sallam I. The role of parenteral antischistosomal therapy in the spread of hepatitis C virus in Egypt. *Lancet* 2000; **355**: 887-891

4 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538

5 **Alter MJ**, Hutin YJ, Armstrong GL. Epidemiology of hepatitis C. In: Liang TJ, Hoofnagle JH, editors. Hepatitis C. San Diego: Academic Press, 2000: 169-183

6 **Alter MJ**, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999; **341**: 556-562

7 **Armstrong GL**, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705-714

8 **Law MG**, Dore GJ, Bath N, Thompson S, Crofts N, Dolan K, Giles W, Gow P, Kaldor J, Loveday S, Powell E, Spencer J, Wodak A. Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. *Int J Epidemiol* 2003; **32**: 717-724

9 **Alter MJ**. Prevention of spread of hepatitis C. *Hepatology* 2002; **36**: S93-S98

10 **Recommendations for prevention and control of hepatitis C virus (HCV) infection and HCV-related chronic disease.** Centers for Disease Control and Prevention. *MMWR Recomm Rep* 1998; **47**: 1-39

11 **Dore GJ**, MacDonald M, Law MG, Kaldor JM. Epidemiology of hepatitis C virus infection in Australia. *Aust Fam Physician* 2003; **32**: 796-798

12 **Gérard C**, Delwaide J, Vaira D, Bastens B, Servais B, Wain E, Bataille C, Daenen G, Belaïche J. Evolution over a 10 year period of the epidemiological profile of 1,726 newly diagnosed HCV patients in Belgium. *J Med Virol* 2005; **76**: 503-510

13 **Payan C**, Roudot-Thoraval F, Marcellin P, Bled N, Duverlie G, Fouchard-Hubert I, Trimoulet P, Couzigou P, Cointe D, Chaput C, Henquell C, Abergel A, Pawlotsky JM, Hezode C, Coudé M, Blanchi A, Alain S, Loustaud-Ratti V, Chevallier P, Trepo C, Gerolami V, Portal I, Halfon P, Bourlière M, Bogard M, Plouvier E, Laffont C, Agius G, Silvain C, Brodard V, Thiefin G, Buffet-Janvresse C, Riachi G, Grattard F, Bourlet T, Stoll-Keller F, Doffoel M, Izopet J, Barange K, Martinot-Peignoux M, Branger M, Rosenberg A, Sogni P, Chaix ML, Pol S, Thibault V, Opolon P, Charrois A, Serfaty L, Fouqueray B, Grange JD, Lefrère JJ, Lunel-Fabiani F. Changing of hepatitis C virus genotype patterns in France at the beginning of the third millenium: The GEMHEP GenoCII Study. *J Viral Hepat* 2005;**12**:405-413

14 **Domínguez A**, Bruguera M, Vidal J, Plans P, Salleras L. Community-based seroepidemiological survey of HCV infection in Catalonia, Spain. *J Med Virol* 2001; **65**: 688-693

15 **Sun CA**, Chen HC, Lu SN, Chen CJ, Lu CF, You SL, Lin SH. Persistent hyperendemicity of hepatitis C virus infection in Taiwan: the important role of iatrogenic risk factors. *J Med Virol* 2001; **65**: 30-34

16 **Sun CA**, Chen HC, Lu CF, You SL, Mau YC, Ho MS, Lin SH, Chen CJ. Transmission of hepatitis C virus in Taiwan: prevalence and risk factors based on a nationwide survey. *J Med Virol* 1999; **59**: 290-296

17 **Campello C**, Poli A, Dal MG, Besozzi-Valentini F. Seroprevalence, viremia and genotype distribution of hepatitis C virus: a community-based population study in northern Italy. *Infection* 2002; **30**: 7-12

18 **Sagnelli E**, Stroffolini T, Mele A, Almasio P, Coppola N, Ferrigno L, Scolastico C, Onofrio M, Imparato M, Filippini P. The importance of HCV on the burden of chronic liver disease in Italy: a multicenter prevalence study of 9,997 cases. *J Med Viro*l 2005; **75**: 522-527

19 **Zhang M**, Sun XD, Mark SD, Chen W, Wong L, Dawsey SM, Qiao YL, Fraumeni JF, Taylor PR, O'Brien TR. Hepatitis C virus infection, Linxian, China. *Emerg Infect Dis* 2005; **11**: 17-21

20 **Di Stefano R**, Stroffolini T, Ferraro D, Usticano A, Valenza LM, Montalbano L, Pomara G, Craxì A. Endemic hepatitis C virus infection in a Sicilian town: further evidence for iatrogenic transmission. *J Med Virol* 2002; **67**: 339-344

21 **Maio G**, d'Argenio P, Stroffolini T, Bozza A, Sacco L, Tosti ME, Intorcia M, Fossi E, d'Alessio G, Kondili LA, Rapicetta M, Mele A. Hepatitis C virus infection and alanine transaminase levels in the general population: a survey in a southern Italian town. *J Hepatol* 2000; **33**: 116-120

22 **Okayama A**, Stuver SO, Tabor E, Tachibana N, Kohara M, Mueller NE, Tsubouchi H. Incident hepatitis C virus infection in a community-based population in Japan. *J Viral Hepat* 2002; **9**: 43-51

23 **Abdel-Aziz F**, Habib M, Mohamed MK, Abdel-Hamid M, Gamil F, Madkour S, Mikhail NN, Thomas D, Fix AD, Strickland GT, Anwar W, Sallam I. Hepatitis C virus (HCV) infection in a community in the Nile Delta: population description and HCV prevalence. *Hepatology* 2000; **32**: 111-115

24 **Medhat A**, Shehata M, Magder LS, Mikhail N, Abdel-Baki L, Nafeh M, Abdel-Hamid M, Strickland GT, Fix AD. Hepatitis c in a community in Upper Egypt: risk factors for infection. *Am J Trop Med Hyg* 2002; **66**: 633-638

25 **Hagan H**, Snyder N, Hough E, Yu T, McKeirnan S, Boase J, Duchin J. Case-reporting of acute hepatitis B and C among injection drug users. *J Urban Health* 2002; **79**: 579-585

26 **Robotin MC**, Copland J, Tallis G, Coleman D, Giele C, Carter L, Spencer J, Kaldor JM, Dore GJ. Surveillance for newly acquired hepatitis C in Australia. *J Gastroenterol Hepatol* 2004; **19**: 283-288

27 **Spada E**, Mele A, Ciccozzi M, Tosti ME, Bianco E, Szklo A, Ragni P, Gallo G, Balocchini E, Sangalli M, Lopalco PL, Moiraghi A, Stroffolini T. Changing epidemiology of parenterally transmitted viral hepatitis: results from the hepatitis surveillance system in Italy. *Dig Liver Dis* 2001; **33**: 778-784

28 **Alter MJ**, Hadler SC, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Moyer LA, Fields HA, Bradley DW. Risk factors for acute non-A, non-B hepatitis in the United States and association with hepatitis C virus infection. *JAMA* 1990; **264**: 2231-2235

29 **Armstrong GL**, Alter MJ, McQuillan GM, Margolis HS. The past incidence of hepatitis C virus infection: implications for the future burden of chronic liver disease in the United States. *Hepatology* 2000; **31**: 777-782

30 **Alter MJ**. Epidemiology of hepatitis C. *Hepatology* 1997; **26**: 62S-65S

31 **Prati D**, Capelli C, Silvani C, De Mattei C, Bosoni P, Pappalettera M, Mozzi F, Colombo M, Zanella A, Sirchia G. The incidence and risk factors of community-acquired hepatitis C in a cohort of Italian blood donors. *Hepatology* 1997; **25**: 702-704

32 **Deuffic S**, Buffat L, Poynard T, Valleron AJ. Modeling the hepatitis C virus epidemic in France. *Hepatology* 1999; **29**: 1596-1601

33 **Fukuizumi K**, Sata M, Suzuki H, Nakano H, Tanikawa K. Hepatitis C virus seroconversion rate in a hyperendemic area of HCV in Japan: a prospective study. *Scand J Infect Dis* 1997; 29: 345-347

34 **Mohamed MK**, Abdel-Hamid M, Mikhail NN, Abdel-Aziz F, Medhat A, Magder LS, Fix AD, Strickland GT. Intrafamilial transmission of hepatitis C in Egypt. *Hepatology* 2005; **42**: 683-687

35 **Perz JF**, Alter MJ. The coming wave of HCV-related liver disease: dilemmas and challenges. *J Hepatol* 2006; 44: 441-443

36 **Deuffic S**, Poynard T, Valleron AJ. Correlation between hepatitis C virus prevalence and hepatocellular carcinoma mortality in Europe. *J Viral Hepat* 1999; **6**: 411-443

37 **Deuffic-Burban S**, Mohamed MK, Larouze B, Carrat F, Valleron AJ. Expected increase in hepatitis C-related mortality in Egypt due to pre-2000 infections. *J Hepatol* 2006; **44**: 455-461

38 **Puro V**, Petrosillo N, Ippolito G. Risk of hepatitis C

seroconversion after occupational exposures in health care workers. Italian Study Group on Occupational Risk of HIV and Other Bloodborne Infections. *Am J Infect Control* 1995; **23**: 273-277

39 **Roberts EA**, Yeung L. Maternal-infant transmission of hepatitis C virus infection. *Hepatology* 2002; **36**: S106-S113

40 **Terrault NA**. Sexual activity as a risk factor for hepatitis C. *Hepatology* 2002; **36**: S99-105

41 **Williams IT**, Perz JF, Bell BP. Viral hepatitis transmission in ambulatory health care settings. *Clin Infect Dis* 2004; **38**: 1592-1598

42 **Hagan H**, Thiede H, Weiss NS, Hopkins SG, Duchin JS, Alexander ER. Sharing of drug preparation equipment as a risk factor for hepatitis C. *Am J Public Health* 2001; **91**: 42-46

43 **Kamili S**, Krawczynski K, McCaustland K, Li X, Alter MJ. Infectivity of hepatitis C virus in plasma after drying and storing at room temperature. *Infect Control Hosp Epidemiol* 2007;**28**:519-524

44 **Bond WW**, Favero MS, Petersen NJ, Gravelle CR, Ebert JW, Maynard JE. Survival of hepatitis B virus after drying and storage for one week. *Lancet* 1981; **1**: 550-551

45 **Gilbert N**, Corden S, Ijaz S, Grant PR, Tedder RS, Boxall EH. Comparison of commercial assays for the quantification of HBV DNA load in health care workers: calibration differences. *J Virol Methods* 2002; **100**: 37-47

46 **Busch MP**, Glynn SA, Stramer SL, Strong DM, Caglioti S, Wright DJ, Pappalardo B, Kleinman SH. A new strategy for estimating risks of transfusion-transmitted viral infections based on rates of detection of recently infected donors. *Transfusion* 2005; **45**: 254-264

47 **Hladik W**, Kataaha P, Mermin J, Purdy M, Otekat G, Lackritz E, Alter MJ, Downing R. Prevalence and screening costs of hepatitis C virus among Ugandan blood donors. *Trop Med Int Health* 2006; **11**: 951-954

48 **Hauri AM**, Armstrong GL, Hutin YJ. The global burden of disease attributable to contaminated injections given in health care settings. *Int J STD AIDS* 2004; **15**: 7-16

49 **Des Jarlais DC**, Diaz T, Perlis T, Vlahov D, Maslow C, Latka M, Rockwell R, Edwards V, Friedman SR, Monterroso E, Williams I, Garfein RS. Variability in the incidence of human immunodeficiency virus, hepatitis B virus, and hepatitis C virus infection among young injecting drug users in New York City. *Am J Epidemiol* 2003; **157**: 467-471

50 **Murray JM**, Law MG, Gao Z, Kaldor JM. The impact of behavioural changes on the prevalence of human immunodeficiency virus and hepatitis C among injecting drug users. *Int J Epidemiol* 2003; **32**: 708-714

51 **Thorpe LE**, Ouellet LJ, Hershow R, Bailey SL, Williams IT, Williamson J, Monterroso ER, Garfein RS. Risk of hepatitis C virus infection among young adult injection drug users who share injection equipment. *Am J Epidemiol* 2002; **155**: 645-653

52 **Alter MJ**. Hepatitis C: fact versus fiction. In: Margolis HS, Alter MJ, Liang J, Dienstag J, editors. Viral Hepatitis and Liver Disease. Atlanta: International Medical Press, 2002: 310-315

53 **Yazdanpanah Y**, De Carli G, Migueres B, Lot F, Campins M, Colombo C, Thomas T, Deuffic-Burban S, Prevot MH, Domart M, Tarantola A, Abiteboul D, Deny P, Pol S, Desenclos JC, Puro V, Bouvet E. Risk factors for hepatitis C virus transmission to health care workers after occupational exposure: a European case-control study. *Clin Infect Dis* 2005; **41**: 1423-1430

54 **Beltrami EM**, Kozak A, Williams IT, Saekhou AM, Kalish ML, Nainan OV, Stramer SL, Fucci MC, Frederickson D, Cardo DM. Transmission of HIV and hepatitis C virus from a nursing home patient to a health care worker. *Am J Infect Control* 2003; **31**: 168-175

55 **Mast EE**, Hwang LY, Seto DS, Nolte FS, Nainan OV, Wurtzel H, Alter MJ. Risk factors for perinatal transmission of hepatitis C virus (HCV) and the natural history of HCV infection acquired in infancy. *J Infect Dis* 2005; **192**: 1880-1889

56 **Alter MJ**, Gerety RJ, Smallwood LA, Sampliner RE, Tabor E, Deinhardt F, Frösner G, Matanoski GM. Sporadic non-A, non-B hepatitis: frequency and epidemiology in an urban U.S. population. *J Infect Dis* 1982; **145**: 886-893

57 **Alter MJ**, Coleman PJ, Alexander WJ, Kramer E, Miller JK, Mandel E, Hadler SC, Margolis HS. Importance of heterosexual activity in the transmission of hepatitis B and non-A, non-B hepatitis. *JAMA* 1989; **262**: 1201-1205

58 **Mele A**, Stroffolini T, Tosti ME, Corona R, Santonastasi F, Gallo G, Ragni P, Balocchini E, Bernacchia R, Moiraghi A. Heterosexual transmission of hepatitis C in Italy. *J Med Virol* 1999; **57**: 111-113

59 **Browne R**, Asboe D, Gilleece Y, Atkins M, Mandalia S, Gazzard B, Nelson M. Increased numbers of acute hepatitis C infections in HIV positive homosexual men; is sexual transmission feeding the increase? *Sex Transm Infect* 2004; **80**: 326-327

60 **Gambotti L**, Batisse D, Colin-de-Verdiere N, Delaroque-Astagneau E, Desenclos JC, Dominguez S, Dupont C, Duval X, Gervais A, Ghosn J, Larsen C, Pol S, Serpaggi J, Simon A, Valantin MA, Velter A. Acute hepatitis C infection in HIV positive men who have sex with men in Paris, France, 2001-2004. *Euro Surveill* 2005; **10**: 115-117

61 **Götz HM**, van Doornum G, Niesters HG, den Hollander JG, Thio HB, de Zwart O. A cluster of acute hepatitis C virus infection among men who have sex with men--results from contact tracing and public health implications. *AIDS* 2005; **19**: 969-974

62 **Rauch A**, Rickenbach M, Weber R, Hirschel B, Tarr PE, Bucher HC, Vernazza P, Bernasconi E, Zinkernagel AS, Evison J, Furrer H. Unsafe sex and increased incidence of hepatitis C virus infection among HIV-infected men who have sex with men: the Swiss HIV Cohort Study. *Clin Infect Dis* 2005; **41**: 395-402

63 **Hwang LY**, Kramer JR, Troisi C, Bull L, Grimes CZ, Lyerla R, Alter MJ. Relationship of cosmetic procedures and drug use to hepatitis C and hepatitis B virus infections in a low-risk population. *Hepatology* 2006; **44**: 341-351

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Cell culture systems for the hepatitis C virus

Gilles Duverlie, Czeslaw Wychowski

Gilles Duverlie, Virology Laboratory, Amiens University Hospital, Amiens, F-80054 Amiens cedex 1, France and Hepatitis C Unit, Institut de Biologie de Lille, 1 rue du Pr Calmette, BP 447, F-59021 Lille cedex, France
Czeslaw Wychowski, Hepatitis C Unit, Institut de Biologie de Lille, 1 rue du Pr Calmette, BP 447, F-59021 Lille cedex, France
Correspondence to: Gilles Duverlie, Laboratoire de Virologie, Hôpital Sud, CHU d'Amiens, 80054 Amiens cedex 1, France. gilles.duverlie@u-picardie.fr
Telephone: +33-322-827917 Fax: +33-322-827928
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Abstract

Since the discovery of HCV in 1989, the lack of a cell culture system has hampered research progress on this important human pathogen. No robust system has been obtained by empiric approaches, and HCV cell culture remained hypothetical until 2005. The construction of functional molecular clones has served as a starting point to reconstitute a consensus infectious cDNA that was able to transcribe infectious HCV RNAs as shown by intrahepatic inoculation in a chimpanzee. Other consensus clones have been selected and established in a human hepatoma cell line as replicons, i.e. self-replicating subgenomic or genomic viral RNAs. However, these replicons did not support production of infectious virus. Interestingly, some full-length replicons could be established without adaptive mutations and one of them was able to replicate at very high levels and to release virus particles that are infectious in cell culture and *in vivo*. This new cell culture system represents a major breakthrough in the HCV field and should enable a broad range of basic and applied studies to be achieved.

Key words: Hepatitis C virus; Biology; Cell Culture System; *In vitro* models

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INTRODUCTION

Since the discovery of the hepatitis C virus (HCV) *via* molecular cloning in 1989[1], its propagation in cell culture has been a major goal for virologists worldwide. All human hepatitis viruses are very difficult to grow in cell culture, and indeed no such system is currently available for the hepatitis B virus (HBV). This problem is also linked to the nature of the host cell, since highly differentiated human hepatocytes are very difficult to maintain in cell culture. However, it has been demonstrated that blood or serum from chronically infected HCV patients is infectious *in vivo* and does contain infectious virions suitable for the *in vitro* infection of cultured hepatocytes.

IN VITRO INFECTION BY HCV STRAINS FROM PATIENTS

In 1992, Shimizu *et al*[2] were the first to report successful HCV infection of the human T-lymphocyte MOLT-4Ma and HPB-Ma lines which had been pre-infected with murine retroviruses. In 1995, Kato *et al*[3] reported similar results with the MT-2 cell line pre-infected with HTLV-I. Use of clone 10-2 of the HPB-Ma cell line enabled a one-year follow-up of the infection, with neutralization by antibodies, inhibition of replication by interferon, visualisation of 50 nm particles by immuno-electronmicroscopy and analysis of the virion density[4]. However, in these and other cell lines (Daudi, PBMC, *etc*.), viral replication could be only intermittently detected by RT-PCR. The human hepatoma cell lines Huh-7 and Hep-G2 could support HCV replication, as did other hepatocytic cell lines immortalized by the SV-40 T antigen[5]. Ito *et al*[6] have cultured primary hepatocytes from chronically infected HCV patients and have obtained relatively high viral titers in both the cells and the supernatant medium. Primary chimpanzee and human hepatocytes are permissive to HCV for the very limited time during which these cells are usable[7-9]. These data indicate that HCV replication (at least) was possible in hepatocyte and lymphocyte cell lines. However, these systems were not reliable and could not be used as models for studying the viral cycle in detail or for screening antiviral drugs. These goals should be achieved by using viral and cellular clones under stable infection conditions[10]. Reverse genetics studies are also very helpful, and it is noteworthy that viral genomes are generally small enough to be engineered by today's oligonucleotide ligation techniques, marking the passage from macromolecular chemistry to "life"[11].

FULL-LENGTH, INFECTIOUS HCV CDNA

HCV is a member of the Flaviviridae and harbours an

envelope with two glycoproteins and a nucleocapsid containing a positive single-strand RNA genome of about 9600 nucleotides. The positive viral RNA (vRNA) has the same polarity as mRNA and can be directly translated in order to express the full set of viral proteins and initiate the viral life cycle. The vRNAs are generated by transcription of cloned, complementary DNA (cDNA) obtained by reverse transcription. The first cloned HCV genomes were incomplete, and a novel sequence at the 3' terminus of the hepatitis C virus was only discovered in 1995 by use of oligonucleotide ligation or synthesis at the 3'end of the HCV genome[12,13].

The first "infectious" cDNA from HCV genotype 1 was successfully obtained by two different groups in 1997[14,15]. The HCV genome (called "H77") was cloned from the serum of an infected patient with a high viral titer. The sequences of many different clones were aligned, a master consensus sequence was established and a full-length HCV consensus sequence was then generated. It was supposed that the viral quasispecies contained a majority of functional genomes with a minority of defective ones, as observed for other viruses[16]. Thus, the establishment of a master consensus sequence is one approach to detecting deleterious mutations in defective virions or mutations introduced by amplification and/or molecular cloning. The identification of an infectious cDNA was successfully achieved, since the transcribed RNAs were able to infect the chimpanzee when inoculated intrahepatically. Other full-length genomic HCV cDNAs have since been established but none of these clones have been adapted to cell culture, as only very low levels of replication were reported[15,17,18].

HCV SUBGENOMIC REPLICONS

New strategies based on the subgenomic selectable HCV replicon were developed in order to enable the selection of cell clones containing autonomously replicating HCV RNAs. In 1999, Lohmann *et al*[19] established the first HCV genotype 1b replicon in the Huh-7 hepatoma cell line. Replicons are subgenomic constructs expressing the viral replicase complex and capable of autonomous viral replication. As with other positive RNA viral genomes, the authors constructed bicistronic vectors-allowing the exact transcription of the HCV 5' and 3' non-translated regions (NTRs)-and then used (1) the HCV internal ribosomal entry site (IRES) to ensure translation of the neomycin phosphotransferase gene (neo) and (2) the EMCV IRES to ensure translation of the HCV replicase complex (at least from NS3 to NS5B). Thus, neomycin-resistant cell clones containing replicating HCV RNAs were then selected. This successful approach enabled the selection of highly permissive cell clones (such as Huh-7.5 and Huh-7-Lunet[20,21]) and adaptive viral mutations (involving principally the NS3, NS4B and NS5A regions[22]). Several of these mutations alter the phosphorylation state of NS5A, the hyperphosphorylated form of which appears to be deleterious for efficient HCV replication[23,24]. These replicons have been very valuable tools for studying HCV replication and for testing antiviral drugs against HCV's major target enzymes, i.e. NS3 helicase, NS3/4A protease and NS5B polymerase. Full-length genomic replicons using the consensus approach (such as the wild type Con-1) failed to produce infectious particles when tested, since very low numbers of viral particles were obtained[25]. Indeed, the different markers enabled the selection of replicative genomic sequences but were not appropriate for selecting for viral particle secretion[26]. Selective pressure acts on the replicase complex (involving most of HCV's non-structural proteins) but not on structural proteins. Furthermore, at least some adaptive mutations seemed to optimize viral replication and impair viral particle production. Interestingly, some replicons were reported as being free of adaptive mutations and were able to replicate at a very high rate[27].

VIRAL PRODUCTION IN CELL CULTURE

Eventually, one of the above-mentioned full-length replicons was found to release viral particles[28]. The replicon was a HCV genotype 2a clone called JFH-1, constructed by Takaji Wakita's group and isolated from a Japanese patient with fulminant hepatitis[29]. It is the first authentic HCV clone considered by the scientific community as capable of growing in cell culture. Transfection of the full-length JFH-1 genome into Huh-7 cells leads to the production of HCV particles that are infectious for naive cells and for chimpanzees. Given that full-length genomic RNA derived from other clones of the same genotype or different HCV genotypes was again not infectious in a cell culture system, chimeric viruses were constructed successfully. Hence, the sequences coding for the HCV JFH-1 structural proteins were replaced by the corresponding sequences from different HCV genotypes[30,31]. The HCV chimera's junction was situated in the NS2 protein, just after the first transmembrane domain in a junction region which has also been identified in the rare, naturally-occurring recombinant forms of HCV (generally found in isolates from intravenous drug users[32]). However, the viral titer obtained for most constructs was low, with the exception of the FL-J6-JFH1 chimeric 2a-2a recombinant virus. This homologous, chimeric form was shown to be infectious in the chimpanzee, and the virus recovered from the infected animal was infectious in cell culture. Comparison of the viral titers obtained by Dr. Wakita's group with those observed by other groups indicates that cell culture conditions may influence viral production. Continuous passages of infected cells or successive infections of naive cells with the supernatant of infected cells leads to an increase in viral production. This finding suggests that certain mutations are probably selected during the infection. Determinants present in the structural proteins may be important for viral release and infection in cell culture. Thus, a detailed study of the key features enabling operation of the complete HCV cell cycle should enable researchers to cultivate other isolates. Very recently, a genotype 1a (H77) virus production system producing low viral titers has been described[33].

Another improvement concerns the cell line used for HCV culturing. Certain clones of the Huh-7 cell line

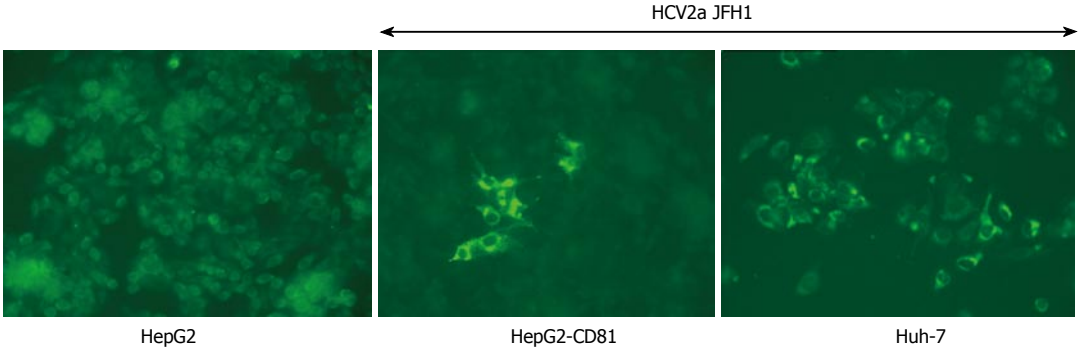

Figure 1 Infection of different hepatoma cell lines by JFH1 virus. HepG2 not expressing the CD81 receptor, HepG2 expressing the CD81 receptor and Huh-7 cell- lines were used for the infection. Cells seeded at between 3 and 5. 10^5 cells per well in 24-well plates were incubated for 2 h at 37℃ with 200 mL of culture medium containing infectious JFH1 virus (obtained after successive re-infection of naive Huh-7 cells). Cells were processed and immunostained for the detection of capsid, as previously described (Rouillé *et al*., 2006, *J Virol*, 80). The Figure shows the detection of HCV capsid protein in HepG2-CD81 and Huh-7 cells but not in HepG2 cells. Re-infection of naive HepG2-CD81 and Huh-7 cells is effective with the resulting supernatant from each infection.

were found to far better than others for growing HCV. "HCV-cured clones" produced by antiviral agents and Huh-7 subclones (such as Huh-7.5 and now Huh-7.5.1[34] or Huh-7-Lunet) support more efficient viral replication and production. Detailed studies have shown that some important features (such as high levels of CD81 receptor expression or perhaps SR-B1 or claudin-1 very recently identified by the Rice's group) could increase viral production and spreading. Indeed, as shown in Figure 1, HepG2 cells which are not usually susceptible to HCV can be infected by the JFH1 virus when they express CD81[30]. Furthermore, defects in innate immunity (such as deficiencies in interferon induction or production) could increase the cell line's permissivity[35,36]. Identification of new cell lines of hepatic (or perhaps lymphocyte) origin could improve our understanding of certain physiopathological aspects of hepatitis C. Equally, HCV production in cell lines from other species (such as murine cell lines) could help to establish a small animal model of HCV infection.

CONCLUSION

The new cell culture system represents a major breakthrough in the HCV field and should enable a broad range of fundamental and applied studies to be achieved, including the production of classic HCV vaccines for efficacy testing. Detailed cellular, molecular and reverse genetics studies are also needed to complete our knowledge of all aspects of HCV's biology.

The identity of the infectious form of HCV in "real life" remains open to debate because HCV in sera is strongly associated with lipoproteins; certain features (such as the infectious form's density) appear to differ from those seen in the HCV particles produced in cell culture systems (HCVcc). New information concerning the receptor(s) involved and other important determinants of permissivity will be valuable for improving the culture system. These new insights could enable researchers to grow clinical HCV isolates and study their particular features.

REFERENCES

1 **Choo QL**, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359-362
2 **Shimizu YK**, Iwamoto A, Hijikata M, Purcell RH, Yoshikura H. Evidence for in vitro replication of hepatitis C virus genome in a human T-cell line. *Proc Natl Acad Sci USA* 1992; **89**: 5477-5481
3 **Kato N**, Nakazawa T, Mizutani T, Shimotohno K. Susceptibility of human T-lymphotropic virus type I infected cell line MT-2 to hepatitis C virus infection. *Biochem Biophys Res Commun* 1995; **206**: 863-869
4 **Shimizu YK**, Purcell RH, Yoshikura H. Correlation between the infectivity of hepatitis C virus in vivo and its infectivity *in vitro*. *Proc Natl Acad Sci USA* 1993; **90**: 6037-6041
5 **Seipp S**, Mueller HM, Pfaff E, Stremmel W, Theilmann L, Goeser T. Establishment of persistent hepatitis C virus infection and replication *in vitro*. *J Gen Virol* 1997; **78**(Pt 10): 2467-2476
6 **Ito T**, Mukaigawa J, Zuo J, Hirabayashi Y, Mitamura K, Yasui K. Cultivation of hepatitis C virus in primary hepatocyte culture from patients with chronic hepatitis C results in release of high titre infectious virus. *J Gen Virol* 1996; **77**(Pt 5): 1043-1054
7 **Lanford RE**, Sureau C, Jacob JR, White R, Fuerst TR. Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. *Virology* 1994; **202**: 606-614
8 **Iacovacci S**, Manzin A, Barca S, Sargiacomo M, Serafino A, Valli MB, Macioce G, Hassan HJ, Ponzetto A, Clementi M, Peschle C, Carloni G. Molecular characterization and dynamics of hepatitis C virus replication in human fetal hepatocytes infected *in vitro*. *Hepatology* 1997; **26**: 1328-1337
9 **Fournier C**, Sureau C, Coste J, Ducos J, Pageaux G, Larrey D, Domergue J, Maurel P. In vitro infection of adult normal human hepatocytes in primary culture by hepatitis C virus. *J Gen Virol* 1998; **79**(Pt 10): 2367-2374
10 **Bartenschlager R**. Hepatitis C virus molecular clones: from cDNA to infectious virus particles in cell culture. *Curr Opin Microbiol* 2006; **9**: 416-422
11 **Cello J**, Paul AV, Wimmer E. Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. *Science* 2002; **297**: 1016-1018
12 **Tanaka T**, Kato N, Cho MJ, Shimotohno K. A novel sequence found at the 3' terminus of hepatitis C virus genome. *Biochem Biophys Res Commun* 1995; **215**: 744-749
13 **Kolykhalov AA**, Feinstone SM, Rice CM. Identification of a highly conserved sequence element at the 3' terminus of hepatitis C virus genome RNA. *J Virol* 1996; **70**: 3363-3371

14 **Kolykhalov AA**, Agapov EV, Blight KJ, Mihalik K, Feinstone SM, Rice CM. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. *Science* 1997; **277**: 570-574

15 **Yanagi M**, Purcell RH, Emerson SU, Bukh J. Transcripts from a single full-length cDNA clone of hepatitis C virus are infectious when directly transfected into the liver of a chimpanzee. *Proc Natl Acad Sci USA* 1997; **94**: 8738-8743

16 **Moormann RJ**, van Gennip HG, Miedema GK, Hulst MM, van Rijn PA. Infectious RNA transcribed from an engineered full-length cDNA template of the genome of a pestivirus. *J Virol* 1996; **70**: 763-770

17 **Beard MR**, Abell G, Honda M, Carroll A, Gartland M, Clarke B, Suzuki K, Lanford R, Sangar DV, Lemon SM. An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 1999; **30**: 316-324

18 **Lanford RE**, Lee H, Chavez D, Guerra B, Brasky KM. Infectious cDNA clone of the hepatitis C virus genotype 1 prototype sequence. *J Gen Virol* 2001; **82**: 1291-1297

19 **Lohmann V**, Körner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110-113

20 **Blight KJ**, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 2002; **76**: 13001-13014

21 **Friebe P**, Boudet J, Simorre JP, Bartenschlager R. Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. *J Virol* 2005; **79**: 380-392

22 **Bartenschlager R**, Frese M, Pietschmann T. Novel insights into hepatitis C virus replication and persistence. *Adv Virus Res* 2004; **63**: 71-180

23 **Evans MJ**, Rice CM, Goff SP. Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. *Proc Natl Acad Sci USA* 2004; **101**: 13038-13043

24 **Neddermann P**, Quintavalle M, Di Pietro C, Clementi A, Cerretani M, Altamura S, Bartholomew L, De Francesco R. Reduction of hepatitis C virus NS5A hyperphosphorylation by selective inhibition of cellular kinases activates viral RNA replication in cell culture. *J Virol* 2004; **78**: 13306-13314

25 **Ikeda M**, Yi M, Li K, Lemon SM. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. *J Virol* 2002; **76**: 2997-3006

26 **Bukh J**, Pietschmann T, Lohmann V, Krieger N, Faulk K, Engle RE, Govindarajan S, Shapiro M, St Claire M, Bartenschlager R. Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc Natl Acad Sci USA* 2002; **99**: 14416-14421

27 **Yi M**, Bodola F, Lemon SM. Subgenomic hepatitis C virus replicons inducing expression of a secreted enzymatic reporter protein. *Virology* 2002; **304**: 197-210

28 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796

29 **Kato T**, Furusaka A, Miyamoto M, Date T, Yasui K, Hiramoto J, Nagayama K, Tanaka T, Wakita T. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. *J Med Virol* 2001; **64**: 334-339

30 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626

31 **Pietschmann T**, Kaul A, Koutsoudakis G, Shavinskaya A, Kallis S, Steinmann E, Abid K, Negro F, Dreux M, Cosset FL, Bartenschlager R. Construction and characterization of infectious intragenotypic and intergenotypic hepatitis C virus chimeras. *Proc Natl Acad Sci USA* 2006; **103**: 7408-7413

32 **Kalinina O**, Norder H, Mukomolov S, Magnius LO. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. *J Virol* 2002; **76**: 4034-4043

33 **Yi M**, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA* 2006; **103**: 2310-2315

34 **Zhong J**, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299

35 **Sumpter R**, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 2005; **79**: 2689-2699

36 **Windisch MP**, Frese M, Kaul A, Trippler M, Lohmann V, Bartenschlager R. Dissecting the interferon-induced inhibition of hepatitis C virus replication by using a novel host cell line. *J Virol* 2005; **79**: 13778-13793

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

The interferon inducing pathways and the hepatitis C virus

Eliane F Meurs, Adrien Breiman

Eliane F Meurs, Pasteur Institute, Hepacivirus Unit, F-75015 Paris cedex, France
Adrien Breiman, Imperial College, Faculty of Medicine, Department of Virology, London W2 1PG, United Kingdom
Supported by grants from the Agence Nationale pour la Recherche contre le SIDA
Correspondence to: Eliane F Meurs, Hepacivirus Unit, Department of Virology, Pasteur Institute, 28 rue du Dr Roux, 75724 Paris Cedex 15, France. emeurs@pasteur.fr
Telephone: +33-1-45688777 Fax: +33-1-40613012
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Abstract

The innate immune response is triggered by a variety of pathogens, including viruses, and requires rapid induction of type I interferons (IFN), such as IFNβ and IFNα. IFN induction occurs when specific pathogen motifs bind to specific cellular receptors. In non-professional immune, virally-infected cells, IFN induction is essentially initiated after the binding of dsRNA structures to TLR3 receptors or to intracytosolic RNA helicases, such as RIG-I /MDA5. This leads to the recruitment of specific adaptors, such as TRIF for TLR3 and the mitochondrial-associated IPS-1/VISA/MAVS/CARDIF adapter protein for the RNA helicases, and the ultimate recruitment of kinases, such as MAPKs, the canonical IKK complex and the TBK1/IKKε kinases, which activate the transcription factors ATF-2/c-jun, NF-κB and IRF3, respectively. The coordinated action of these transcription factors leads to induction of IFN and of pro-inflammatory cytokines and to the establishment of the innate immune response. HCV can cleave both the adapters TRIF and IPS-1/VISA/MAVS/CARDIF through the action of its NS3/4A protease. This provokes abrogation of the induction of the IFN and cytokine pathways and favours viral propagation and presumably HCV chronic infection.

Key words: Toll-like receptor; RNA helicase; Mitochondrial adapter Cardif; TBK1/IKKepsilon; Interferon induction; HCV NS3A protease

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INTRODUCTION

The innate immune response is triggered in response to a variety of pathogens, such as bacteria and viruses, and is essential for a rapid limitation in the spread or action of these pathogens. The typeI interferons, represented by IFNβ and by different subtypes of IFNα, play an important role in this process as they can mount an immediate antiviral response and stimulate adaptative immunity[1] TypeI interferons are secreted proteins that exert their function after binding to specific IFNAR receptors and after activation of the JAK/STAT signalling pathway[2]. They are potent and can induce over 300 genes, collectively referred to as interferon stimulated genes (ISGs)[3]. Because of their antiviral, antiproliferative and immunomodulatory activities, IFNs are used for the treatment of different tumors and viral infections, such as HBV and HCV. Under normal physiological and health conditions, however, IFNs are expressed at a minimum level and their induction in response to a pathogen involves a complex and well orchestrated cellular machinery.

Microbial agents are recognized through some of their motifs or pathogen-associated molecular patterns (PAMPS) by specific cellular receptors, referred to as PRRs (pathogen-recognition receptors). The PAMPs responsible for IFN induction can be bacterial external compounds, such as lipopolysacharides (LPS), viral envelopes and nucleic acids including dsRNA, ssRNA and DNA. The PRRs are members of the toll-like receptors (TLRs) family, located either at cellular (TLR4 for LPS and viral envelops) or endosomal membranes (TLR3 for dsRNA, TLR7/8 for ssRNA and TLR9 for DNA)[4-7]. In addition to this, another route of induction takes place in the cytosol through activation of specific RNA helicases, such as RIG-I and MDA5[8,9]. The cellular type plays an important role in the specificity of induction, since IFN is induced in the immune plasmacytoid cells through TLR7/8 (ssRNA) and TLR9 (DNA), while its induction in the non-professional immune cells, including dendritic cells (DCs), requires the endosomal TLR3 or the intracytoplasmic RNA helicase[10].

At the transcriptional level, IFN induction requires the conjugated action of the three transcription factors: ATF2/c-jun, NF-κB and IRF3. ATF2/c-jun and NF-κB are activated in response to various stimuli, such as growth factors and pro-inflammatory molecules, by phosphorylation through the MAPK cascade and the canonical IKK α/β/γ complex, respectively. IRF3 phosphorylation is triggered by

a viral infection, after incubation of cells with dsRNA or after introduction of dsRNA by transfection[11]. The kinases responsible for IRF3 phosphorylation, which were referred to as VAK for virus-activated kinases for some years[11], were identified in 2003 as the two non-canonical IκB kinases: TBK1 (Tank binding kinase 1, also known as NAK for NF-κB activating kinase) and IKKε[12,13].

THE TLR3/TRIF PATHWAY

After binding to their respective PAMPs, the TLRs recruit adaptor proteins through homotypic interactions with their cyoplasmic TIR (Toll/IL-1 receptor) domain. All TLRs, except TLR3, recruit MyD88, which links them to the NF-κB and MAPK pathways through activation of the IRAKs (IL-1 receptor associated kinases) and TRAF6 (TNF receptor associated factor 6). In contrast, TLR3, upon dsRNA binding, recruits a different adapter, named TRIF (TIR domain-containing adapter inducing IFN-β) or TICAM (TIR-containing adaptor molecule-1)[14,15]. TLR4 can also induce IFN through TRIF, but, in that case, it recruits TRIF indirectly through interaction with another adaptor called TRAM (TRIF related adaptor molecule) or TICAM-2[16]. TRIF interacts with a number of signaling molecules, such as TRAF6, which in turn activate the NF-κB and MAPK pathways[17,18]. In addition, it can also activate NF-κB through interaction with the kinase RIP-1 (receptor interacting protein-1)[19]. Importantly, TRIF is involved in IRF3 activation by recruiting TBK1 and, presumably, also IKKε, although this remains controversial[13,17]. TRIF can also recruit TRAF6, which activates the NF-κB and the MAPK pathways[17,18], NAP-1 (NAK associated protein-1) and TRAF3, which are both involved in IFN induction, although their exact role still needs to be clarified[20,22]. Through all these different interactions, TRIF triggers induction of IFN and pro-inflammatory cytokines (Figure 1).

In accord with a role for TLR3 in IFN induction in response to dsRNA, TLR3 deficient mice present a strong reduction in their ability to induce IFN and proinflammatory cytokines when injected with synthetic or viral dsRNA. These mice are also more susceptible to infection by MCMV[5]. TLR3 is expressed in endosomal compartments and is abundant in conventional dendritic cells, therefore allowing immediate activation of the immune response. TLR3 has also been shown to be important in crosspriming, therefore allowing CD8+ T-cell response at the site of virally-infected tissue cells[23]. However, the role of TLR3 in the antiviral response is probably more complex. For instance, infection of TLR3-deficient mice with West Nile virus (WNV) resulted in a better survival of these mice to the infection than the TLR3-wt mice, in which the infection provoked a TLR3-dependent inflammatory response, associated with brain penetration of the virus and neuronal injury[24]. Similarly, TLR3-deficient mice had an unexpected survival advantage to Influenza A virus (IAV), which is a highly contagious acute respiratory disease, despite a higher viral production in the lungs, because these animals displayed significantly reduced inflammatory mediators than the wt animals in the bronchoalveolar airspace[25].

Like TLR3, the TLR 7, 8 and 9 are expressed in endosomal compartments but their expression is restricted to a subtype of dendritic cells, which are the plasmacytoid dendritic cells (pDCs). These latter cells represent less than 1% of the circulating PBMCs but can release massive amounts of IFN-α in the blood. They were previously also named NIPC for Natural Interferon Producing Cells[1]. Induction of IFN by these TLRs, in response to ssRNA (TLR7.8) or DNA (TLR9) is exclusively MyD88-dependent and does not require TRIF. In this situation, the signaling events leading to IFN induction involve recruitment of TRAF6, IRAK4 and the transcription factor IRF7. This provokes IRF-7 phosphorylation and direct induction of IFNα. This signaling process is possible because of the constitutive presence of IRF7 in the pDCs[26,28].

THE RNA HELICASE PATHWAY

TLR3- and TRIF-deficient mice are impaired in their ability to induce IFN in response to dsRNA and they may become sensitive to viral infections[5,29]. However, their response to dsRNA is not totally abolished and they are still resistant to some viral infections, such as VSV (vesicular stomatitis virus) or SeV (Sendai virus)[30]. This suggested the existence of novel IFN-inducing pathways, which were independent of the TLR3/TRIF axis. A role for the dsRNA-dependent protein kinase PKR was unlikely since earlier studies showed that this kinase was not required for IRF3 or IRF7 phosphorylation[31]. Furthermore, the induction of T cell stimulatory molecules was normal and induction of IFN-β was only slightly suppressed in DC from PKR-deficient mice[32].

Human K562 cells lack the entire IFN-encoding locus and do not activate IRF3 after virus infection, unless they are treated with IFNβ. An expression cDNA library generated from IFNβ-treated K562 cells was therefore generated and screened for stimulation of the transcription of an IRF-dependent reporter in the presence of the synthetic dsRNA poly(I)-poly(C), after transfection in murine L929 cells. This led to the isolation of one clone encoding for the caspase activation recruitment domain (CARD)-containing the N terminal domain of the DexD/H box helicase RIG-I [8]. The presence of CARD allows recruitment of proteins through homotypic interactions. RIG-I was initially described as being induced by Retinoic Acid (RIG = retinoic acid inducible gene)[33]. It was also shown to be induced by IFNγ[34], TNF-α and IFNα[35]. RIG-I belongs to a family of RNA helicases that also contains MDA-5 (melanoma differentiation associated gene-5)[36] and LGP2[37]. MDA-5 is highly homologous to RIG-I (23% identity in the CARD and 35% identity in the helicase domain). Both proteins bind dsRNA and transmit signaling through their RNA helicase/ATPase domain, probably by a conformational change, which enables their N-terminal CARD domain to initiate the downstream signaling events leading to ATF2/C-jun, NF-κB and IRF3 activation. In contrast, LGP2, which shows 31% and 41% identity with the RNA helicase domains of RIG-I and MDA5, respectively, lacks the CARD domain and thus probably has a negative regulatory role on the RIG-I /MDA5 pathway[38]. It is interesting to note that all three RNA helicases, i.e., RIG-I , MDA5 and

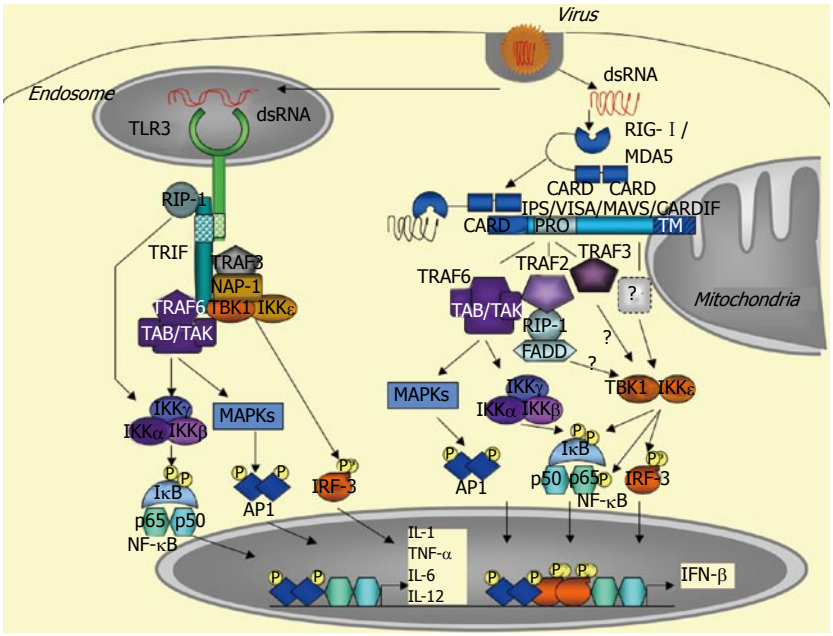

Figure 1 Schematic representation of the dsRNA-activated TLR3 and RNA helicase IFN inducing pathways. dsRNA: double-stranded RNA; TLR3: Toll-like receptor 3; RIP-1 Receptor interacting protein -1; TRIF: TIR (Toll/IL-1-like receptor) domain-containing adapter inducing IFN-β; TRAF2, TRAF3 and TRAF6: TNFR-associated factor; NAP-1: NAK (NF-κB activating kinase) Associated Protein-1; TBK1: TANK (TRAF family member-associated NFκB activator) Binding Kinase 1 (also known as NAK); IKKε: IkB kinase epsilon; TAB: TAK binding protein; TAK: TGFβ-associated kinase; IKKγ: also known as NEMO (NF-κB Essential Modulator), associates with the IKKα and IKKβ kinases; IKKα IκB Kinase α; IKKβ IκB Kinase β; MAPK: Mitogen activated protein kinase; IkB: Inhibitor NF-κB; P65: NF-κB subunit; P50: NF-κB subunit; AP1: ATF2/c-jun transcription factor; IRF3: Interferon regulatory factor 3; RIG-I: Retinoic inducible gene-1; MDA5: Melanoma differentiation associated gene 5; CARD: Caspase association recognition domain; IPS-1: Interferon-β promoter stimulator 1; MAVS: Mitochondrial antiviral signaling; VISA: Virus-induced signaling adaptor; CARDIF: CARD adapter inducing IFNβ; PRO: Proline rich domain; FADD: FAS associated protein *via* death domain. IL-1, IL-6 and IL-12: Interleukines, TNF-α: Tumor necrosis factor α;

LGP2, are IFN-inducible proteins. Yet, they are directly involved in the very first step of IFN induction, at least for RIG-I and MD-5. This indicates that the early steps of IFN induction already require significant cytosolic expression of these RNA helicases. Accordingly, RIG-I can be independently induced in response to different stimuli, such as TNF-α[35]. It is therefore possible that virally-induced inflammatory processes are important to generate the required amounts of RIG-I necessary for triggering IFN induction. Although RIG-I and MDA5 present strong similarities, they apparently do not have similar functions in cells. For instance, deletion of the RIG-I gene is lethal with most of the embryos dying between 12 and 14 d and with mice born alive dying after 3 wk. The developmental defect of the RIG-I -/- embryos was linked to massive liver degeneration[10]. In contrast, MDA-5 deficient mice are healthy[39]. Most cell types derived from the RIG-I deficient embryo are unable to produce type I IFN and inflammatory cytokines, such as IL-6, upon SeV infection[10]. Interestingly, systematic comparison of RIGI and MDA5 deficient MEFs showed that the two RNA helicases differ in their specificity for IFN induction. RIG-I was required for IFN induction by *in vitro* transcribed RNAs and by the following viruses: SeV, NDV, influenza virus, VSV or JEV, while MDA5 was required for IFN induction by poly(I)-poly(C) and the picornaviruses EMCV, Theiler and Mengo[39,40]. The reason for this discrepancy was recently solved independently by two groups who showed that the ligand for RIG-I is an uncapped 5' triphosphate RNA, which is a situation found in viruses of the Flaviviridae family, including HCV, and non segmented viruses, such as Paramyxoviruses and rhabdoviruses. In contrast, MDA5 recognize viruses with protected 5' RNA ends, such as in the case of picornaviruses[41,42].

THE IPS-1/VISA/MAVS/Cardif ADAPTER

In 2005, four groups independently reported the identification of an adapter protein that links the RNA helicase to the downstream MAPK, NF-κB and IRF3 signaling pathways. This protein is referred to as IPS-1 (Interferon-β Promoter Stimulator 1[43]), VISA (Virus-Induced Signaling Adaptor[44]), MAVS (Mitochondrial AntiViral Signaling[45]) and CARDIF (CARD adapter inducing IFNβ[46]). This protein was previously identified in a cDNA library screen as a NF-κB activating molecule[47]. In the absence of a consensus name for this protein, it will be referred to here as CARDIF, in acknowledgment of the group that first presented the sensitivity of this protein to cleavage by the HCV NS3/4A protease (46; see below). The particularity of this adapter protein is localization to the mitochondrial membrane through a specific transmembrane domain located at its C terminus[45]. The 540 residue CARDIF protein associates with the tandem CARD domain of RIG-I through its own N-terminal 1-77 CARD-like domain. Coprecipitation assays showed that CARDIF associates strongly with RIG-I and weakly with MDA5[46]. Another important feature of CARDIF is a proline rich region (103-173 residues) near the N terminus, through which it interacts with several signaling components including TRAF6, TRAF2[44], RIP1, FADD[43] and more recently, TRAF3[48] (Figure 1). It is still unclear how TBK1 and IKKε associate with CARDIF. In one report, TBK1 was found to associate with CARDIF and IKKε was not examined[44]. In another study, neither of these kinases was found to associate with CARDIF but the data were presented only for TBK1[43]. In contrast, a strong association of CARDIF with IKKε and no interaction with TBK1 was presented[46]. In accord with the latter, recent confocal microscopy analysis demonstrated a tight colocalization for IKKε with the mitochondrial protein CARDIF, whereas TBK1 was associated with other vesicles[49]. Using coprecipitation techniques, our group also recently confirmed association of CARDIF with IKKε but not with TBKI (Vitour *et al*, unpublished observations). The CARDIF deficient mice are viable and fertile. Upon viral infection, such as VSV, they can produce IFNα and

IFNβ in their sera, as measured by ELISA, presumably through TLR activation, for instance in pDCs. However, they failed to produce IFNα, IFNβ and IL-6 after poly(I)-poly(C) injection, which is reminiscent of the defect in MDA5 deficient mice to respond to poly(I)-poly(C)[39]. Infection of the CARDIF -/- and -/+ mice with different concentrations of VSV showed a VSV-induced mortality that was both dependent on the CARDIF gene dosage and viral titer. Since VSV was shown to induce IFN through RIG-I , these *in vivo* experiments firmly demonstrate that CARDIF is involved in both the IFN inducing pathways mediated by RIG-I and MDA5[50].

THE TBK1 AND IKKε KINASES

The TBK1 and IKKε kinases play an essential role in the induction of IFN and inflammatory cytokines through their ability to phosphorylate serine residues at the C terminus of both IRF3 and IRF7. This provokes a change in the conformation of these transcription factors, promoting their dimerization and then their binding to their DNA consensus binding sites[12,13,51]. The two kinases are enzymatically similar with strong sequence identity. Accordingly, they behave similarly in their ability to activate IRF3 and IRF7 and in their ability to phosphorylate the IκBα inhibitor of NF-κB at its Ser 36 residue, whereas the two structurally related IKKα and IKKβ kinases phosphorylate IκBα at residues Ser32 and Ser36[52]. Although very similar, TBK1 and IKKε present some differences that may be of importance. For instance, deletion of the TBK1 gene leads to embryonic lethality at d 15[53], wheras IKKε deficient mice are viable[54]. Another difference is the fact that IKKε is more closely associated with CARDIF than TBK1[46,49]. Finally, both IKKε and TBK1 were shown to sustain the NF-κB transcriptional activity through the phosphorylation of specific serine residues at the C terminal transactivation domain of the cRel[55] or RelA p65 subunit[56,57]. Interestingly, however, IKKε was found to play a more critical role than TBK1 in controlling the basal/constitutive p65 phosphorylation[57]. This new finding coupled to the fact that IKKε can sustain its own expression *via* NF-κB and c/EBPδ transcription factors[58,59], whereas expression of TBK1 is constitutive, provides a link to suspect a role for IKKε in controlling the proliferation of certain cancer cells. In contrast, TBK1 could play a major role in IFNβ induction. Indeed, studies with TBK1 and IKKε murine deficient cells pointed out a more important role for TBK1 than for IKKε in IFN induction in response to LPS, dsRNA (delivered intracytoplasmically) and to virus infection. However, use of the IKKε/TBK1 doubly deficient cells revealed a complete abolition of IFNβ induction[54].

THE HCV NS3/4A PROTEASE AND THE IFN INDUCING PATHWAYS

The current treatment against HCV, a combination of pegylated IFN and ribavirin, leads to viral clearance in 50% to 80% of cases. The efficacy of treatment depends on several factors, such as age and sex of the patients, viral parameters, such as genotypes and viral load, and host immune parameters[60]. HCV can interfere with the cellular response to IFN through some of its proteins, which can target the JAK/STAT signaling pathway that is activated in response to the binding of IFN to its receptor[61-63], or through other interactions leading to inhibition of the induction or the function of some ISGs[64]. In 2003, the HCV NS3/4A protease was shown to interfere with IFN induction by preventing IRF3 phosphorylation[65]. This important finding was achieved at the same time as the identification of the two kinases leading to IRF3-phosphorylating kinases, TBK1 and IKKε[12,13].

The HCV 70 Kda NS3 protein presents a serine proteinase domain at its N terminus (aa 1-180) and an RNA helicase domain at its C-terminus (aa 181-631) (reviewed in[66]). Its helicase activity is coordinated by ATP and allows NS3 to move along the RNA like an inchworm to catalyse RNA unwinding[67]. This activity is important for HCV replication. The protease activity catalyses the following cleavages of the viral polyprotein: NS3-NS4A, NS4A-NS4B, NS4B-NS5A and NS5A-NS5B with the following efficiency: NS5A/5B > NS4A/4B > NS4B/5A. Cleavage between NS3 and NS4A is an intramolecular reaction and the rest of the cleavages are mediated *in trans*[68]. NS4A is a small protein of 54 aa that acts as a cofactor to enhance the NS3 protease activity. For this reason, and also because the NS3 requires a noncatalytic structural zinc ion for its protease activity, this enzyme is unique among the other members of the trypsin superfamily to which it belongs[66]. The catalytic domain of NS3 is formed by a triad of three important residues, S139, H57 and D81. After cleavage of the NS3/4A junction, the C-terminus of NS3 forms a β-strand that occupies the proteinase active site and thus protects it. In contrast with other proteinases, the substrate binding site of NS3 is shallow and solvent-exposed and its selective recognition of substrates requires extended contact surface. The active site of NS3 is well conserved among the different genotypes and the HCV sequences that are cleaved by NS3/4A have the consensus sequence D/E-XXXX-C/T ↓S/A-XX-L/W/Y (Table 1). The cleavage sequence and protease specificity of NS3/4A protease have been well characterized since 1993 and it was conceivable that, due to its ability to cleave in trans, the NS3/4A protease may be able to cleave cellular proteins in addition to the processing of the viral proteins.

Analysis of the mechanism(s) by which NS3/4A was inhibiting the IRF3 phosphorylation allowed determine that this protease was acting upstream of the two TBK1/IKKε kinases and was affecting both the TLR3/TRIF pathway and the RIG-I helicase pathway[69,70]. Disruption of IFN induction through TLR3/TRIF was shown to be due to the cleavage of the TLR3 adapter TRIF by NS3/4A[71], while disruption of IFN induction through the RIG-I pathway was due to the cleavage of the mitochondrial adapter protein IPS-1/MAVS/VISA/CARDIF[46,49,72]. Cleavage of TRIF occurs between its Cys372 and Ser373 residues, which separates its TIR domain from the TBK1-interacting N terminus domain. It is interesting to note that the TRIF cleavage site (PSSTPC↓SAHLT) differs from

Table 1 Sequence of the NS3/4A -mediated cleavages in HCV polyprotein from different genotypes

Name	Genotype (GI)	NS3/4A	NS4A/4B	NS4B/5A	NS5A/5B	Reference
H77	1a (GI: 2316097)	MSADLEVVT STWVLVGG	QEFDEMEEC SQHLPYIE	ISSECTTPC SGSWLRDI	ADTEDVVCC SSYSWTGA	Kolykhalov *et al*, 1997
HCV-N	1b (GI: 23957856)	MSADLEVVT STWVLVGG	REFDEMEEC ASHLPYIE	INEDCSTPC SGSWLRDV	EAGESVVCC SMSYTWTG	Beard *et al*, 1999
JFH1	2a (GI: 13122261)	MQADLEVMT STWVLAGG	EAFDEMEEC ASRAALIE	ITEDCPIPC SGSWLRDV	EEDDTTVCC SMSYSWTG	Kato *et al*, 2001
NZL1	3a (GI: 514395)	MSADLEVTT STWVLLGG	QQYDEMEEC SQAAPYIE	INEDYPSPC SDDWLRTI	SEEQSVVCC SMSYSWTG	Sakamoto *et al*, 1994
ED43	4a (GI: 2252489)	MSADLEVVT STWVLVGG	QQFDEMEEC SKHLPLVE	INEDCSTPC STPCAESW	SGSEDVVCC SMSYSWTG	Chamberlain *et a*l, 1997
SA13	5a (GI: 3660725)	MSADLEVIT STWVLVGG	QQFDEMEEC SASLPYMD	IGEDYSTPC DGTWLRAI	SDEDSVVCC SMSYSWTG	Bukh *et al*, 1998
6a33	6a (GI: 57791993)	MSADLEVIT STWVLVGG	QQFDEMEEC SRHIPYLAE	VNEDTATPC ATSWLRDV	SDQDDVVCC SMSYSWTG	Zhou *et al*, 2004

The sequence of the junction between the non structural proteins NS3 and NS4A (NS3/4A) , NS4A and NS4B (NS4A/4B), NS4B and NS5A (NS4B/5A) and NS5A and NS5B (NS5A/5B) is given for 6 different HCV genotypes. The gene accession number (GI) for each is given in the Genotype column. For each sequence, the space indicates the NS3/4A-cleavage site. The consensus cleavage site is given in the text.

the HCV consensus cleavage site by a proline at the P6 position instead of an acidic residue. Strikingly, this proline is preceded by a stretch of 7 prolines and it is thought that this particular sequence may enhance the affinity of TRIF for the NS3 protease[73]. This stretch of proline, which can form a left-handed polyproline II helix may compensate for the absence of acidic residue at the P6 position, which normally is contributing to enhance the Km values in the viral natural substrates. A helix composed of hydrophobic residues was identified in the NS3 protease domain, not far from the protease active site and may represent a possible site to anchor TRIF near the active site of NS3[73]. The CARDIF cleavage site at the 508 residue, EREVPC↓ HRPS, presents more similarity with the HCV consensus cleavage site, except that there is a histidine residue at position P'1 instead of a serine or alanine[46]. Because of this, it is possible to conceive that several cellular substrates for NS3/4A exist. However, they may be difficult to depict, based solely on sequence examination, if, similarly to TRIF and CARDIF, they diverge from the consensus NS3/4A cleavage sequence. The highly specific product-based macrocyclic NS3 protease inhibitor BILN 2061[74] and the less toxic new generation of another class of NS3 inhibitors, referred to as electrophilic or serine-trap inhibitors, such as VX-950[75] and SCH6[76], probably exert their high inhibitory effect on HCV infection, not only by preventing HCV expression[77] but also by preventing NS3/4A to interfere with IFN induction.

HCV INFECTION AND THE RIG-I /Cardif/ TBK1/IKKε PATHWAY

The ability of HCV to inhibit the early events of IFN induction emphasizes the importance of the IFN signalling pathways and may therefore represent one of the mechanisms by which this virus compromises the host immune response and favours its propagation. Indeed, the *in vitro* propagation of infectious particles of HCV genotype 2a[77-79] and HCV of genotype 1a[80] is now highly promoted by infecting a Huh7 cellular clone that was previously isolated for its high susceptibility to HCV replicons[81]. The particularity of this clone, known as Huh7.5, is to contain a mutation in the first CARD domain of RIG-I . This mutation does not prevent the binding of RIG-I to dsRNA but abolishes its ability to activate downstream elements and is likely the one responsible for the inability of this clone to induce IFNβ and early ISGs in response to a viral or dsRNA stimulus. Indeed, complementation of Huh7.5 cells with a plasmid expressing RIG-I restores ISG56 induction in response to SeV or with *in vitro* transcribed HCV dsRNA-containing structures, such as its NTRs (non translated regions)[82]. The 5' and 3' HCV NTRs were proposed as the HCV elements required for RIG-I activation immediately after internalisation of the viral genome into the cytosol[82]. This was confirmed recently with a study showing the importance of 5' triphosphate in blunt-end dsRNA to signals to IFN induction and allowing the cell to discriminate between self and non self[83] and with the identification of 5' triphosphate RNAs as being the ligand for RIG-I [41,42].

The possibility of using the JFH1 recombinant virus of genotype 2a to infect cell cultures *in vitro* now gives the possibility to analyse the interaction of HCV with IFN induction pathways in more natural conditions of infection. Indeed, specific cleavage of CARDIF, but not of CARDIF C508A in which the NS3/4A cleavage site has been abolished, could be demonstrated after transient transfection in JFH1-infected Huh7.5 cells[46]. In support of cleavage of CARDIF by NS3/4A, subcellular redistribution of endogenous CARDIF from the mitochondria to the cytosol could be demonstrated in COS cells after transient transfection with NS3/4A-encoding plasmids, in HCV replicon cells[84], in JFH1-infected Huh7 cells and in a liver biopsy from a patient with chronic HCV infection[84]. Much work is needed yet to fully understand the exact relationship between the RNA helicase/CARDIF/kinases pathway and the ability of HCV to escape the cellular defense. For instance, the NS3/4A inhibitor BILN2061 was shown to restore the IFN induction in response to ectopically added CARDIF in JFH1-infected cells[85] but definite proof that the NS3/4A inhibitor BILN2061 can restore IFN induction

in HCV-infected cells remains to be established. JFH1 was reported to infect Huh7 cells with a viral progeny reaching similar levels to those obtained from Huh7.5 cells, after a lag of 7 d. This delay was first explained by the ability of JFH1 to induce an antiviral response in the Huh7 cells, but not in the Huh7.5 cells where the RIG-I pathway is defective and cannot recruit the downstream CARDIF adapter[79]. In a follow-up study, however, it was shown that JFH1 fails to induce IFNβ in the Huh7 cells, as well as early ISGs, such as ISG15 or ISG56, from the onset of infection. Interestingly, these authors could demonstrate that JFH1 was blocking IFNβ induction upstream of the TBK1/IKKε kinases, presumably through CARDIF cleavage. However, overexpression of CARDIF in those infected-cells could not restore dsRNA-induced IFN-β promoter activity and RIG-I overexpression could only partially restore it. A current hypothesis is that, in addition to cleaving CARDIF through its NS34A protease activity, HCV infection also provokes RIG-I inactivation through a process independent of NS34A. This suggests the existence of a RIG-I dependent signaling pathway that could by-pass CARDIF to trigger IFNβ expression, and thus represents an additional threat for the virus[85]. In support of this, in a recent study, we showed that expression levels of RIG-I (and of the other RNA helicases MDA5 and LGP2) were down-regulated in liver biopsies from HCV chronically-infected patients. In these biopsies, the expression levels of IKKε-, but not those of TBK1, were also down-regulated[86]. Interestingly, IKKε, when overexpressed, can provoke inhibition of HCV expression in a replicon system and we demonstrated that its antiviral action can occur rapidly, in the absence of IFN induction, through the action of one or several genes induced through activation of IRF3, NF-κB and c/EBPδ[70,86]. RIG-I belongs to the genes induced by IKKε and it is possible to hypothesize that HCV chronic infection thrives in an environment with low RIG-I and IKKε expresssion and/or activity. In line with the down-regulation of IKKε, decreased expression of the NF-κB RelA subunit, one of IKKε susbtrates, was found to be associated with enhanced fibrosis progression in the liver of patients with chronic hepatitis C[87].

CONCLUSIONS

The recent identification of different partners from the TLR- and RNA helicase-IFN inducing pathways, coupled with the possibility of using cell culture systems infected with recombinant HCV, now allows rapid progress in the comprehension of the relative importance of these pathways in cellular defence and in their ability to interfere with HCV propagation.

REFERENCES

1 **Le Bon A**, Tough DF. Links between innate and adaptive immunity via type I interferon. *Curr Opin Immunol* 2002; **14**: 432-436

2 **Brierley MM**, Fish EN. Stats: multifaceted regulators of transcription. *J Interferon Cytokine Res* 2005; **25**: 733-744

3 **de Veer MJ**, Holko M, Frevel M, Walker E, Der S, Paranjape JM, Silverman RH, Williams BR. Functional classification of interferon-stimulated genes identified using microarrays. *J Leukoc Biol* 2001; **69**: 912-920

4 **Hemmi H**, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. *Nature* 2000; **408**: 740-745

5 **Alexopoulou L**, Holt AC, Medzhitov R, Flavell RA. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 2001; **413**: 732-738

6 **Diebold SS**, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 2004; **303**: 1529-1531

7 **Heil F**, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 2004; **303**: 1526-1529

8 **Yoneyama M**, Kikuchi M, Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 2004; **5**: 730-737

9 **Andrejeva J**, Childs KS, Young DF, Carlos TS, Stock N, Goodbourn S, Randall RE. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proc Natl Acad Sci USA* 2004; **101**: 17264-17269

10 **Kato H**, Sato S, Yoneyama M, Yamamoto M, Uematsu S, Matsui K, Tsujimura T, Takeda K, Fujita T, Takeuchi O, Akira S. Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 2005; **23**: 19-28

11 **Servant MJ**, Grandvaux N, Hiscott J. Multiple signaling pathways leading to the activation of interferon regulatory factor 3. *Biochem Pharmacol* 2002; **64**: 985-992

12 **Sharma S**, tenOever BR, Grandvaux N, Zhou GP, Lin R, Hiscott J. Triggering the interferon antiviral response through an IKK-related pathway. *Science* 2003; **300**: 1148-1151

13 **Fitzgerald KA**, McWhirter SM, Faia KL, Rowe DC, Latz E, Golenbock DT, Coyle AJ, Liao SM, Maniatis T. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 2003; **4**: 491-496

14 **Yamamoto M**, Sato S, Mori K, Hoshino K, Takeuchi O, Takeda K, Akira S. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J Immunol* 2002; **169**: 6668-6672

15 **Oshiumi H**, Matsumoto M, Funami K, Akazawa T, Seya T. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 2003; **4**: 161-167

16 **Oshiumi H**, Sasai M, Shida K, Fujita T, Matsumoto M, Seya T. TIR-containing adapter molecule (TICAM)-2, a bridging adapter recruiting to toll-like receptor 4 TICAM-1 that induces interferon-beta. *J Biol Chem* 2003; **278**: 49751-49762

17 **Sato S**, Sugiyama M, Yamamoto M, Watanabe Y, Kawai T, Takeda K, Akira S. Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 2003; **171**: 4304-4310

18 **Jiang Z**, Mak TW, Sen G, Li X. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. *Proc Natl Acad Sci USA* 2004; **101**: 3533-3538

19 **Meylan E**, Burns K, Hofmann K, Blancheteau V, Martinon F, Kelliher M, Tschopp J. RIP1 is an essential mediator of Toll-like receptor 3-induced NF-kappa B activation. *Nat Immunol* 2004; **5**: 503-507

20 **Sasai M**, Oshiumi H, Matsumoto M, Inoue N, Fujita F, Nakanishi M, Seya T. Cutting Edge: NF-kappaB-activating kinase-associated protein 1 participates in TLR3/Toll-IL-1 homology domain-containing adapter molecule-1-mediated

IFN regulatory factor 3 activation. *J Immunol* 2005; **174**: 27-30
21 **Häcker H**, Redecke V, Blagoev B, Kratchmarova I, Hsu LC, Wang GG, Kamps MP, Raz E, Wagner H, Häcker G, Mann M, Karin M. Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 2006; **439**: 204-207
22 **Oganesyan G**, Saha SK, Guo B, He JQ, Shahangian A, Zarnegar B, Perry A, Cheng G. Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. *Nature* 2006; **439**: 208-211
23 **Schulz O**, Diebold SS, Chen M, Näslund TI, Nolte MA, Alexopoulou L, Azuma YT, Flavell RA, Liljeström P, Reis e Sousa C. Toll-like receptor 3 promotes cross-priming to virus-infected cells. *Nature* 2005; **433**: 887-892
24 **Wang T**, Town T, Alexopoulou L, Anderson JF, Fikrig E, Flavell RA. Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. *Nat Med* 2004; **10**: 1366-1373
25 **Le Goffic R**, Balloy V, Lagranderie M, Alexopoulou L, Escriou N, Flavell R, Chignard M, Si-Tahar M. Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2006; **2**: e53
26 **Kawai T**, Sato S, Ishii KJ, Coban C, Hemmi H, Yamamoto M, Terai K, Matsuda M, Inoue J, Uematsu S, Takeuchi O, Akira S. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nat Immunol* 2004; **5**: 1061-1068
27 **Honda K**, Yanai H, Mizutani T, Negishi H, Shimada N, Suzuki N, Ohba Y, Takaoka A, Yeh WC, Taniguchi T. Role of a transductional-transcriptional processor complex involving MyD88 and IRF-7 in Toll-like receptor signaling. *Proc Natl Acad Sci USA* 2004; **101**: 15416-15421
28 **Uematsu S**, Sato S, Yamamoto M, Hirotani T, Kato H, Takeshita F, Matsuda M, Coban C, Ishii KJ, Kawai T, Takeuchi O, Akira S. Interleukin-1 receptor-associated kinase-1 plays an essential role for Toll-like receptor (TLR)7- and TLR9-mediated interferon-{alpha} induction. *J Exp Med* 2005; **201**: 915-923
29 **Yamamoto M**, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 2003; **301**: 640-643
30 **Edelmann KH**, Richardson-Burns S, Alexopoulou L, Tyler KL, Flavell RA, Oldstone MB. Does Toll-like receptor 3 play a biological role in virus infections? *Virology* 2004; **322**: 231-238
31 **Smith EJ**, Marié I, Prakash A, García-Sastre A, Levy DE. IRF3 and IRF7 phosphorylation in virus-infected cells does not require double-stranded RNA-dependent protein kinase R or Ikappa B kinase but is blocked by Vaccinia virus E3L protein. *J Biol Chem* 2001; **276**: 8951-8957
32 **Honda K**, Sakaguchi S, Nakajima C, Watanabe A, Yanai H, Matsumoto M, Ohteki T, Kaisho T, Takaoka A, Akira S, Seya T, Taniguchi T. Selective contribution of IFN-alpha/beta signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc Natl Acad Sci USA* 2003; **100**: 10872-10877
33 **Sun YW**. RIG-I, a human homolog gene of RNA helicase is induced by Retinoic Acid during the differentiation of acute promyelocytic leukemia cell. Thesis Shangai second Medical Univ; 1997
34 **Cui XF**, Imaizumi T, Yoshida H, Borden EC, Satoh K. Retinoic acid-inducible gene-I is induced by interferon-gamma and regulates the expression of interferon-gamma stimulated gene 15 in MCF-7 cells. *Biochem Cell Biol* 2004; **82**: 401-405
35 **Matikainen S**, Sirén J, Tissari J, Veckman V, Pirhonen J, Severa M, Sun Q, Lin R, Meri S, Uzé G, Hiscott J, Julkunen I. Tumor necrosis factor alpha enhances influenza A virus-induced expression of antiviral cytokines by activating RIG-I gene expression. *J Virol* 2006; **80**: 3515-3522
36 **Kang DC**, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM, Fisher PB. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proc Natl Acad Sci USA* 2002; **99**: 637-642
37 **Rothenfusser S**, Goutagny N, DiPerna G, Gong M, Monks BG, Schoenemeyer A, Yamamoto M, Akira S, Fitzgerald KA. The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 2005; **175**: 5260-5268
38 **Yoneyama M**, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, Taira K, Foy E, Loo YM, Gale M, Akira S, Yonehara S, Kato A, Fujita T. Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 2005; **175**: 2851-2858
39 **Kato H**, Takeuchi O, Sato S, Yoneyama M, Yamamoto M, Matsui K, Uematsu S, Jung A, Kawai T, Ishii KJ, Yamaguchi O, Otsu K, Tsujimura T, Koh CS, Reis e Sousa C, Matsuura Y, Fujita T, Akira S. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006; **441**: 101-105
40 **Gitlin L**, Barchet W, Gilfillan S, Cella M, Beutler B, Flavell RA, Diamond MS, Colonna M. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proc Natl Acad Sci USA* 2006; **103**: 8459-8464
41 **Hornung V**, Ellegast J, Kim S, Brzózka K, Jung A, Kato H, Poeck H, Akira S, Conzelmann KK, Schlee M, Endres S, Hartmann G. 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 2006; **314**: 994-997
42 **Pichlmair A**, Schulz O, Tan CP, Näslund TI, Liljeström P, Weber F, Reis e Sousa C. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* 2006; **314**: 997-1001
43 **Kawai T**, Takahashi K, Sato S, Coban C, Kumar H, Kato H, Ishii KJ, Takeuchi O, Akira S. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 2005; **6**: 981-988
44 **Xu LG**, Wang YY, Han KJ, Li LY, Zhai Z, Shu HB. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Mol Cell* 2005; **19**: 727-740
45 **Seth RB**, Sun L, Ea CK, Chen ZJ. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. *Cell* 2005; **122**: 669-682
46 **Meylan E**, Curran J, Hofmann K, Moradpour D, Binder M, Bartenschlager R, Tschopp J. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature* 2005; **437**: 1167-1172
47 **Matsuda A**, Suzuki Y, Honda G, Muramatsu S, Matsuzaki O, Nagano Y, Doi T, Shimotohno K, Harada T, Nishida E, Hayashi H, Sugano S. Large-scale identification and characterization of human genes that activate NF-kappaB and MAPK signaling pathways. *Oncogene* 2003; **22**: 3307-3318
48 **Saha SK**, Pietras EM, He JQ, Kang JR, Liu SY, Oganesyan G, Shahangian A, Zarnegar B, Shiba TL, Wang Y, Cheng G. Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *EMBO J* 2006; **25**: 3257-3263
49 **Lin R**, Lacoste J, Nakhaei P, Sun Q, Yang L, Paz S, Wilkinson P, Julkunen I, Vitour D, Meurs E, Hiscott J. Dissociation of a MAVS/IPS-1/VISA/Cardif-IKKepsilon molecular complex from the mitochondrial outer membrane by hepatitis C virus NS3-4A proteolytic cleavage. *J Virol* 2006; **80**: 6072-6083
50 **Sun Q**, Sun L, Liu HH, Chen X, Seth RB, Forman J, Chen ZJ. The specific and essential role of MAVS in antiviral innate immune responses. *Immunity* 2006; **24**: 633-642
51 **Servant MJ**, Grandvaux N, tenOever BR, Duguay D, Lin R, Hiscott J. Identification of the minimal phosphoacceptor site required for in vivo activation of interferon regulatory factor 3 in response to virus and double-stranded RNA. *J Biol Chem* 2003; **278**: 9441-9447
52 **Peters RT**, Liao SM, Maniatis T. IKKepsilon is part of a novel PMA-inducible IkappaB kinase complex. *Mol Cell* 2000; **5**: 513-522

53 **Bonnard M**, Mirtsos C, Suzuki S, Graham K, Huang J, Ng M, Itié A, Wakeham A, Shahinian A, Henzel WJ, Elia AJ, Shillinglaw W, Mak TW, Cao Z, Yeh WC. Deficiency of T2K leads to apoptotic liver degeneration and impaired NF-kappaB-dependent gene transcription. *EMBO J* 2000; **19**: 4976-4985

54 **Hemmi H**, Takeuchi O, Sato S, Yamamoto M, Kaisho T, Sanjo H, Kawai T, Hoshino K, Takeda K, Akira S. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *J Exp Med* 2004; **199**: 1641-1650

55 **Harris J**, Olière S, Sharma S, Sun Q, Lin R, Hiscott J, Grandvaux N. Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKK epsilon. *J Immunol* 2006; **177**: 2527-2535

56 **Mattioli I**, Geng H, Sebald A, Hodel M, Bucher C, Kracht M, Schmitz ML. Inducible phosphorylation of NF-kappa B p65 at serine 468 by T cell costimulation is mediated by IKK epsilon. *J Biol Chem* 2006; **281**: 6175-6183

57 **Adli M**, Baldwin AS. IKK-i/IKKepsilon controls constitutive, cancer cell-associated NF-kappaB activity via regulation of Ser-536 p65/RelA phosphorylation. *J Biol Chem* 2006; **281**: 26976-26984

58 **Kravchenko VV**, Mathison JC, Schwamborn K, Mercurio F, Ulevitch RJ. IKKi/IKKepsilon plays a key role in integrating signals induced by pro-inflammatory stimuli. *J Biol Chem* 2003; **278**: 26612-26619

59 **Wang N**, Ahmed S, Haqqi TM. Genomic structure and functional characterization of the promoter region of human IkappaB kinase-related kinase IKKi/IKKvarepsilon gene. *Gene* 2005; **353**: 118-133

60 **Hoofnagle JH**. Course and outcome of hepatitis C. *Hepatology* 2002; **36**: S21-S29

61 **Heim MH**, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J Virol* 1999; **73**: 8469-8475

62 **Larrea E**, Aldabe R, Molano E, Fernandez-Rodriguez CM, Ametzazurra A, Civeira MP, Prieto J. Altered expression and activation of signal transducers and activators of transcription (STATs) in hepatitis C virus infection: in vivo and in vitro studies. *Gut* 2006; **55**: 1188-1196

63 **Melén K**, Fagerlund R, Nyqvist M, Keskinen P, Julkunen I. Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. *J Med Virol* 2004; **73**: 536-547

64 **Pavio N**, Lai MM. The hepatitis C virus persistence: how to evade the immune system? *J Biosci* 2003; **28**: 287-304

65 **Foy E**, Li K, Wang C, Sumpter R, Ikeda M, Lemon SM, Gale M. Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. *Science* 2003; **300**: 1145-1148

66 **De Francesco R**, Tomei L, Altamura S, Summa V, Migliaccio G. Approaching a new era for hepatitis C virus therapy: inhibitors of the NS3-4A serine protease and the NS5B RNA-dependent RNA polymerase. *Antiviral Res* 2003; **58**: 1-16

67 **Dumont S**, Cheng W, Serebrov V, Beran RK, Tinoco I, Pyle AM, Bustamante C. RNA translocation and unwinding mechanism of HCV NS3 helicase and its coordination by ATP. *Nature* 2006; **439**: 105-108

68 **Bartenschlager R**. The NS3/4A proteinase of the hepatitis C virus: unravelling structure and function of an unusual enzyme and a prime target for antiviral therapy. *J Viral Hepat* 1999; **6**: 165-181

69 **Foy E**, Li K, Sumpter R, Loo YM, Johnson CL, Wang C, Fish PM, Yoneyama M, Fujita T, Lemon SM, Gale M. Control of antiviral defenses through hepatitis C virus disruption of retinoic acid-inducible gene-I signaling. *Proc Natl Acad Sci USA* 2005; **102**: 2986-2991

70 **Breiman A**, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, Fujita T, Hiscott J, Meurs EF. Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. *J Virol* 2005; **79**: 3969-3978

71 **Li K**, Foy E, Ferreon JC, Nakamura M, Ferreon AC, Ikeda M, Ray SC, Gale M, Lemon SM. Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 2005; **102**: 2992-2997

72 **Li XD**, Sun L, Seth RB, Pineda G, Chen ZJ. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proc Natl Acad Sci USA* 2005; **102**: 17717-17722

73 **Ferreon JC**, Ferreon AC, Li K, Lemon SM. Molecular determinants of TRIF proteolysis mediated by the hepatitis C virus NS3/4A protease. *J Biol Chem* 2005; **280**: 20483-20492

74 **Lamarre D**, Anderson PC, Bailey M, Beaulieu P, Bolger G, Bonneau P, Bos M, Cameron DR, Cartier M, Cordingley MG, Faucher AM, Goudreau N, Kawai SH, Kukolj G, Lagacé L, LaPlante SR, Narjes H, Poupart MA, Rancourt J, Sentjens RE, St George R, Simoneau B, Steinmann G, Thibeault D, Tsantrizos YS, Weldon SM, Yong CL, Llinàs-Brunet M. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 2003; **426**: 186-189

75 **Lin K**, Perni RB, Kwong AD, Lin C. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCv replicon cells. *Antimicrob Agents Chemother* 2006; **50**: 1813-1822

76 **Bogen SL**, Arasappan A, Bennett F, Chen K, Jao E, Liu YT, Lovey RG, Venkatraman S, Pan W, Parekh T, Pike RE, Ruan S, Liu R, Baroudy B, Agrawal S, Chase R, Ingravallo P, Pichardo J, Prongay A, Brisson JM, Hsieh TY, Cheng KC, Kemp SJ, Levy OE, Lim-Wilby M, Tamura SY, Saksena AK, Girijavallabhan V, Njoroge FG. Discovery of SCH446211 (SCH6): a new ketoamide inhibitor of the HCV NS3 serine protease and HCV subgenomic RNA replication. *J Med Chem* 2006; **49**: 2750-2757

77 **Lindenbach BD**, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623-626

78 **Wakita T**, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791-796

79 **Zhong J**, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294-9299

80 **Yi M**, Villanueva RA, Thomas DL, Wakita T, Lemon SM. Production of infectious genotype 1a hepatitis C virus (Hutchinson strain) in cultured human hepatoma cells. *Proc Natl Acad Sci USA* 2006; **103**: 2310-2315

81 **Blight KJ**, McKeating JA, Rice CM. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 2002; **76**: 13001-13014

82 **Sumpter R**, Loo YM, Foy E, Li K, Yoneyama M, Fujita T, Lemon SM, Gale M. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *J Virol* 2005; **79**: 2689-2699

83 **Marques JT**, Devosse T, Wang D, Zamanian-Daryoush M, Serbinowski P, Hartmann R, Fujita T, Behlke MA, Williams BR. A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat Biotechnol* 2006; **24**: 559-565

84 **Loo YM**, Owen DM, Li K, Erickson AK, Johnson CL, Fish PM, Carney DS, Wang T, Ishida H, Yoneyama M, Fujita T, Saito T, Lee WM, Hagedorn CH, Lau DT, Weinman SA, Lemon SM, Gale M. Viral and therapeutic control of IFN-beta promoter stimulator 1 during hepatitis C virus infection. *Proc Natl Acad Sci USA* 2006; **103**: 6001-6006

85 **Cheng G**, Zhong J, Chisari FV. Inhibition of dsRNA-induced signaling in hepatitis C virus-infected cells by NS3 protease-dependent and -independent mechanisms. *Proc Natl Acad Sci USA* 2006; **103**: 8499-8504

86 **Vilasco M**, Larrea E, Vitour D, Dabo S, Breiman A, Regnault B, Riezu JI, Eid P, Prieto J, Meurs EF. The protein kinase IKKepsilon can inhibit HCV expression independently of IFN and its own expression is downregulated in HCV-infected livers. *Hepatology* 2006; **44**: 1635-1647

87 **Boya P**, Larrea E, Sola I, Majano PL, Jiménez C, Civeira MP, Prieto J. Nuclear factor-kappa B in the liver of patients with chronic hepatitis C: decreased RelA expression is associated with enhanced fibrosis progression. *Hepatology* 2001; **34**: 1041-1048

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Iunel Fabiani, Professor, *Series Editor*

Hepatocellular carcinoma in patients with hepatitis C virus-related chronic liver disease

Jean-Claude Trinchet, Nathalie Ganne-Carrié, Pierre Nahon, Gisèle N'kontchou, Michel Beaugrand

Jean-Claude Trinchet, Nathalie Ganne-Carrié, Pierre Nahon, Gisèle N'kontchou, Michel Beaugrand, Service d'Hépato-Gastroentérologie, Hôpital Jean Verdier, Assistance Publique-Hôpitaux de Paris, UPRES EA 3409 and UPRES EA 3410, UFR SMBH, Université Paris 13, Bondy, France
Correspondence to: Jean-Claude Trinchet, MD, PhD, Professor, Service d'Hépato-Gastroentérologie, Hôpital Jean Verdier-93143 Bondy Cedex,
France. jean-claude.trinchet@jvr.aphp.fr
Telephone: +33-1-48026280 Fax: +33-1-48026202
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Abstract

Hepatitis C virus (HCV) is a major cause of hepatocellular carcinoma (HCC) worldwide due to the high prevalence of HCV infection and the high rate of HCC occurrence in patients with HCV cirrhosis. A striking increase in HCC incidence has been observed during the past decades in most industrialized countries, partly related to the growing number of patients infected by HCV. HCC is currently the main cause of death in patients with HCV-related cirrhosis, a fact that justifies screening as far as curative treatments apply only in patients with small tumors. As a whole, treatment options are similar in patients with cirrhosis whatever the cause. Chemoprevention could be also helpful in the near future. It is strongly suggested that antiviral treatment of HCV infection could prevent HCC occurrence, even in cirrhotic patients, mainly when a sustained virological response is obtained.

Key words: Hepatocellular carcinoma; Cirrhosis; Hepatitis C virus ; Epidemiology; Screening; Treatment; Prevention

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world, with more than 600 000 new cases yearly. Most patients with HCC have an underlying chronic liver disease (often cirrhosis), resulting mainly from chronic infection by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, and often an association of these causes. HCC has recently gained more interest due to its increasing incidence in industrialized countries[1,2].

A link between non-A non-B hepatitis and HCC occurrence was firstly suspected in Japan more than 20 years ago due to contrast between simultaneous increase in HCC incidence and decline of HBV infection in this country[1]. This relationship was rapidly confirmed in the early 1990s when specific serum testing for HCV infection became available. HCV was subsequently classified as a human carcinogen in 1994[1]. Almost all cases of HCV-related HCC occur in patients with cirrhosis, which is a well-established precancerous state[3]. However a direct carcinogenetic role of HCV is strongly suspected in humans and supported by experimental models showing the oncogenic effect of viral core and NS3 proteins[4,5].

In this review, we will discuss different aspects of HCV-related carcinogenesis and HCC treatment, focusing on similarities and differences with tumors related to other causes of chronic liver diseases, particularly HBV and alcohol.

EPIDEMIOLOGY

HCV is a major cause of HCC worldwide due to high prevalence of chronic infection affecting about 170 millions of people. In industrialized countries, HCV infection and excessive alcohol consumption are the two leading causes of cirrhosis and HCC is often associated[1,2]. However regional differences have been shown. In Japan, HCV is currently the main cause of HCC accounting for more than 70% of cases. A similar rate is observed in Southern Europe such as Italy or Spain, even if some studies have reported a higher role of alcohol consumption[6]. By contrast, in other countries such as France[7] or Belgium[8], excessive alcohol intake remains the leading cause of cirrhosis and HCC (accounting for more than 60% of cases), HCV being only the second cause (about 25%-30% of cases).

A striking increase in HCC incidence has been observed for more than 20 years in most industrialized countries. This fact has been clearly established from epidemiological

surveillance databases in Japan[9], UK[10], Italy[11], France[12] and the USA[13]. In France, the age-standardised mortality rates per 100 000 people (which reflects incidence due to short life expectancy of patients) due to primary liver cancer (mostly HCC) has increased from 3.2 to 11.1 in men and from 1.2 to 2.5 in women between 1979 and 1994[12]. It is generally admitted that this increase is related to the growing number of patients contaminated by HCV several decades ago who reached the stage of cirrhosis and consequently developed cancer[1,2]. Epidemiological models suggest that this trend would likely persist 10-15 years without strong improvement in treatment efficacy[14]. However additional factors may also contribute to increase HCC incidence such as improved management or prevention of other lethal complications of cirrhosis, oesophageal haemorrhage[15] and bacterial infections resulting in longer survival of patients at high risk. Additionally prevalence of overweight and diabetes type 2 is increasing in developed countries, a factor that favors HCC occurrence[13].

The situation in industrialized countries is in contrast with developing areas such as Eastern Asia and sub-Saharan Africa. In these regions, HBV infection (often associated with aflatoxin exposure) is still the leading cause of chronic liver disease and HCC, accounting for more than 80% of cases[1,2]. The incidence of HCC is notably higher than in industrialized countries and seems to be stable or decreasing, likely due to better control of aflatoxin exposure or HBV infection[9]. However, even in these regions HCV infection has a significant prevalence and is also an important cause of HCC[9].

As a whole HCV-related HCC is currently a major public health problem in many countries worldwide, leading to increased financial burden over time[16].

RISK (OR PREDICTIVE) FACTORS

Almost all the patients with HCV-related HCC have cirrhosis at the time of diagnosis[3]. Therefore cirrhosis, which is associated with genetic alterations predisposing to cancer[17], is the main risk factor for HCC occurrence in these patients. Cases of HCC in patients with chronic hepatitis without cirrhosis have been reported but remains very scarce[18]. A similar feature has been found in patients with alcohol-related liver disease but is markedly different in patients chronically infected by HBV, where HCC occurs before cirrhosis in up to 40% of cases[1]. In cohorts of patients with HCV-related cirrhosis, the risk of HCC occurrence over time is high and roughly linear, between 2% and 8% yearly as a whole[19]. A higher rate has been reported in Japan (4%-8%) than in Western countries (2%-4%) suggesting the influence of yet unknown epidemiological factors[19]. Moreover this risk seems to be higher in HCV-related cirrhosis than in alcohol or HBV-related cirrhosis[19].

Obviously the risk of HCC occurrence is not similar among all patients with HCV cirrhosis. Numerous studies have been performed to identify risk factors on an individual basis[19]. Identifying such factors could lead to important progress in terms of clinical management (see Screening) and understanding of hepatocarcinogenesis[20]. Numerous predictive factors have been identified ranging from simple epidemiological parameters such as age or sex[21] to most sophisticated ones identified by molecular biology[22,23]. However most of the studies have assessed simultaneously a limited number of factors (sometimes only one) precluding global interpretation, and have included patients with different causes of liver diseases although risk factors might be different from a cause to another. The more commonly identified predictive factors (most of them not specific to HCV patients) are age higher than 50, male sex, advanced cirrhosis (reflected by low platelet count or oesophageal varices), high basal alpha-fetoprotein (AFP) serum levels[21,24,25], and more recently overweight and diabetes[26,27]. Factors can be combined in scores or indexes allowing to split patients between different categories of HCC risk. In a recent Japanese study performed in 183 patients with HCV cirrhosis, the estimated risk of HCC at 5-year ranged between 9% and 64% according to the value of a score combining age, sex, serum AFP and platelet count[21]. The influence of tobacco smoking or HCV genotype remains controversial[19]. Histological lesions such as large cell dysplasia[25] and regenerative and proliferative changes[28] have been also reported to increase the risk of cancer, but their assessment requires liver biopsy interpreted by an experienced observer and therefore their practical relevance is less important. Iron overload does not seem to influence carcinogenesis in HCV patients conversely to alcoholics[29]. The association of other causes of liver diseases, such as concomitant HBV infection or high alcohol intake, markedly enhances the risk of HCC in patients with HCV cirrhosis. A potential role of occult HBV infection in HCV-related HCC has also been suspected[30].

NATURAL HISTORY

The characteristics of patients at the time of HCC diagnosis are influenced by the aetiology and the status of the underlying liver disease. They may influence prognosis and the choice of treatments. Patients with HCV-related HCC are usually older than those with alcoholic or HBV-related cirrhosis, a difference likely due to the age at exposition to causative agents (childhood for HBV, adulthood for HCV) or in the rate of progression to cirrhosis (faster for alcoholics)[11,31,32]. The sex ratio is close to one, in contrast with the high prevalence of males in patients with HCC due to alcohol[6]. Tumors seem to be more often unique and small-sized (that is to say slow growing) in patients with HCV cirrhosis in comparison with HBV, even if this fact remains controversial[6]. Liver function is usually more preserved in patients with viral cirrhosis than in those with alcoholic cirrhosis[6]. However whether those differences are related to the natural history of the disease or to the management of patients or both is not determined. As an example compliance to HCC screening (see below) is markedly lower in patients with alcoholic cirrhosis[8].

As a whole survival remains low in patients with HCC (median < 6-12 mo), reflecting the currently high number of advanced tumors at diagnosis and the subsequent

poor efficacy of treatment. However marked differences are observed among etiological groups and individual patients. The characteristics of cancer as well as of the underlying liver disease influence survival as reflected by parameters relevant in prognostic classifications[33]. HCC is clearly the main cause of death in patients with HCV-related cirrhosis[34,35]. In a recent Italian prospective study including 214 patients[34], HCC developed in 32%, ascites in 23%, jaundice in 17%, upper gastrointestinal bleeding in 6%, and encephalopathy in 1% during a follow-up of 17 years. This outcome differs markedly from patients with alcohol-related cirrhosis, the complications of liver failure and portal hypertension being still the leading cause of death[36], a difference that could be in part explained by compliance of patients to clinical management. For instance, the rates of endoscopic screening of oesophageal varices and preventive measures for haemorrhage or bacterial infection is not clearly stated in most published studies[37].

TREATMENT

In patients with HCC, treatment decision depends on several factors including size and extension of tumor, liver function, and general condition of the patient (such as age, comorbidities and general status)[38,39]. There is currently no medically validated therapy, even if recent characterisation of molecular pathways of hepatocarcinogenesis raises some hope for the future[23]. Intra-arterial chemoembolization has been claimed to result in survival improvement in selected cases but contra-indications, mainly due to liver failure, are frequent and the number of eligible patients who would really benefit from it is low[40,41]. Despite these reservations, patients with underlying viral disease seem to have a more favourable outcome and a lesser rate of post-procedure complications than patients with alcoholic cirrhosis.

Important advances have occurred concerning curative treatment of small tumors, even if randomized trials are lacking to definitely establish their benefit[39]. Milan criteria, defined as either one nodule less than 5 cm in diameter or 2 or 3 nodules each less than 3 cm in diameter, help select patients eligible for transplantation but increasingly also for other curative options[42]. Liver transplantation, which is the best curative option for the long term as it is able to remove the tumor and the underlying cirrhosis (preventing therefore the occurrence of new tumors), can be performed in only limited number of well-selected patients[42]. Other curative options, resection and percutaneous ablation mostly by radiofrequency, are able to cure the tumor mostly when small and well circumscribed[43]. Due to a lesser mortality and morbidity and a less deleterious influence on liver function, radiofrequency is to be performed in an increasingly larger number of patients with cirrhosis as new techniques allow now to treat tumors more than 3 cm in diameter. Nevertheless tumor recurrence rate is high (10%-20% per year) in cases of resection or radiofrequency due to either intrahepatic metastasis or occurrence of a new HCC, a case that is particularly frequent in patients with HCV infection. This fact justifies post-therapeutic surveillance of patients and the search for preventive treatments[42] (see Prevention).

The choice of therapy in patients with HCC is largely independent of the aetiology of the disease. However, as previously stated, patients with HCV-related HCC have a better liver function than alcoholic patients. Therefore they are less prone to develop liver failure after aggressive procedures. This fact might explain the better results of arterial chemoembolization in patients with viral disease compared with patients with alcoholic cirrhosis[41]. This might also partially explain the lesser mortality and morbidity of patients after hepatectomy in Asian countries in contrast with Western and their better overall outcome[42]. Conversely it has also been suggested that hepatic recurrences after surgery or ablative therapy are more frequent in patients with HCV-related HCC, possibly due to a higher incidence of new tumors, but this point needs confirmation[44].

SCREENING

The failure to improve survival in patients with advanced HCC has led to the development of screening strategies aiming to detect small tumors treatable by curative methods. Screening is currently based on regular periodic ultrasonography (US) in patients with cirrhosis[2]. A focal lesion discovered by screening indicates recall procedures, mainly imaging methods with vascular injection of contrast media (TDM, MRI, US)[45] and (in restricted cases) liver biopsy. This is especially important in case of a nodule below 1-2 cm in diameter because as it might be a non cancerous macronodule (which may remain stable or even disappear spontaneously over time) in more than fifty percent of cases[46]. Cholangiocarcinomas and liver lymphomas may also occur in patients with HCV cirrhosis, even if rare[46]. A diagnostic algorithm based on focal lesion size has been proposed by the international Barcelona conference in 2000[38] and recently modified[39]. The usefulness of serum AFP or other serum markers for screening is doubtful[2] due to high rates of false negatives (serum AFP is rarely increased in patients with small HCC) and false positive results, particularly in HCV cirrhosis[47]. The periodicity of screening is not established, an interval of 6 mo being usually recommended[39].

HCC screening is now recommended for every patient with cirrhosis, whatever the aetiology (and also for some patients with HBV infection without cirrhosis)[48]. However, as previously stated, HCC occurrence is strongly influenced by the cause of liver disease and numerous individual risk factors[19]. It is likely that non selective screening may lead to unjustified medical burden and costs. Therefore there is an urgent need to predictive scores using parameters recordable at bedside[21,27] and to validate them in prospective studies performed in well-defined populations, particularly according to the aetiology of cirrhosis. If HCV patients with bridging fibrosis at biopsy (stage F3 in Metavir classification) are candidates; screening is still debatable, even if sometimes recommended.

Even if a survival benefit has not been established (randomized trials being ethically questionable), HCC screening is now largely performed in industrialized

countries[49,50], leading to a better knowledge of liver carcinogenesis and an increasing rate of small HCC at diagnosis[51].

PREVENTION

The goal of primary prevention is to avoid or delay the occurrence of HCC by using medical treatments[52,53]. Chemoprevention is obviously a complementary method to screening to improve survival. At present there is a growing set of data suggesting the preventive role of antiviral drugs in patients with HCV-related chronic liver disease[52,53], alfa interferon being the most extensively studied drug. In patients with chronic hepatitis and only mild fibrosis, a virtual eradication of long term risk of HCC (and other complications) is associated to sustained virological response. However this effect remains debated in patients with established cirrhosis, mainly because most studies were retrospective and non randomized, and subjected to bias. Only two small randomized trials have been published with conflicting results[54,55]. Two metaanalyses[56,57] have suggested a moderate preventive effect on HCC occurrence in patients treated by alfa interferon by comparison to untreated patients. This effect was largely independent of virological response. However the studies included in those metaanalyses have been performed using monotherapy by standard alfa interferon with very low rates of sustained virological response. Some recent studies including larger cohorts of patients[58] treated by bitherapy[59] suggest a higher preventive effect, particularly in patients with sustained virological response. Those results are to be confirmed but encourage treating patients with compensated cirrhosis. The efficacy of long term low doses of pegylated interferon need to be established by ongoing randomized trials that could also identify predictive factors of a favourable result[52,53].

Another concern is the extremely high rate of recurrences following local curative treatment either by resection or percutaneous ablation of a first HCC. Secondary prevention should be aimed at decreasing either local recurrences (corresponding to treatment failure) and/or distant recurrences (corresponding to metastasis or to new HCC)[52,53]. Randomized trials performed in patients with cirrhosis mostly due to HCV infection have suggested a benefit in recurrence rate and survival using polyprenoic acid (a non commercialized retinoid derivative), adoptive immunotherapy, continuous or intermittent interferons alfa and beta treatments, and intraarterial radioactive iodine injection mixed with lipiodol[52,53]. These studies need confirmation because of their small sample size and have not lead up to now to important practical progress. Similarly to primary chemoprevention, post-procedure antiviral treatment using pegylated interferon alone or pegylated interferon and ribavirin might reduce recurrences if sustained viral response is obtained. Well-designed (randomized) trials involving large numbers of patients are still needed.

CONCLUSION

Some major questions should be addressed in the near future, particularly (but not only) in patients with HCV cirrhosis. It is still difficult to identify patients with compensated cirrhosis who require specific management including HCC screening and chemoprevention. Many of them are asymptomatic and undiagnosed. Even in patients suspected of having cirrhosis, a reliable confirmation requires liver biopsy, an ill-accepted and costly procedure. Non invasive methods of assessment of liver fibrosis, such as elastometry (Fibroscan®, Echosens, Paris, France)[60] or blood tests[61,62], might facilitate early diagnosis of cirrhosis, particularly in HCV infected patients. Future prospective studies concerning risk factors of HCC and predictive scores should take into account the periodic evaluation of those parameters. Thirdly the preventive effect of antiviral treatment in patients with HCV cirrhosis, including new molecules in development[63], should be precisely assessed in prospective trials.

REFERENCES

1 **McGlynn KA**, London WT. Epidemiology and natural history of hepatocellular carcinoma. *Best Pract Res Clin Gastroenterol* 2005; **19**: 3-23
2 **Sherman M**. Hepatocellular carcinoma: epidemiology, risk factors, and screening. *Semin Liver Dis* 2005; **25**: 143-154
3 **Liang TJ**, Heller T. Pathogenesis of hepatitis C-associated hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S62-S71
4 **Branda M**, Wands JR. Signal transduction cascades and hepatitis B and C related hepatocellular carcinoma. *Hepatology* 2006; **43**: 891-902
5 **Kew MC**. Interaction between hepatitis B and C viruses in hepatocellular carcinogenesis. *J Viral Hepat* 2006; **13**: 145-149
6 **Gelatti U**, Donato F, Tagger A, Fantoni C, Portolani N, Ribero ML, Martelli C, Trevisi P, Covolo L, Simonati C, Nardi G. Etiology of hepatocellular carcinoma influences clinical and pathologic features but not patient survival. *Am J Gastroenterol* 2003; **98**: 907-914
7 **Chevret S**, Trinchet JC, Mathieu D, Rached AA, Beaugrand M, Chastang C. A new prognostic classification for predicting survival in patients with hepatocellular carcinoma. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. *J Hepatol* 1999; **31**: 133-141
8 **Henrion J**, Libon E, De Maeght S, Schapira M, Ghilain JM, Maisin JM, Heller FR. Surveillance for hepatocellular carcinoma: compliance and results according to the aetiology of cirrhosis in a cohort of 141 patients. *Acta Gastroenterol Belg* 2000; **63**: 5-9
9 **Kao JH**, Chen DS. Changing disease burden of hepatocellular carcinoma in the Far East and Southeast Asia. *Liver Int* 2005; **25**: 696-703
10 **Taylor-Robinson SD**, Foster GR, Arora S, Hargreaves S, Thomas HC. Increase in primary liver cancer in the UK, 1979-1994. *Lancet* 1997; **350**: 1142-1143
11 **Stroffolini T**, Andreone P, Andriulli A, Ascione A, Craxi A, Chiaramonte M, Galante D, Manghisi OG, Mazzanti R, Medaglia C, Pilleri G, Rapaccini GL, Simonetti RG, Taliani G, Tosti ME, Villa E, Gasbarrini G. Characteristics of hepatocellular carcinoma in Italy. *J Hepatol* 1998; **29**: 944-952
12 **Deuffic S**, Poynard T, Buffat L, Valleron AJ. Trends in primary liver cancer. *Lancet* 1998; **351**: 214-215
13 **El-Serag HB**. Hepatocellular carcinoma: recent trends in the United States. *Gastroenterology* 2004; **127**: S27-S34
14 **Deuffic S**, Buffat L, Poynard T, Valleron AJ. Modeling the hepatitis C virus epidemic in France. *Hepatology* 1999; **29**: 1596-1601
15 **Carbonell N**, Pauwels A, Serfaty L, Fourdan O, Lévy VG, Poupon R. Improved survival after variceal bleeding in patients with cirrhosis over the past two decades. *Hepatology* 2004; **40**: 652-659

16 **El-Serag HB**, Siegel AB, Davila JA, Shaib YH, Cayton-Woody M, McBride R, McGlynn KA. Treatment and outcomes of treating of hepatocellular carcinoma among Medicare recipients in the United States: a population-based study. *J Hepatol* 2006; **44**: 158-166

17 **Laurent-Puig P**, Legoix P, Bluteau O, Belghiti J, Franco D, Binot F, Monges G, Thomas G, Bioulac-Sage P, Zucman-Rossi J. Genetic alterations associated with hepatocellular carcinomas define distinct pathways of hepatocarcinogenesis. *Gastroenterology* 2001; **120**: 1763-1773

18 **Grando-Lemaire V**, Guettier C, Chevret S, Beaugrand M, Trinchet JC. Hepatocellular carcinoma without cirrhosis in the West: epidemiological factors and histopathology of the non-tumorous liver. Groupe d'Etude et de Traitement du Carcinome Hépatocellulaire. *J Hepatol* 1999; **31**: 508-513

19 **Fattovich G**, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. *Gastroenterology* 2004; **127**: S35-S50

20 **Fattovich G**, Llovet JM. Risk factors for hepatocellular carcinoma in HCV-cirrhosis: what we know and what is missing. *J Hepatol* 2006; **44**: 1013-1016

21 **Ikeda K**, Arase Y, Saitoh S, Kobayashi M, Someya T, Hosaka T, Akuta N, Suzuki Y, Suzuki F, Sezaki H, Kumada H, Tanaka A, Harada H. Prediction model of hepatocarcinogenesis for patients with hepatitis C virus-related cirrhosis. Validation with internal and external cohorts. *J Hepatol* 2006; **44**: 1089-1097

22 **Cha C**, Dematteo RP. Molecular mechanisms in hepatocellular carcinoma development. *Best Pract Res Clin Gastroenterol* 2005; **19**: 25-37

23 **Roberts LR**, Gores GJ. Hepatocellular carcinoma: molecular pathways and new therapeutic targets. *Semin Liver Dis* 2005; **25**: 212-225

24 **Velázquez RF**, Rodríguez M, Navascués CA, Linares A, Pérez R, Sotorríos NG, Martínez I, Rodrigo L. Prospective analysis of risk factors for hepatocellular carcinoma in patients with liver cirrhosis. *Hepatology* 2003; **37**: 520-527

25 **Ganne-Carrié N**, Chastang C, Chapel F, Munz C, Pateron D, Sibony M, Dény P, Trinchet JC, Callard P, Guettier C, Beaugrand M. Predictive score for the development of hepatocellular carcinoma and additional value of liver large cell dysplasia in Western patients with cirrhosis. *Hepatology* 1996; **23**: 1112-1118

26 **El-Serag HB**, Hampel H, Javadi F. The association between diabetes and hepatocellular carcinoma: a systematic review of epidemiologic evidence. *Clin Gastroenterol Hepatol* 2006; **4**: 369-380

27 **N'Kontchou G**, Paries J, Htar MT, Ganne-Carrie N, Costentin L, Grando-Lemaire V, Trinchet JC, Beaugrand M. Risk factors for hepatocellular carcinoma in patients with alcoholic or viral C cirrhosis. *Clin Gastroenterol Hepatol* 2006; **4**: 1062-1068

28 **Azzaroli F**, Colecchia A, Lodato F, Trerè D, Bacchi Reggiani ML, Festi D, Prati GM, Accogli E, Casanova S, Derenzini M, Roda E, Mazzella G. A statistical model predicting high hepatocyte proliferation index and the risk of developing hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *Aliment Pharmacol Ther* 2006; **24**: 129-136

29 **Nahon P**, Sutton A, Pessayre D, Rufat P, Ziol M, Ganne-Carrie N, Charnaux N, Trinchet JC, Gattegno L, Beaugrand M. Manganese superoxide dismutase dimorphism and iron overload, hepatocellular carcinoma, and death in hepatitis C virus–infected patients. *Clin Gastroenterol Hepatol* 2007; **5**: 630-635

30 **Squadrito G**, Pollicino T, Cacciola I, Caccamo G, Villari D, La Masa T, Restuccia T, Cucinotta E, Scisca C, Magazzu D, Raimondo G. Occult hepatitis B virus infection is associated with the development of hepatocellular carcinoma in chronic hepatitis C patients. *Cancer* 2006; **106**: 1326-1330

31 **Miyagawa S**, Kawasaki S, Makuuchi M. Comparison of the characteristics of hepatocellular carcinoma between hepatitis B and C viral infection: tumor multicentricity in cirrhotic liver with hepatitis C. *Hepatology* 1996; **24**: 307-310

32 **Shiratori Y**, Shiina S, Imamura M, Kato N, Kanai F, Okudaira T, Teratani T, Tohgo G, Toda N, Ohashi M. Characteristic difference of hepatocellular carcinoma between hepatitis B- and C- viral infection in Japan. *Hepatology* 1995; **22**: 1027-1033

33 **Sala M**, Forner A, Varela M, Bruix J. Prognostic prediction in patients with hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 171-180

34 **Sangiovanni A**, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, Del Ninno E, Morabito A, Colombo M. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. *Hepatology* 2006; **43**: 1303-1310

35 **Imazeki F**, Yokosuka O, Fukai K, Kawai S, Kanda T, Kojima H, Saisho H. Lower incidence of hepatic failure than hepatocellular carcinoma in Japanese patients with chronic hepatitis C. *Liver Int* 2005; **25**: 772-778

36 **Pessione F**, Ramond MJ, Peters L, Pham BN, Batel P, Rueff B, Valla DC. Five-year survival predictive factors in patients with excessive alcohol intake and cirrhosis. Effect of alcoholic hepatitis, smoking and abstinence. *Liver Int* 2003; **23**: 45-53

37 **Giannini EG**, Risso D, Testa R, Trevisani F, Di Nolfo MA, Del Poggio P, Benvegnù L, Ludovico Rapaccini G, Farinati F, Zoli M, Borzio F, Caturelli E. Prevalence and prognostic significance of the presence of esophageal varices in patients with hepatocellular carcinoma. *Clin Gastroenterol Hepatol* 2006; **4**: 1378-1384

38 **Bruix J**, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, Christensen E, Pagliaro L, Colombo M, Rodés J. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001; **35**: 421-430

39 **Bruix J**, Sherman M. Management of hepatocellular carcinoma. *Hepatology* 2005; **42**: 1208-1236

40 **Bruix J**, Sala M, Llovet JM. Chemoembolization for hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S179-S188

41 **Trinchet JC**, Ganne-Carrie N, Beaugrand M. Review article: intra-arterial treatments in patients with hepatocellular carcinoma. *Aliment Pharmacol Ther* 2003; **17** Suppl 2: 111-118

42 **Llovet JM**, Schwartz M, Mazzaferro V. Resection and liver transplantation for hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 181-200

43 **Beaugrand M**, N'kontchou G, Seror O, Ganne N, Trinchet JC. Local/regional and systemic treatments of hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 201-211

44 **Sasaki Y**, Yamada T, Tanaka H, Ohigashi H, Eguchi H, Yano M, Ishikawa O, Imaoka S. Risk of recurrence in a long-term follow-up after surgery in 417 patients with hepatitis B- or hepatitis C-related hepatocellular carcinoma. *Ann Surg* 2006; **244**: 771-780

45 **Colli A**, Fraquelli M, Casazza G, Massironi S, Colucci A, Conte D, Duca P. Accuracy of ultrasonography, spiral CT, magnetic resonance, and alpha-fetoprotein in diagnosing hepatocellular carcinoma: a systematic review. *Am J Gastroenterol* 2006; **101**: 513-523

46 **Caturelli E**, Bartolucci F, Biasini E, Vigliotti ML, Andriulli A, Siena DA, Attino V, Bisceglia M. Diagnosis of liver nodules observed in chronic liver disease patients during ultrasound screening for early detection of hepatocellular carcinoma. *Am J Gastroenterol* 2002; **97**: 397-405

47 **Di Bisceglie AM**, Sterling RK, Chung RT, Everhart JE, Dienstag JL, Bonkovsky HL, Wright EC, Everson GT, Lindsay KL, Lok AS, Lee WM, Morgan TR, Ghany MG, Gretch DR. Serum alpha-fetoprotein levels in patients with advanced hepatitis C: results from the HALT-C Trial. *J Hepatol* 2005; **43**: 434-441

48 **Sherman M**. Screening for hepatocellular carcinoma. *Best Pract Res Clin Gastroenterol* 2005; **19**: 101-118

49 **Ganne-Carrié N**, Chevret S, Barbare JC, Chaffaud C, Grando V, Vogt AM, Beaugrand M, Trinchet JC. Practical screening and early treatment of hepatocellular carcinoma. Results of a French survey. *Gastroenterol Clin Biol* 2002; 26: 570-577

50 **Chalasani N**, Horlander JC, Said A, Hoen H, Kopecky KK, Stockberger SM, Manam R, Kwo PY, Lumeng L. Screening for hepatocellular carcinoma in patients with advanced cirrhosis. *Am J Gastroenterol* 1999; **94**: 2988-2993

51 **Toyoda H**, Kumada T, Kiriyama S, Sone Y, Tanikawa M, Hisanaga Y, Yamaguchi A, Isogai M, Kaneoka Y, Washizu J. Impact of surveillance on survival of patients with initial hepatocellular carcinoma: a study from Japan. *Clin Gastroenterol Hepatol* 2006; **4**: 1170-1176

52 **Heathcote EJ**. Prevention of hepatitis C virus-related hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S294-S302

53 **Colombo M**, Donato MF. Prevention of hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 155-161

54 **Valla DC**, Chevallier M, Marcellin P, Payen JL, Trepo C, Fonck M, Bourliere M, Boucher E, Miguet JP, Parlier D, Lemonnier C, Opolon P. Treatment of hepatitis C virus-related cirrhosis: a randomized, controlled trial of interferon alfa-2b versus no treatment. *Hepatology* 1999; **29**: 1870-1875

55 **Nishiguchi S**, Shiomi S, Nakatani S, Takeda T, Fukuda K, Tamori A, Habu D, Tanaka T. Prevention of hepatocellular carcinoma in patients with chronic active hepatitis C and cirrhosis. *Lancet* 2001; **357**: 196-197

56 **Papatheodoridis GV**, Papadimitropoulos VC, Hadziyannis SJ. Effect of interferon therapy on the development of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis: a meta-analysis. *Aliment Pharmacol Ther* 2001; **15**: 689-698

57 **Cammà C**, Giunta M, Andreone P, Craxì A. Interferon and prevention of hepatocellular carcinoma in viral cirrhosis: an evidence-based approach. *J Hepatol* 2001; **34**: 593-602

58 **Shiratori Y**, Ito Y, Yokosuka O, Imazeki F, Nakata R, Tanaka N, Arakawa Y, Hashimoto E, Hirota K, Yoshida H, Ohashi Y, Omata M. Antiviral therapy for cirrhotic hepatitis C: association with reduced hepatocellular carcinoma development and improved survival. *Ann Intern Med* 2005; **142**: 105-114

59 **Hung CH**, Lee CM, Lu SN, Wang JH, Hu TH, Tung HD, Chen CH, Chen WJ, Changchien CS. Long-term effect of interferon alpha-2b plus ribavirin therapy on incidence of hepatocellular carcinoma in patients with hepatitis C virus-related cirrhosis. *J Viral Hepat* 2006; **13**: 409-414

60 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Lédinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54

61 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075

62 **Calès P**, Oberti F, Michalak S, Hubert-Fouchard I, Rousselet MC, Konaté A, Gallois Y, Ternisien C, Chevailler A, Lunel F. A novel panel of blood markers to assess the degree of liver fibrosis. *Hepatology* 2005; **42**: 1373-1381

63 **Reesink HW**, Zeuzem S, Weegink CJ, Forestier N, van Vliet A, van de Wetering de Rooij J, McNair L, Purdy S, Kauffman R, Alam J, Jansen PL. Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* 2006; **131**: 997-1002

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Hepatitis C virus: Virology, diagnosis and management of antiviral therapy

Stéphane Chevaliez, Jean-Michel Pawlotsky

Stéphane Chevaliez, Jean-Michel Pawlotsky, French National Reference Center for Viral Hepatitis B, C and delta,Department of Virology & INSERM U841, Henri Mondor Hospital, University of Paris XII, Créteil, France
Correspondence to: Jean-Michel Pawlotsky, MD, PhD, Professor, Department of Virology, Hôpital Henri Mondor, 51 avenue du Maréchal de Lattre de Tassigny, 94010 Créteil, France. jean-michel.pawlotsky@hmn.aphp.fr
Telephone: +33-1-49812827 Fax: +33-1-49814831
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Abstract

Hepatitis C virus (HCV) infects approximately 170 million individuals worldwide. Prevention of HCV infection complications is based on antiviral therapy with the combination of pegylated interferon alfa and ribavirin. The use of serological and virological tests has become essential in the management of HCV infection in order to diagnose infection, guide treatment decisions and assess the virological response to antiviral therapy. Anti-HCV antibody testing and HCV RNA testing are used to diagnose acute and chronic hepatitis C. The HCV genotype should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure. HCV RNA monitoring during therapy is used to tailor treatment duration in HCV genotype 1 infection, and molecular assays are used to assess the end-of-treatment and, most importantly the sustained virological response, i.e. the endpoint of therapy.

Key words: Hepatitis C virus; serological tests; Hepatitis C virus genotype; HCV RNA quantification; Interferon alpha; Ribavirin

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INTRODUCTION

Hepatitis C virus (HCV) infects approximately 170 million individuals worldwide. Chronic HCV infection has been estimated to be responsible for approximately 250 000 to 350 000 deaths per year, essentially related to decompensation of cirrhosis, end-stage liver disease and hepatocellular carcinoma. Prevention of HCV infection complications can be achieved by antiviral therapy based on the use of a combination of pegylated interferon (IFN) alfa and ribavirin, that yields a sustained eradication of infection in 40% to 50% of cases[1]. The use of serological and virological tests has become essential in the management of HCV infection in order to diagnose infection, and most importantly guide treatment decisions and assess the virological response to antiviral therapy.

HEPATITIS C VIRUS VIROLOGY

Hepatitis C virus and its genotypes

HCV is a member of the Flaviviridae family, genus Hepacivirus[2]. Six HCV genotypes[1-6] and a large number of subtypes (1a, 1b, 1c, *etc.*) have been identified so far[2]. HCV only natural host is man. All HCV genotypes have a common ancestor virus. However, HCV genotypes 1, 2, and 4 emerged and diversified in Central and Western Africa, genotype 5 in South Africa, and genotypes 3 and 6 in China, South-East Asia and the Indian subcontinent. In these areas, a large number of subtypes of these genotypes are found[2]. The rest of the world, in particular industrialized areas, harbor a small number of HCV subtypes that could widely spread because they met an efficient route of transmission, such as blood transfusion or the intravenous use of drugs. They include genotypes 1a, 1b, 2a, 2b, 2c, 3a, 4a and 5a[2].

HCV virion and lifecycle

The HCV virion is made of a single-stranded positive RNA genome, contained into an icosahedral capsid, itself enveloped by a lipid bilayer within which two different glycoproteins are anchored[3]. The genome contains three distinct regions: (1) a short 5' non-coding region that contains two domains, a stem-loop structure involved in positive-strand priming during HCV replication and the internal ribosome entry site (IRES), the RNA structure responsible for attachment of the ribosome and polyprotein translation; (2) a long, unique open reading frame (ORF) of more than 9000 nucleotides which is translated into a precursor polyprotein, secondarily cleaved to give birth to the structural proteins (the capsid protein

C and the two envelope glycoproteins E1 and E2) and to the non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The functions of the non-structural proteins have been elucidated by a large number of studies and by analogy to related viruses; only NS4B and NS5A have no well-defined functions to date; (3) a short 3' non-coding region principally involved in minus-strand priming during HCV replication[3]. The 3' UTR has been divided into three regions: a variable sequence of approximately 40 bases, a variable length poly-UC rich tract, and a highly conserved 98 base region.

The HCV lifecycle starts with virion attachment to its specific receptor[3]. Several candidate molecules have been suggested to play a role in the receptor complex, including tetraspanin CD81, the scavenger receptor BI (SR-BI), the adhesion molecules DC-SIGN and L-SIGN and the low-density lipoprotein (LDL) receptor. The HCV lifecyle is poorly known because of the lack, until very recently, of a productive culture system. It is supposed, by analogy with the Flaviviridae, that the virus linked to its receptor complex is internalized, and that the nucleocapsid is released into the cytoplasm. The virus is decapsidated, and the genomic HCV RNA is used both for polyprotein translation and replication in the cytoplasm. Replication and post-translational processing appear to take place in a membranous web made of the non-structural proteins and host cell proteins called "replication complex", located in close contact with perinuclear membranes. Genome encapsidation appears to take place in the endoplasmic reticulum and nucleocapsids are enveloped and matured into the Golgi apparatus before newly produced virions are released in the pericellular space by exocytosis[3].

VIROLOGICAL TOOLS

Serological assays

Anti-HCV antibody detection: The detection of anti-HCV antibodies in plasma or serum is based on the use of third-generation enzyme immunoassays (EIAs), that detect antibodies directed against various HCV epitopes. Recombinant antigens are used to capture circulating anti-HCV antibodies onto the wells of microtiter plates, microbeads, or specific holders adapted to closed automated devices. The presence of anti-HCV antibodies is revealed by anti-antibodies labeled with an enzyme that catalyzes the transformation of a substrate into a colored compound. The optical density (OD) ratio of the reaction (sample OD/internal control OD) is proportional to the amount of antibodies in the serum or plasma sample[4]. The specificity of third-generation EIAs for anti-HCV is greater than 99%[5]. Their sensitivity is more difficult to determine, given the lack of a gold standard method, but it is excellent in HCV-infected immunocompetent patients. EIAs can be fully automated and are well adapted to large volume testing. Immunoblot tests are nowadays clinically obsolete given the good performance of third-generation anti-HCV EIAs[6].

Serological determination of the HCV genotype: The HCV genotype can be determined by seeking for antibodies directed to genotype-specific HCV epitopes with a competitive EIA. The currently available assay (Murex HCV serotyping 1-6 HC02, Abbott Laboratories, North Chicago, Illinois) identifies the type (1 to 6), but does not discriminate among the subtypes, and provides interpretable results in approximately 90% of chronically infected immunocompetent patients[7]. Mixed serological reactivities can be observed that could be related to mixed infection although cross-reactivity or recovery from one genotype infection and persistence of viremia with another genotype cannot be ruled out.

Detection and quantification of HCV RNA

For many years, the HCV RNA quantitative units used in the various assays did not represent the same amount of HCV RNA in a clinical sample. The World Health Organization (WHO) has established an international standard for universal standardization of HCV RNA quantification units. An HCV RNA international unit (IU) has been defined, which is currently used in all of the commercial HCV RNA quantitative assays and should be preferred to any other quantitative unit. Indeed, the use of standardized IUs for HCV RNA quantification allows deriving recommendations and guidelines from clinical trials and applying them in clinical practice with any HCV RNA assay.

Qualitative, non-quantitative HCV RNA detection: Qualitative detection assays are based on the principle of target amplification using either "classic" polymerase chain reaction (PCR), "real-time" PCR or "transcription-mediated amplification"(TMA)[8]. HCV RNA is extracted and reverse transcribed into a single-stranded complementary DNA (cDNA), which is subsequently processed into a cyclic enzymatic reaction leading to the generation of a large number of detectable copies. Double-stranded DNA copies of HCV genome are synthesized in PCR-based assays, whereas single-stranded RNA copies are generated in TMA. Detection of amplified products is achieved by hybridizing the produced amplicons onto specific probes after the reaction in "classic" PCR or TMA techniques[8]. In "real-time" PCR, each round of amplification leads to the emission of a fluorescent signal and the number of signals per cycle is proportional to the amount of HCV RNA in the starting sample[8-10]. Qualitative detection assays must detect 50 HCV RNA IU/mL or less, and have equal sensitivity for the detection of all HCV genotypes. The lower limit of detection of the qualitative, nonquantitative reverse-transcriptase PCR-based assay Amplicor® HCV v2.0, or of its semi-automated version Cobas® Amplicor® HCV v2.0 (Roche Molecular Systems, Pleasanton, California) is 50 IU/mL, whereas that of the TMA-based assay Versant® HCV RNA Qualitative Assay (Siemens, Tarrytown, New York) is 10 IU/mL. Real-time PCR assays, which are also able to quantify HCV RNA, have lower limits of detection of 15 IU/mL (Cobas Ampliprep®-Cobas Taqman® (CAP-CTM) HCV Test, Roche Molecular Systems) and of 12-30 IU/mL according to the amount of blood tested (Abbott RealTime™ HCV Assay, Abbott Diagnostic) when they are used as purely qualitative, nonquantitative assays.

HCV RNA quantification: HCV RNA can be quantified

by means of target amplification techniques (competitive PCR or real-time PCR) or signal amplification techniques [branched DNA (bDNA) assay][8]. Five standardized assays are commercially available. Two of them are based on competitive PCR: Amplicor HCV Monitor® v2.0 and its semi-automated version Cobas® Amplicor HCV Monitor® v2.0 (Roche Molecular Systems), and LCx® HCV RNA Quantitative Assay (Abbott Laboratories); one is based on bDNA technology, Versant® HCV RNA 3.0 Assay (Siemens); and two are based on real-time PCR amplification, Cobas® TaqMan HCV Test, which can be coupled with automated extraction in Cobas Ampliprep® (CAP-CTM, Roche Molecular Systems), and Abbott RealTime™ HCV assay (Abbott Diagnostics), which uses the Abbott *m*2000RT system and can also be coupled with an automated extraction procedure in *m*2000SP (*m*2000 Real-Time PCR System). HCV RNA levels falling above the upper limit of quantification of the assay are underestimated and the samples must be retested after 1/10 to 1/100 dilution in order to achieve accurate quantification. The Cobas® TaqMan HCV Test has been shown to underquantify some HCV genotype 4 and, less often, HCV genotype 2 samples[11]. In addition, differences in calibration of the assays relative to the primary WHO HCV RNA standard lead to slight differences between the results given in the same samples by different assays in spite of the use of international units as a quantification unit[11]. However, the most promising approach for the future is fully automated real-time PCR assays.

Molecular determination of the HCV genotype (genotyping)

The reference method for HCV genotype determination is direct sequencing of the NS5B or E1 regions of HCV genome by means of "in-house" techniques, followed by sequence alignment with prototype sequences and phylogenetic analysis[2,12]. In clinical practice, HCV genotype can be determined by various commercial kits, using direct sequence analysis of the 5' noncoding region (Trugene® 5' NC HCV Genotyping Kit, Bayer HealthCare) or reverse hybridization analysis using genotype-specific probes located in the 5' noncoding region (commercialized as INNO-LiPA HCV II, Innogenetics, Ghent, Belgium, or Versant® HCV Genotyping Assay, Bayer HealthCare)[13-16]. Mistyping is rare with these techniques, but mis-subtyping may occur in 10% to 25% of cases, related to the studied region (5' noncoding region) rather than the technique used. These errors have no clinical consequences, because only the type is used for therapeutic decision-making. An assay based on direct sequencing of the NS5B region is currently in development (Trugene® NS5B HCV Genotyping Kit, Bayer HealthCare).

DIAGNOSIS OF HCV INFECTION

Acute hepatitis C

Patients with a suspicion of acute hepatitis C should be tested for both anti-HCV antibodies by EIA and HCV RNA with a sensitive technique, i.e. an HCV RNA assay with a lower limit of detection of 50 IU/mL or less[4]. Four marker profiles can be observed according to the presence or absence of either marker. The presence of HCV RNA in the absence of anti-HCV antibodies is strongly indicative of acute HCV infection, which will be confirmed by seroconversion (i.e. the appearance of anti-HCV antibodies) a few days to week later. Acutely infected patients can also have both HCV RNA and anti-HCV antibodies at the time of diagnosis. It is difficult, in this case, to distinguish acute hepatitis C from an acute exacerbation of chronic hepatitis C or an acute hepatitis of another cause in a patient with chronic hepatitis C.

Acute hepatitis C is very unlikely if both anti-HCV antibodies and HCV RNA are absent or if anti-HCV antibodies are present without HCV RNA. The latter patients should however be retested after a few week because HCV RNA can be temporarily undetectable, due to transient, partial control of viral replication before infection becomes chronic[17]. Apart from such cases, the presence of anti-HCV antibodies in the absence of HCV RNA is generally seen in patients who have recovered from a past HCV infection. Nevertheless, this pattern cannot be differentiated from a false positive EIA result, the exact prevalence of which is unknown.

Chronic hepatitis C

In patients with clinical or biological signs of chronic liver disease, chronic hepatitis C is certain when both anti-HCV antibodies and HCV RNA are present[6,18]. Detectable HCV replication in the absence of anti-HCV antibodies is exceptional with the current enzyme immunoassays, almost exclusively observed in profoundly immunodepressed patients, hemodialysis patients or agammaglobulinemic subjects[19,20].

In patients who have no indication for therapy or have a contra-indication to the use of antiviral drugs, virological tests have no prognostic value. Thus, they cannot be used to predict the natural course of infection or the onset of extrahepatic manifestations. In untreated patients, the severity of liver inflammation and fibrosis must be evaluated every three to five years by means of a liver biopsy or non-invasive serological or ultrasound-based testing[1].

MANAGEMENT OF ANTIVIRAL THERAPY

The current standard treatment for chronic hepatitis C is the combination of pegylated interferon (IFN) alfa and ribavirin[1]. The efficacy endpoint of hepatitis C treatment is the "sustained virological response" (SVR), defined by the absence of detectable HCV RNA in serum as assessed by an HCV RNA assay with a lower limit of detection of 50 IU/mL or less 24 wk after the end of treatment[1]. The advent of new, more sensitive assays with different lower limits of HCV RNA detection may create some confusion as to whether patients have "undetectable" HCV RNA during therapy.

Initiation of therapy

Only patients with detectable HCV RNA should be considered for pegylated IFN alfa and ribavirin combination therapy[1]. The decision to treat patients

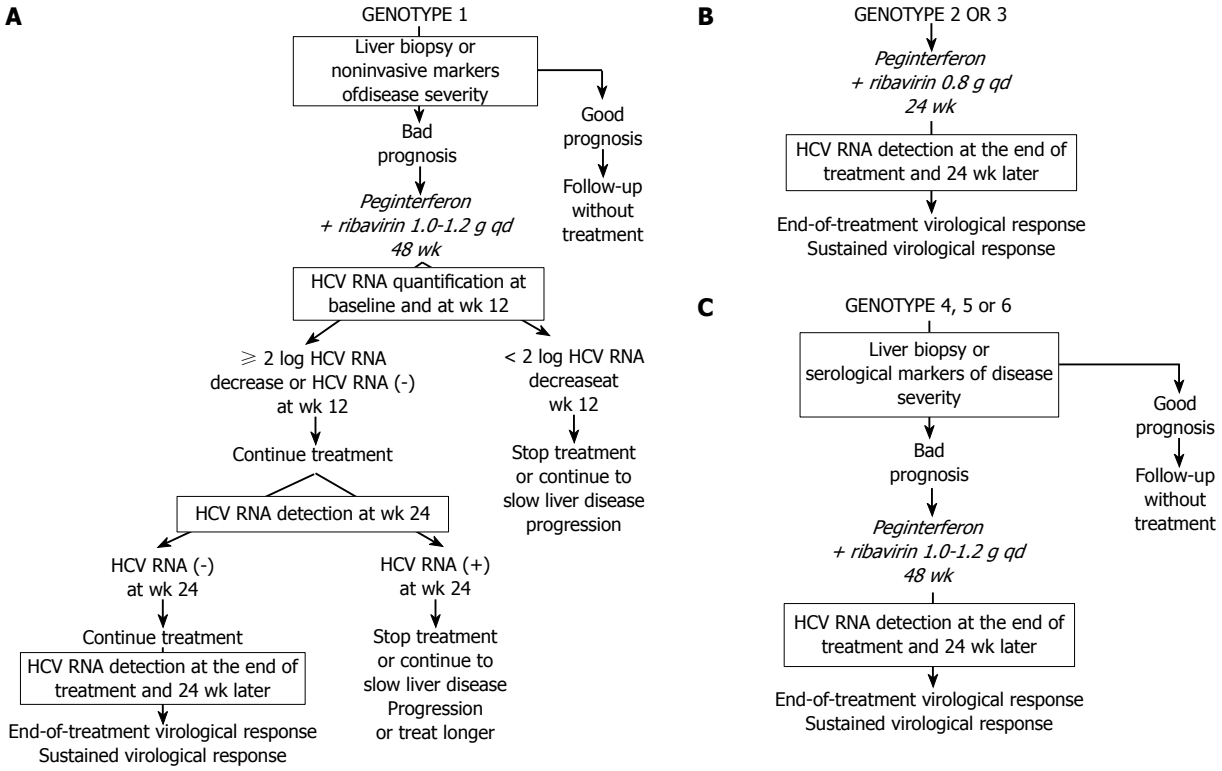

Figure 1 Current algorithms for the use of HCV virological tools in the treatment of chronic hepatitis C, according to the HCV genotype: genotype 1 (**A**), genotypes 2 and 3 (**B**), and genotypes 4, 5 and 6 (**C**). Adapted from[32], with permission.

with chronic hepatitis C depends on multiple parameters, including a precise assessment of the severity of liver disease and of its foreseeable outcome, the presence of absolute or relative contra-indications to therapy, and the patient's willingness to be treated.

The HCV genotype should be systematically determined before treatment, as it determines the indication, the duration of treatment, the dose of ribavirin and the virological monitoring procedure[21].

HCV genotype 1

Given the likelihood of a sustained virological response, of the order of 40% to 50%, a precise assessment of liver disease prognosis by means of a liver biopsy or a non-invasive method based on serological markers of fibrosis or ultrasound-based testing[22,23] must be performed in order to help with the treatment decision (Figure 1A). It is recommended not to treat patients with mild lesions and to re-assess their liver disease after 3 to 5 years. The patients with inflammation and/or fibrosis (Metavir score A $\geqslant$ 2 and/or F $\geqslant$ 2) have an indication for therapy[1].

The approved dose of pegylated IFN alfa-2a is 180 μg per week, independent of body weight, whereas that of pegylated IFN alfa-2b is weight-adjusted at 1.5 μg/kg per week, identical for all HCV genotypes. Patients infected with HCV genotype 1 should receive a high dose of ribavirin, i.e. 1000 to 1200 mg daily, based on body weight less than or greater than 75 kg. The heaviest patients could benefit from a higher ribavirin dose, up to 1600 mg daily. Genotype 1-infected patients theoretically require 48 wk of treatment (Figure 1A)[1]. Monitoring of HCV RNA load decrease during therapy must be performed in order to avoid treating patients with no likelihood of a subsequent SVR[24,25]. For this purpose, HCV RNA quantification must be performed at baseline and after 12 wk of treatment (Figure 1A) with the same technique in order to ensure comparability of the results at the two time points[1]. Treatment must be continued when there is a 2-log drop in HCV RNA level, i.e. when baseline HCV RNA level is divided by 100 or more, or when HCV RNA is undetectable at wk 12[1]. In these patients, it is recommended to assess the presence of HCV RNA with a sensitive technique (lower limit of detection: 50 IU/mL or less) at wk 24. If HCV RNA is undetectable at wk 24, treatment must be continued until wk 48, with a high likelihood of an SVR. It was recently suggested that 24 wk of therapy might be sufficient for patients with a low baseline viral load, a 2-log or more decline at wk 12 and undetectable HCV RNA at wk 24[26]. Rapid virological responders, defined as patients with no detectable HCV RNA at wk 4 of therapy could also benefit from shorter therapy. Ongoing trials will feed future guidelines.

In contrast, if HCV RNA is still detectable at wk 24, the likelihood of an SVR is virtually nil and treatment can be stopped or continued with the only aim to slow liver disease progression in patients with a severe prognosis, without any hope to eradicate infection (Figure 1A)[1,24]. Ongoing trials are studying whether a prolonged antiviral treatment or maintenance therapy with pegylated IFN alfa monotherapy could be beneficial in these patients. When treatment is continued until wk 48, the end-of-treatment and sustained virological responses should be assessed by

means of a sensitive HCV RNA assay, with a lower limit of detection of 50 IU/mL or less[1]. HCV RNA detection at the end of therapy is highly predictive of a post-treatment relapse (the more sensitive assays allow earlier identification of relapser patients), whereas the absence of HCV RNA at the end of treatment indicates a virological response. The latter patients must be retested for HCV RNA with a sensitive method 24 wk later in order to assess the SVR, i.e. the endpoint of therapy[1,4]. HCV infection appears to be definitively cured in the vast majority of sustained virological responders.

The lack of a 12-wk virological response (no change or an HCV RNA decrease of less than 2 logs at wk 12) is associated with a virtually nil probability of a subsequent sustained virological response[24,25]. Treatment can thus be stopped at wk 12 in these patients, or continued to slow liver disease progression without clearing the virus (Figure 1A). The benefits of maintenance therapy on the outcome of HCV-associated liver disease are currently under investi-gation. This "stopping rule", based on monitoring of HCV RNA load reduction at wk 12, was recently shown to also apply to patients co-infected with HCV and human immunodeficiency virus[27-29].

HCV genotypes 2 and 3

Patients infected with HCV genotypes 2 or 3 have a 70%-80% likelihood of an SVR with a low dose of ribavirin and only 24 wk of treatment[21,25,30]. Thus, in the absence of contra-indications, these patients should be treated regardless of the severity of their liver disease (Figure 1B). The recommended dose of pegylated IFN alfa-2a or alfa-2b is the same as for HCV genotype 1. The fixed recommended dose of ribavirin is 800 mg per day (Figure 1B)[1]. It is possible that lower doses of ribavirin and/or shorter duration of treatment could be sufficient to achieve an SVR in certain subgroups of patients with genotype 2 or 3 infection, such as those with a low baseline viral load and no extensive fibrosis or cirrhosis, as suggested by recent preliminary data[31]. One should be careful in patients who combine several baseline parameters of non-response, such as extensive fibrosis, an old age and a male gender, who might need 48 wk of therapy to clear infection.

No monitoring of HCV RNA level changes during therapy is recommended in the patients with genotype 2 or 3 infection, because the vast majority of them become HCV RNA-negative early during treatment. Like in HCV genotype 1-infected patients, the virological response must be assessed by means of a sensitive HCV RNA assay at the end of therapy and 24 wk later in order to determine whether the virological response is sustained (Figure 1B)[1,4].

HCV genotypes 4, 5 and 6

In the absence of any clinical trial including a sufficient number of patients, the likelihood of an SVR and the optimal treatment schedule remain unknown for the patients infected with HCV genotypes 4, 5 or 6. It is thus recommended to treat them like those infected with HCV genotype 1, i.e. with pegylated IFN alfa at the usual dose, combined with a high dose of ribavirin (1000-1200 mg per day, according to body weight less or greater than 75 kg) (Figure 1C). In the absence of published data, no stopping rules have been defined and it is recommended to treat these patients for a total of 48 wk. The virological response must be assessed by means of a sensitive HCV RNA assay (lower limit of detection of 50 IU/mL or less) at the end of therapy and 24 wk later[1,4].

CONCLUSION

Virological tools have nowadays become mandatory at every step of HCV infection treatment. Algorithms have been derived that allow the clinician to tailor treatment schedules to the individual patient and his/her virological response to therapy, in order to optimize the results of pegylated IFN-ribavirin therapy. This approach is cost-effective, because treatment dose and duration are adapted to the patient's needs and administration can be stopped when the likelihood of a sustained virological response is nil.

In the future, more sensitive HCV RNA assays will be available. They will better differentiate responder patients who will subsequently relapse from those who will not. They will also detect the virological relapse earlier after the end of therapy. A number of ongoing studies are currently assessing the capacity of viral load measurements at earlier time points to predict the sustained virological response or non-response. New algorithms will be developed soon, based on the assessment of viral load reductions at wk 4 of therapy, or eventually earlier. Too complicated algorithms applied to a too high number of patient subgroups may however make therapy more and more difficult on an every day basis. In addition, early predictions based on frequent viral load measurements may not always be easily feasible in the clinical setting. Overall, it will be important to keep treatment algorithms as simple and feasible as possible in order to offer the best chance of success to the individual patients.

REFERENCES

1 **NIH Consensus Statement on Management of Hepatitis C: 2002.** *NIH Consens State Sci Statements* 2002; **19**: 1-46
2 **Simmonds P**, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H, Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ, Widell A. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. *Hepatology* 2005; **42**: 962-973
3 **Penin F**, Dubuisson J, Rey FA, Moradpour D, Pawlotsky JM. Structural biology of hepatitis C virus. *Hepatology* 2004; **39**: 5-19
4 **Pawlotsky JM**. Use and interpretation of virological tests for hepatitis C. *Hepatology* 2002; **36**: S65-S73
5 **Colin C**, Lanoir D, Touzet S, Meyaud-Kraemer L, Bailly F, Trepo C. Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. *J Viral Hepat* 2001; **8**: 87-95
6 **Pawlotsky JM**, Lonjon I, Hezode C, Raynard B, Darthuy F, Remire J, Soussy CJ, Dhumeaux D. What strategy should be used for diagnosis of hepatitis C virus infection in clinical laboratories? *Hepatology* 1998; **27**: 1700-1702
7 **Pawlotsky JM**, Prescott L, Simmonds P, Pellet C, Laurent-Puig

P, Labonne C, Darthuy F, Remire J, Duval J, Buffet C, Etienne JP, Dhumeaux D, Dussaix E. Serological determination of hepatitis C virus genotype: comparison with a standardized genotyping assay. *J Clin Microbiol* 1997; **35**: 1734-1739

8 **Pawlotsky JM**. Molecular diagnosis of viral hepatitis. *Gastroenterology* 2002; **122**: 1554-1568

9 **Martell M**, Gómez J, Esteban JI, Sauleda S, Quer J, Cabot B, Esteban R, Guardia J. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* 1999; **37**: 327-332

10 **Komurian-Pradel F**, Paranhos-Baccalà G, Sodoyer M, Chevallier P, Mandrand B, Lotteau V, André P. Quantitation of HCV RNA using real-time PCR and fluorimetry. *J Virol Methods* 2001; **95**: 111-119

11 **Chevaliez S**, Bouvier-Alias M, Brillet R, Pawlotsky JM. Overestimation and underestimation of hepatitis C virus RNA levels in a widely used real-time polymerase chain reaction-based method. Hepatology 2007;46:22-31

12 **Simmonds P**. Viral heterogeneity of the hepatitis C virus. *J Hepatol* 1999; **31** Suppl 1: 54-60

13 **Germer JJ**, Vandenameele JN, Mitchell PS, Harmsen WS, Yao JD. Automated sample preparation for the TRUGENE HIV-1 genotyping kit using the MagNA pure LC instrument. *Diagn Microbiol Infect Dis* 2004; **49**: 59-61

14 **Stuyver L**, Wyseur A, van Arnhem W, Hernandez F, Maertens G. Second-generation line probe assay for hepatitis C virus genotyping. *J Clin Microbiol* 1996; **34**: 2259-2266

15 **Stuyver L**, Wyseur A, van Arnhem W, Lunel F, Laurent-Puig P, Pawlotsky JM, Kleter B, Bassit L, Nkengasong J, van Doorn LJ. Hepatitis C virus genotyping by means of 5'-UR/core line probe assays and molecular analysis of untypeable samples. *Virus Res* 1995; **38**: 137-157

16 **Zheng X**, Pang M, Chan A, Roberto A, Warner D, Yen-Lieberman B. Direct comparison of hepatitis C virus genotypes tested by INNO-LiPA HCV II and TRUGENE HCV genotyping methods. *J Clin Virol* 2003; **28**: 214-216

17 **Lavillette D**, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J Virol* 2005; **79**: 6023-6034

18 **EASL International Consensus Conference on Hepatitis C.** Paris, 26-28, February 1999, Consensus Statement. European Association for the Study of the Liver. *J Hepatol* 1999; **30**: 956-961

19 **Lok AS**, Chien D, Choo QL, Chan TM, Chiu EK, Cheng IK, Houghton M, Kuo G. Antibody response to core, envelope and nonstructural hepatitis C virus antigens: comparison of immunocompetent and immunosuppressed patients. *Hepatology* 1993; **18**: 497-502

20 **Thio CL**, Nolt KR, Astemborski J, Vlahov D, Nelson KE, Thomas DL. Screening for hepatitis C virus in human immunodeficiency virus-infected individuals. *J Clin Microbiol* 2000; **38**: 575-577

21 **Hadziyannis SJ**, Sette H, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355

22 **Castéra L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Lédinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350

23 **Poynard T**, Imbert-Bismut F, Munteanu M, Ratziu V. FibroTest-FibroSURE: towards a universal biomarker of liver fibrosis? *Expert Rev Mol Diagn* 2005; **5**: 15-21

24 **Davis GL**, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645-652

25 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, Häussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982

26 **Zeuzem S**, Buti M, Ferenci P, Sperl J, Horsmans Y, Cianciara J, Ibranyi E, Weiland O, Noviello S, Brass C, Albrecht J. Efficacy of 24 weeks treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C infected with genotype 1 and low pretreatment viremia. *J Hepatol* 2006; **44**: 97-103

27 **Chung RT**, Andersen J, Volberding P, Robbins GK, Liu T, Sherman KE, Peters MG, Koziel MJ, Bhan AK, Alston B, Colquhoun D, Nevin T, Harb G, van der Horst C. Peginterferon Alfa-2a plus ribavirin versus interferon alfa-2a plus ribavirin for chronic hepatitis C in HIV-coinfected persons. *N Engl J Med* 2004; **351**: 451-459

28 **Pawlotsky JM**. Treating hepatitis C in "difficult-to-treat" patients. *N Engl J Med* 2004; **351**: 422-423

29 **Torriani FJ**, Rodriguez-Torres M, Rockstroh JK, Lissen E, Gonzalez-García J, Lazzarin A, Carosi G, Sasadeusz J, Katlama C, Montaner J, Sette H, Passe S, De Pamphilis J, Duff F, Schrenk UM, Dieterich DT. Peginterferon Alfa-2a plus ribavirin for chronic hepatitis C virus infection in HIV-infected patients. *N Engl J Med* 2004; **351**: 438-450

30 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965

31 **Dalgard O**, Bjøro K, Hellum KB, Myrvang B, Ritland S, Skaug K, Raknerud N, Bell H. Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. *Hepatology* 2004; **40**: 1260-1265

32 **Pawlotsky JM**. Therapy of hepatitis C: from empiricism to eradication. *Hepatology* 2006; **43**: S207-S220

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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TOPIC HIGHLIGHT

Francoise Lunel Fabiani, Professor, *Series Editor*

Hepatitis C virus-related lymphoproliferative disorders: An overview

Anna Linda Zignego, Carlo Giannini, Clodoveo Ferri

Anna Linda Zignego, Carlo Giannini, Department of Internal Medicine, Center for Systemic Manifestations of Hepatitis Viruses (MASVE) and Center for Research, Transfer and High Education DENOThe, University of Florence, Medical School, Florence, Italy
Clodoveo Ferri, Rheumatology Unit, Department of Internal Medicine, Medical School, University of Modena and Reggio Emilia, Modena, Italy
Correspondence to: Anna Linda Zignego, MD, PhD, Professor of Medicine, Department of Internal Medicine, University of Florence, Viale Morgagni 85, 50134 Florence, Italy. a.zignego@dmi.unifi.it
Telephone: +39-055-4296483 Fax: +39-055-417123
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Abstract

Hepatitis C virus (HCV) is a global health problem affecting 3% of the world's population (about 180 million) and a cause of both hepatic and extrahepatic diseases. B-cell lymphoproliferative disorders, whose prototype is mixed cryoglobulinemia, represent the most closely related as well as the most investigated HCV-related extrahepatic disorder. The association between extrahepatic (lymphoma) as well as hepatic malignancies (hepatocellular carcinoma) has justified the inclusion of HCV among human cancer viruses. HCV-associated manifestations also include porphyria cutanea tarda, lichen planus, nephropathies, thyreopathies, sicca syndrome, idiopathic pulmonary fibrosis, diabetes, chronic polyarthritis, sexual dysfunctions, cardiopathy/atherosclerosis, and psychopathological disorders. A pathogenetic link between HCV virus and some lymphoproliferative disorders was confirmed by their responsiveness to antiviral therapy, which is now considered the first choice treatment. The aim of the present paper is to provide an overview of extrahepatic manifestations of HCV infection with particular attention to B-cell lymphoproliferative disorders. Available pathogenetic hypotheses and suggestions about the most appropriate, currently available, therapeutic approaches will also be discussed.

Key words: Hepatitis C virus; Extrahepatic manifestations; Lymphoproliferative disorders; Mixed cryoglobulinemia; Lymphoma

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INTRODUCTION

Hepatitis C virus (HCV) is associated with several extrahepatic disorders (extrahepatic manifestations of HCV = EHMs-HCV)[1,2]. These latter may be classified into four main categories including: (1) EHMs-HCV that are characterized by a very strong association as demonstrated by both epidemiological and pathogenetic evidence; (2) disorders for which a significant association with HCV infection is supported by substantial data; (3) associations that still require confirmation and/or a more detailed characterization compared to similar pathologies of different etiology or idiopathic nature; and (4) anecdotal observations (Table 1)[3]. B-cell lymphoproliferative disorders (LPDs) represent the most closely related as well as the most investigated forms and should be considered an ideal model for both clinico-therapeutic and pathogenetic deductions.

HCV-RELATED LYMPHOPROLIFERATIVE DISORDERS

Mixed Cryoglobulinemia

Mixed cryoglobulinemia (MC) is the most documented HCV-related extrahepatic disorder[4-6]. The strong association between HCV and MC has been confirmed by serological and molecular investigations[4,6,7]. This disorder is defined by the presence of serum immunoglobulins (Igs) that become insoluble below 37℃ and can dissolve by warming serum (cryoglobulins, CGs). According to Brouet *et al*[8], CGs are classified on the basis of their Ig composition: in type I cryoglobulinemia CGs are composed of a pure monoclonal component, usually sustained by an indolent B-cell lymphoma, whereas type II and type III MC are characterized by a mixture of polyclonal IgG and monoclonal IgM or by polyclonal IgG and IgM, respectively. In MC, IgG and IgM with rheumatoid factor (RF) activity participate in the composition of circulating immunocomplexes (CIC). The IgM

Table 1 Classification of extrahepatic manifestations of HCV infection

A: Association defined on the basis of high prevalence and pathogenesis
Mixed cryoglobulinemia (complete or incomplete clinical syndrome)
B: Association defined on the basis of higher prevalences than in controls
B-cell non-Hodgkin's lymphoma
Monoclonal gammopathies
Porphyria cutanea tarda
Lichen planus
C: Associations to be confirmed/characterized
Autoimmune thyroiditis
Thyroid cancer
Sicca syndrome
Alveolitis-Lung fibrosis
Diabetes mellitus
Non-cryoglobulinemic nephropathies
Aortic atherosclerosis
D: Anecdotal observations
Psoriasis
Peripheral/central neuropathies
Chronic polyarthritis
Rheumatoid arthritis
Polyartheritis nodosa
Bechet's syndrome
Poly/dermatomyositis
Fibromyalgia
Chronic urticaria
Chronic pruritus
Kaposi's pseudo-sarcoma
Vitiligo
Cardiomyopathies
Mooren corneal ulcer
Erectile dysfunctions
Necrolytic acral erythema

monoclonal component in type II MC is represented by RF molecules that most frequently display the WA cross-reactive idiotype[9]. Type II (MC II) accounts for 50%-60% of MC, and type III (MC III) for the remaining 30%-40%. Cryoproteins are HCV RNA enriched in comparison with supernatants in HCV-infected patients. Studies performed in unselected populations of chronic HCV-positive subjects showed a high prevalence of serum CGs, ranging from 19% to > 50% according to different studies[10,11]. However, CGs are generally present at low levels and symptoms are generally absent or very mild, whereas clinically evident MC - MC syndrome or MCS would be evident in 10%-15% to 30% of MC subjects and in 5%-10% of all HCV infected patients[10-12].

The most common symptoms of MCS are weakness, arthralgias, and purpura (Meltzer and Franklin triad). Raynaud's phenomenon, peripheral neuropathy, sicca syndrome, renal involvement, lung disorders, fever, and hematocytopenia may also be observed[13]. In a recent study involving 231 Italian MC patients, peripheral neuropathy was observed in the majority of cases, representing the most frequent clinical feature after the triad, followed by sicca syndrome, Raynaud's phenomenon and renal involvement[14].

In MC patients, peripheral neuropathy includes mixed neuropathies, which are prevalently sensitive, axonal, and can manifest themselves as symmetrical distal neuropathies, multiple mononeuritis or mononeuropathies[15-17]. Involvement of the central nervous system is unusual and generally presents as transient dysarthria and hemiplegia. Pathological findings show axonal damage with epineural vasculitic infiltrates and endoneural microangiopathy.

The association between MC and glomerulonephritis has been clearly demonstrated[18,19]. Nephropathy is observed in 20% of patients at MCS diagnosis, and in 35%-60% during follow-up[14,19]. The presence of renal involvement is one of the worst prognostic indices in the natural history of MCS[14]. MC-related nephropathy is clinically characterized by hematuria, proteinuria (sometimes in nephritic range, i.e. > 3 g/24 h), edemas, and renal failure of variable grade. The histological picture is similar to that of idiopathic membranoproliferative glomerulonephritis, but characterized by capillary thrombi consisting of precipitated cryoglobulins under light microscopy and widespread deposits of IgM in capillary loops. Histological analysis shows a thickening of glomerular basal membrane, cellular proliferation and infiltration of circulating macrophages. From a clinical point of view, MC patients frequently present with one or more subclinical signs of renal involvement, including asymptomatic hematuria, without nephrotic proteinuria (< 3 g/24 h), with normal or only fairly reduced renal function (creatinine < 1.5 mg). In 30% of cases, the clinical manifestation of MC may be acute nephrotic syndrome[4]. Hypertension may be seen in 80% of MC patients with renal involvement[20].

Renal manifestations represent a negative prognostic factor in MC, even if their course may vary. However, in the long term, MC-related nephropathy may progress to terminal chronic renal failure requiring dialysis in up to 15% of patients[21].

The association between MC and severe liver damage has been widely discussed[1,2,9,22,23]. Recent studies have shown an epidemiological association between MC and severe liver damage[10,24] as well as between MC and liver steatosis[25]. In the previously cited study performed on 231 MC patients, survival analysis according to the Kaplan-Meier method revealed a significantly lower cumulative 10-year survival, calculated from time of diagnosis, in MC patients when compared with expected death in the age- and sex-matched general population. Moreover, significantly lower survival rates were observed in males and in subjects with renal involvement, the most frequent causes of death being nephropathy (33%), malignancies (23%), liver involvement (13%), and diffuse vasculitis (13%)[14].

No standardized criteria are presently available for diagnosis of MC syndrome. However, valuable classifications have been proposed[26]. In the presence of clear clinical and serological data (i.e., purpura, MC, reduced C4 values, organ involvement), the diagnosis of MCS is relatively easy. However, the diagnosis is frequently only suggested by one or more altered laboratory data (RF-test + and/or mixed cryoglobulinemia and/or reduced C4 values) with or without mild symptoms (arthralgias and/or asthenia). Moreover, some HCV-positive subjects may show clinically evident MCS, though incomplete from a serological point of view, mainly with the temporary absence of circulating CGs. This paradox may be explained

by the fact that the rate of CGs responsible for vasculitic damage in MCS varies over time among different subjects as well as within the same patient, ranging from 0% to 100%[3]. In addition, the difficulty in correctly determining the presence of CGs, due to their thermolability, should be taken into account.

Determination of serum CGs and serum sample collection must be in accordance with laboratory standards as follows: withdrawal of 20 mL of whole blood at warm temperature; centrifugation for 2-3 min at 37℃; rapid collection of serum (supernatant); incubation of serum at 4℃ for one week; evaluation of CGs present at d 7; isolation and washing of CGs by phosphate buffered saline at 4℃; characterization of Ig and IgM monoclonality in the cryoprecipitate. Due to the fact that some mixed CGs are present in low concentrations, differentiation between type II and III CGs often requires a more sensitive method of immunochemical characterization such as electroimmunofixation or western blot, than conventional immunoelectrophoresis[27].

Several data, including the presence of a clonal expansion of B-lymphocytes (BL) in peripheral blood and/or liver infiltrates[28-31], and the histopathological features of the bone-marrow and liver lymphoid infiltrates (see below) confirm the lymphoproliferative nature of MC. Several studies show that a B-cell clonal expansion (in particular of RF B-cells) underlies MC, that this condition is associated with Bcl-2/J_H rearrangement (see below), and that MC-II can evolve into a frank B-cell non-Hodgkin's lymphoma (NHL) in approximately 8%-10% of cases after a long period of time[14].

From a histopathological point of view, the determination of monoclonal lymphoproliferation of uncertain significance (MLDUS) in subjects with clinico-laboratory features of MC-II is typical[3,32-34]. MLDUS represents oligoclonal proliferations of small BL, preferentially located in the bone marrow and liver. In these organs, MLDUS is generally present with phenotypical and histological aspects comparable to indolent B-cell lymphoma. A deeper, immunomorphological analysis reveals two different varieties: a first and more frequent variety, with analogous features to B-cell chronic lymphatic leukemia (CLL)/small cell lymphoma and a second, less frequent, lymphoplasmacytic-like form. The incidence of these histological forms varies in different reports[33,35-40].

LYMPHOMA

HCV-associated lymphatic malignancies may be observed during the course of MC or they may be idiopathic forms. About 8%-10% of MC-II evolve into lymphoma[36,41], generally after long-lasting infection, as demonstrated also by the advanced age of patients who develop HCV-related lymphoma. In a recent survey, MC patients had a 35 times higher risk of NHL than the general population[42].

A significant association between B-cell derived NHL and HCV infection was initially reported in Italian subjects[43-49], and subsequently confirmed by a large majority of international studies[33,42,47,50-54]. However, discordant data appeared in northern European and North American surveys[55-60], and it is now evident that a clear south/north gradient of prevalence exists, in part reflecting different HCV infection prevalence in the general population, and suggesting the contribution of environmental and/or genetic factors[54].

From a histopathological point of view, although all histological types can virtually be found, B-cell derived NHL is the most common of the HCV-related lymphatic malignancies[33,45,47,48,61-64].

Reports in the literature indicate varied incidences of different histotypes that may in part be related to different diagnostic-classification approaches[40,64-66]. However, the most diffuse varieties appear to be peripheral B-cell-derived indolent NHL. According to the REAL/WHO classification[67,68], the most prevalent forms include follicular lymphoma, B-cell chronic lymphocytic leukemia/small lymphocyte lymphoma, lymphoplasmacytic lymphoma, and marginal zone lymphoma[33]. Among the marginal zone lymphomas, a special association with HCV infection was reported for the mucosa-associated lymphoid tissue (MALT) lymphoma[64,69,70], as well as the splenic forms, as confirmed by some reports indicating that marginal splenic lymphoma regressed after antiviral therapy, in spite of previous ineffective chemotherapy[71-72].

Finally, it is of note that a serum monoclonal gammopathy, most frequently type IgM/K, was included among HCV-associated LPDs[73]. In most HCV-positive patients, MG was classified as MGUS (monoclonal gammopathies of uncertain significance), which has to be monitored in order to exclude the possibility (though remote) of evolution into multiple myeloma. Currently, a few HCV-positive patients with monoclonal gammopathy are considered affected by myeloma according to clinico-pathological characteristics[68,74,75].

PATHOGENESIS OF HCV-RELATED LYMPHOPROLIFERATIVE DISORDERS

The pathogenesis of HCV-related lymphoproliferative disorders is at present unknown, although knowledge has accumulated during the last decade which suggests interesting pathogenetic hypotheses.

First, the individuation of HCV lymphotropism at the beginning of the 1900s led to the hypothesis of a causal link between infection of lymphatic cells and autoimmune-lymphoproliferative disorders[76]. In an initial study, it was observed that both HCV positive strand (genomic) and negative strand (antigenomic = replicative intermediates) could be detected in peripheral blood mononuclear cells (PBMC) taken from patients with chronic HCV infection; that both B and T lymphocytes and monocytes/macrophages may score positive and that the mitogen stimulation of PBMC cultures increased the determination of HCV sequences, with particular reference to negative strand ones. In addition, the notion of better detection of HCV lymphatic infection in HIV coinfected patients was introduced[77]. A lot of information about HCV lymphotropism, in both *in vivo* and *in vitro* systems, has accumulated during the past decade. These studies were able to better characterize this viral prerogative by the use of more specific methods or study models[78-84]. However,

it was impossible to obtain a clear scientific confirmation of a direct link between HCV lymphotropism and LPD pathogenesis, mostly due to difficulties in the identification of valuable scientific models, in spite of the demonstration of a stronger involvement of the lymphatic system in HCV infection in patients with MC than in HCV patients without[5,85], and, more recently, the favoring effect of B-cell infection in promoting lymphatic cell proliferation[86]. By contrast, several interesting data suggest a role only indirectly played by HCV infection in LPD pathogenesis through the host's immune response[30,87-90]. Several studies focused on the importance played by sustained antigenic stimulation, partly analogous to mechanisms which may play a key role in lymphomagenesis due to *H pylori*. In this light, the identification of the specific binding between the HCV E2 protein and the CD81 molecule - which is ubiquitous but particularly abundant on the B-cell surface[91] - led to the hypothesis of a possible role played by HCV in the promotion of a consistent polyclonal B-cell response to viral antigens which favor the development of LPDs. According to this working hypothesis, the viral infection will favor lymphomagenesis in a linear, progressive way until the possible malignant transformation.

Contrasting data, showing that HCV may favor mutations of immunoglobulin genes and oncogenes by a "hit and run" mechanism, have recently been obtained both in cell lines and in cultured cells taken from HCV-infected patients[92]. This study was justified by previous observations showing the significant association existing between Bcl-2 rearrangement (14;18 translocation) and chronic HCV infection, especially in those patients developing type II MC[93-98], and MALT lymphoma[99]. In type II MC patients, the analysis of synchronous and metachronous blood samples showed the clonal expansion of B-cells harboring this chromosomal rearrangement[95]. In addition, it was possible to demonstrate the overexpression of the anti-apoptotic Bcl-2 protein with a higher Bcl-2/bax ratio in t(14;18)-positive B-cell samples[95], as well as a modification of detectability of t(14;18) B-cell clones following antiviral treatment[100]. Only virologically effective treatments led to the regression of clones, with consequent lack of t(14;18) - positive cells in peripheral blood at the end of treatment, whereas this effect was not observed in non-responder patients[100]. Regression of B-cell lymphoproliferation after effective antiviral treatments was also observed in different studies which utilized different parameters[101] and was interpreted as a consequence of the dependence of such lymphoproliferation on viral antigenic stimulation.

More recently, the availability of the study model of MC patients experiencing a sustained virological response to antiviral treatments and undergoing long term post-treatment follow-up, has provided new data and, in turn, opened the way for a series of previously unexpected issues. This analysis, that was performed by the use of very sensitive methods for HCV sequence determination, allowed the identification of subjects which, even if scoring persistently HCV RNA-negative in both serum and liver samples, were HCV RNA positive in lymphatic cells and showed persistence of MCS stigmata ([102] and Zignego *et al*, submitted paper). In contrast, this behavior was not observed in patients who showed complete viral clearance, and in whom MCS persistently disappeared. These observations, after more than a decade, strongly suggested the possibility of a role played by HCV lymphotropism, as initially supposed, even if the exact mechanisms involved are at present unknown.

Overall, on the basis of such data, it is possible to suggest that HCV infection may lead to the pathogenesis of LPDs through a complex, multistep pathogenetic mechanism including - as the initial, favoring event-a strong and persistent stimulation of the B-cell compartment by viral epitopes. This would be responsible for sustained polyclonal expansion - physiological, even if abnormal in quantitative terms - which in turn would favor mistakes in immunoglobulin gene rearrangement (VDJ rearrangement) processes such as t(14;18) translocation, thus favoring the abnormal survival of corresponding B-cells. Apart from these mechanisms, only indirectly related to HCV, the role played by the active infection of B cells and the possible intervention of a direct mutagenic effect of HCV by a "hit and run" mechanism following their infection should be taken into account and be the object of future study. In the presence of a background of predisposing constitutional-genetic factors, i.e., a low stimulation threshold of RF-B cells, the existence of factors leading to inhibition of B-cell apoptosis would favor the transition from polyclonal activation and expansion of RF B cells producing polyclonal IgM RF molecules (type III MC), to the emergence of a dominant clone producing a monoclonal IgM RF (type II MC). In other words, the switch from a polyclonal MC secondary to the infection - an epiphenomenal effect - to the oligo-monoclonal one, might evolve into a lymphatic malignancy. In this light, some data exist suggesting that infected B-cells obtained from MC patients may correspond to MC-typical RF B-cells[103], and that t(14;18) positive B cells may correspond to RF B-cells (Giannini *et al*, unpublished results). On the other hand, it is well known that abnormal B-cell survival would *per se* favor the accumulation of genetic mutations, possibly leading to the final neoplastic transformation (Figure 1). Analogous to the association between several different autoimmune and or lymphoproliferative disorders and a known pathogenetic mechanism, it is very plausible that, in some cases, an HCV-associated LPD showing similar clinical features may arise from alternative pathogenetic sources not involving Bcl-2 overexpression and/or, more specifically, Bcl-2 rearrangement[103].

THERAPY OF HCV-RELATED LYMPHOPROLIFERATIVE DISORDERS

Mixed cryoglobulinemia

Most information about treatment of HCV-related LPDs is derived from studies concerning MCS. This syndrome, before the identification of its viral etiology, was interpreted and treated as an "essential" autoimmune/lymphoproliferative disease by using a combination of anti-inflammatory, immunosuppressive drugs, as well as procedures able to reduce the amount of circulating

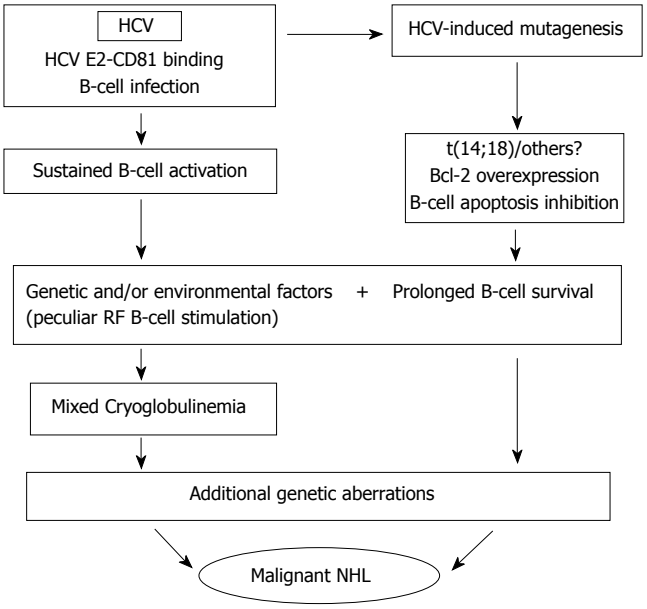

Figure 1 Hypothetical interpretation of the complex relationship between chronic HCV infection, and lymphoproliferative disorders.During chronic infection, HCV is responsible for sustained B-cell proliferation. Factors favoring polyclonal B lymphoproliferation may include the specific binding of HCV E2 protein to CD 81 (a tetraspannin which, on the B-cell surface, is part of an activating molecular complex lowering the threshold of B-cell activation by specific epitopes)[92,95], as well as the specific activation of reactive T-cells by HCV and cytokines. Favoring factors may also be represented by the persistent infection of B-cells[78,95]. Resulting sustained B-cell proliferation would in turn favor the occurrence of t(14;18) translocation and/or other errors during V(D)J rearrangement processes in germinal centers located in secondary lymphoid organs which, in case of HCV infection, would also include the liver[28]. A more direct role played by HCV lymphatic infection *via* viral mutagenic properties cannot be excluded[93]. Bcl-2 antiapoptotic protein overexpression in B-cells would result and lead to abnormally prolonged B-cell life. In predisposed subjects, in presence of unknown environmental, viral and/or genetic factors, HCV infection could lead to sustained production of cryoglobulins. These predisposing factors may include the peculiar susceptibility of IgM RF producing B-cells (RF B-cells) to be activated and/or the presence of particular viral variants bearing epitopes capable of selectively activating RF B-cells. It is tempting to hypothesize that the Bcl-2 rearrangement, by inhibiting B-cell apoptosis, may favor the lack of silencing higher affinity, potentially pathological RF B-cells[143], possibly leading to the development of MC syndrome. In turn, abnormal survival of B-cells would favor the acquisition of additional genetic aberrations which might ultimately lead to transformation to a frank B-cell malignancy[33].

immunocomplexes (cryoglobulins) such as plasma exchange and low antigen content diet (LAC diet). The identification of the viral etiology led to the attempt to eradicate HCV with interferon (IFN)-based treatments. Interestingly, because of its antiproliferative properties, IFN was successfully used in the treatment of MCS even before the identification of HCV[104,105]. Several studies, utilizing different therapeutic protocols, have been carried out (Table 2), and the usefulness of IFN therapy for HCV-related MC is now firmly established. However, an accurate meta-analysis of performed studies is still hampered by the heterogeneity of regimens used so that an optimum regimen has not yet been determined nor have prognostic criteria been accurately delineated.

Antiviral treatment essentially followed the evolution of treatment of HCV-related chronic liver disease, with some latency. In a first series of studies, the effects of IFN monotherapy were tested[22,106-116] (Table 2). When compared with HCV-related chronic hepatitis, treatment of MC was associated with a relatively poorer response and high relapse rate. The frequent relapse of both HCV replication and MC syndrome at the end of IFN treatment suggested the combination with ribavirin (RBV) (Table 3): this therapeutic option appeared valid in several studies[117-120]. Interestingly, it has been shown that RBV monotherapy also decreases transaminase levels and MC-related symptoms, probably due to its immunomodulatory effects[121-123]. Further improvement in the sustained virological response (SVR) rate was obtained by the introduction of pegylated IFNs[124-126] (Table 3). However, additional controlled studies are needed to gain definitive information.

Interestingly, all available studies show that clinico-immunological and virologic response are generally strictly related[112,117,119,125-127]. In recent studies, persistence of isolated lymphatic infection after therapy was significantly associated with persistence of MCS stigmata[102]. By contrast, a long term clinical response was correlated with persistent HCV negativity of different compartments ([102], Zignego *et al*, submitted paper).

Disappearance of B-lymphocyte (BL) monoclonal infiltrate from bone marrow as well as BL expansion in peripheral blood following IFN therapy has been shown. In particular, the antiviral response was shown to be significantly related to the lack of detection of circulating B-cell clones bearing t(14;18) translocation[95,100,128] (see also above). The reappearance of circulating translocated BL clones after virological relapse at the end of treatment, as well as the persistent detection of t(14;18) positive clones in subjects with unmodified viral load after identical therapy, strongly indicates that clonal expansion of translocated cells depends on modifications of viral replication induced by antiviral treatment[100,128]. More recently, long-term analysis of HCV + MCS patients showing SVR after therapy (see above) indicated that occult lymphatic infection and persistence of MCS stigmata were also associated with persistent determination of expanded t(14;18) carrying B-cell clones ([102], and Zignego *et al*, submitted paper). Altogether the current data suggest that IFN treatment, when successful, may also help in preventing the evolution of HCV-related LPDs.

In conclusion, the available data concerning antiviral treatment of MCS show that this therapeutic approach should be the first option because of the antiproliferative and immunomudulatory effects of IFN, the usefulness of antiviral therapy as demonstrated in most available studies, the strict correlation between virological and clinical response, as well as the positive effect of inhibition of viral replication on B-cell clonal expansion that is considered the pathogenetic basis of MC. However, in comparison with antiviral treatment of HCV chronic liver disease, antiviral therapy of MCS is more complex for several reasons including the absence of standardized treatment protocols, the higher frequency of relapse, and generic or MCS-specific contraindications to antiviral treatment (i.e., advanced age, severe liver disease, acute nephritis, widespread vasculitis). In addition, the interpretation

Table 2 IFN monotherapy in HCV-related mixed cryoglobulinemia

Author	Year	No. patients	Treatment	CS	Treatment duration (months)	EOT response	Sustained response
Ferri	1993	15	2 MIU IFN/d (1 m) – 2 MIU IFN × 3/w (5 m)	Yes	6	80%	
Ferri	1993	26	2 MIU IFN/d (1 m) – 2 MIU IFN × 3/w (5 m)	Yes	6	100%	0
Marcellin	1993	2	3 MIU IFN × 3/w		6	50%	
Johnson	1993	4	1-10 MIU IFN	No	2-12	75%[1]	
Misiani	1994	27	1.5 MIU IFN × 3/w (1 w) – 3 MIU IFN × 3/w (23 w)	No	6	60%	0
Dammacco	1994	15	3 MIU IFN × 3/w	No	12	53.30%	25%
		16	3 MIU IFN × 3/w	Yes	12	52.90%	33.30%
Johnson	1994	14	Variable IFN	No		0[1]	
Mazzaro	1994	18	3 MIU IFN × 3/w	No		28%	
Mazzaro	1995	18	3 MIU IFN × 3/w	No	6	28%	11%
		18	3 MIU IFN × 3/w	No	12	39%	22%
Casaril	1996	25	6 MIU IFN × 3/w	No	6	52%[2]	
Cohen	1996	20	3 MIU IFN × 3/w			60%[3]	9%[3]
Akriviadis	1997	20	3-5 MIU IFN × 3/w	No	6-12	65%[2]	33%[2]
Casato	1997	31	3 MIU IFN/d (3 m) – 3 MIU IFN × 3/w (≥ 9 m)	No	≥ 12	62%	

CS: corticosteroids; MIU: millions of international units; d: daily; m: months; w: week; EOT: end of treatment; [s]: Kidney function improvement; [2]: Cryoglobulins disappearance, [3]: both complete and partial MC syndrome response. From: *Zignego A.L.* Postgraduate Course of the 41st Annual Meeting of the EASL, 2006, modified.

Table 3 Combined IFN (recombinant or pegylated) + ribavirin therapy in HCV-related mixed cryoglobulinemia

Author	Year	No. patients	Treatment	CS	Treatment duration (months)	EOT response	Sustained response
Durand	1998	5 NR	RBV	No	10-36	100%	0%
Calleja	1999	18	3 MIU IFN × 3/w	No	12	55%	28%
		8 NR	3 MIU IFN × 3/w + RBV	No	12	63%	38%
Zuckerman	2000	9 NR	3 MIU IFN × 3/w + RBV	No	6	78%	
Cacoub	2002	14	Variable IFN + RBV	variable	6-56		71%
Mazzaro	2003	27 NR or Rel	3 MIU × 3/w + RBV	No	12	85%	
Alric	2004	18	3 MIU × 3/w or Peg-IFN + RBV	No	≥ 18		70%
Cacoub	2005	9	Peg-IFN 1.5 μg Kg^{-1} w^{-1} + RBV		≥ 10		88%
Mazzaro	2005	18	Peg-IFN 1 μg Kg^{-1} w^{-1} + RBV	No	12	89%	44%

CS: corticosteroids; MIU: millions of international units; d: day; m: months; w: week; EOT: end of treatment; NR: non responders; Rel: relapser; RBV: ribavirin. From: *Zignego A.L.* Postgraduate Course of the 41st Annual Meeting of the EASL, 2006.

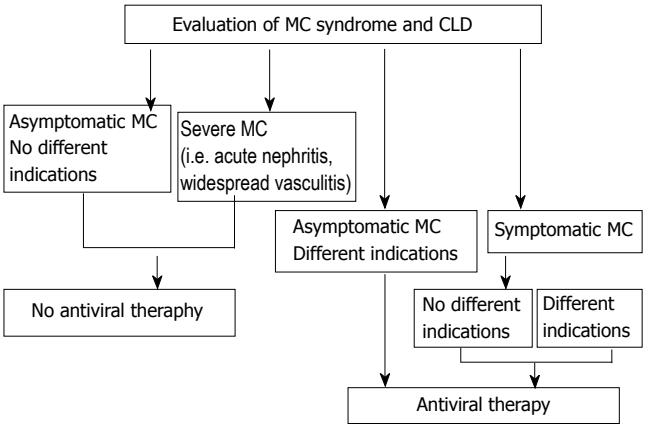

Figure 2 Algorithm for treatment of HCV-positive mixed cryoglobulinemia patients with antiviral therapy according to the evaluation of both mixed cryoglobulinemia syndrome and chronic liver damage. Available data suggest that antiviral therapy should be considered the first choice treatment in mixed cryoglobulinemia, to be performed even in the absence of different indications, excluding only those patients without symptoms and other indications or patients with too severe manifestations such as acute nephritis or widespread vasculitis. (Zignego AL, Postgraduate Course EASL 2006, Vienna and [34], modified)

of results following IFN treatment in MCS patients seems to be much more complex than in MCS-negative chronic HCV infection. In fact, biochemical markers of MC response (cryocrit, RF or complement values) may be more independent of virological response than ALT levels. This may confirm the importance of a multiphase, complex, pathogenetic mechanism in MCS, suggesting the need for precise monitoring of this category of patients and the definition of predictive markers which indicate evolution towards pathogenetic phases of the disease. A possible candidate marker may be the evaluation of BL clonal expansion modifications in PBMC samples, as previously mentioned[95,100,101,128]; further studies are needed to better define its clinical value.

Figure 2 shows a suggested algorithm for treatment of HCV-related MCS with antiviral therapy. At present, antiviral therapy is suggested as the first choice treatment of this condition, to be performed even in the absence of different indications, with the exclusion of patients with only biochemical stigmata of MC (i.e., CGs). Particular attention should also be given to the treatment of severe MCS (i.e., patients with acute nephritis, widespread vasculitis). In these cases no sufficient data are available supporting the safety of IFN administration and a cautious attitude is strongly suggested. It is opportune to use

alternative, more "traditional" therapeutic approaches in all patients in whom antiviral treatment is contraindicated or not tolerated or who are non-responders. Alternatives include corticosteroids, immunosuppressive drugs, FANS, plasmapheresis and a hypo-antigenic diet[34,129]. Treatment should be tailored to the single patient, according to the severity of clinical symptoms, and considering the possible additional factors involved (age, co-morbidity etc.), and limited to the time (weeks or months) required for symptom remission. Any therapeutic approach aimed at improving serological parameters in clinically asymptomatic patients should be avoided.

Corticosteroids are the most commonly used therapy for MCS before HCV identification due to the fact that, even at low doses, they can control the majority of MCS symptoms. On the other hand, corticosteroids may favor HCV replication, may cause several side effects, and do not induce significant modifications in the cryocrit levels or in the natural history of the disease. Cytostatic-immunosuppressive drugs (i.e., cyclophosphamide, chlorambucil and azathioprine) have been used mainly in the absence of response to corticosteroids and/or during the acute phases of MCS (i.e., acute nephritis evolving towards renal failure, hyperviscosity syndrome in association with plasmapheresis). These molecules generally have some severe side effects, including disease progression secondary to the relevant immunosuppressive effect[130]. A special note must be made of new B-cell specific immunosuppressive therapy based on the use of chimeric antibodies against the CD20, a B cell specific surface antigen (rituximab)[131,132]. Several studies showed that rituximab is effective in most patients with MC, leading to marked improvement or resolution of the syndrome - with particular reference to skin lesions - and regression of the expanded B-cell clones[131,132]. However, in spite of the fact that no immediate treatment-induced liver damage has been reported, this drug leads to an increase in HCV replication, explaining interest in its combination with antiviral molecules[34,131-134]. Overall, this therapeutic approach appears to be very promising in the management of MCS patients, however, future prospective, controlled, and randomized studies are still required to establish evidence-based guidelines to treat HCV-related MCS.

Other therapeutic measures are aimed at reducing CG concentration. These include plasmapheresis, and a LAC diet. Plasmapheresis represents the aphaeretic removal of CGs and circulating immunocomplexes. Because of its effectiveness and rapid action, it is especially indicated in the presence of acute manifestations (cryoglobulinemic nephritis, severe sensorimotor neuropathies, cutaneous ulcers, hyperviscosity syndrome). The association with cyclophosphamide has been shown to be effective in reducing the "rebound effect" at the end of aphaeresis. The LAC diet has a reduced content of alimentary macro-molecules with high antigenic properties allowing more efficient removal of CGs by the reticulo-endothelial system. This diet can improve minor manifestations of the disease (purpura, arthralgias, paresthesias), and is generally prescribed at the initial stage of the disease.

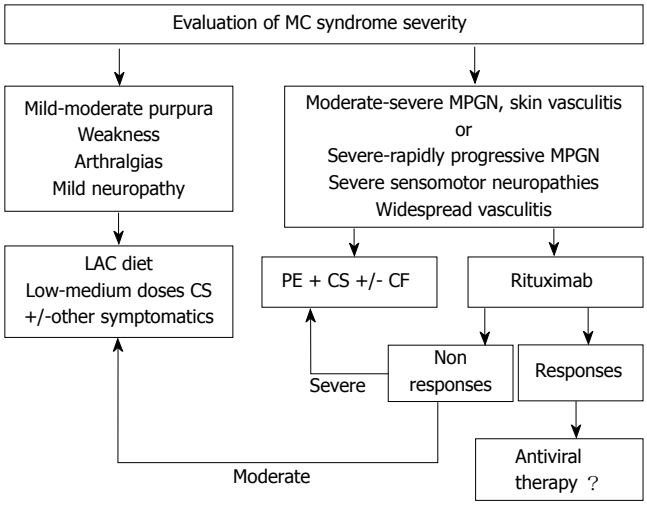

Figure 3 Algorithm for treatment of HCV-positive mixed cryoglobulinemia patients who are non-responders or in whom antiviral treatment is contraindicated or not tolerated. When antiviral treatment is not indicated this algorithm suggests other approaches for the management of mixed cryoglobulinemia, including anti-inflammatory drugs (first corticosteroids, CS), procedures able to lower the concentration of cryoglobulins such as the low antigen content (LAC) diet or plasma exchange (PE) as well as immunosuppressive drugs (cyclophosphamide, CF). In patients with only mild to moderate syndrome, cycles of therapy with anti-inflammatories, the LAC diet or other symptomatic treatments are suggested. Therapy should be adapted to the single patient and limited in time. In patients with more severe syndromes, cycles of plasma exchange plus corticosteroids and/or immunosuppressive drugs are indicated. The use of rituximab, a selective B-cell suppressor, may be an alternative treatment in some cases where antiviral therapy is initially contraindicated. (Zignego AL, Postgraduate Course EASL 2006, Vienna and [34])

Figure 3 is a synthetic algorithm of the management of MCS, when etiological therapy is not possible or is ineffective.

LYMPHOMA

In light of the above observations about MCS, the inclusion of antiviral therapy seems to be rational in therapeutic schemes for HCV-positive NHL. This appears to be confirmed by recent studies, performed in low-grade lymphoma[135] and, in particular, in marginal zone lymphomas[71,136]. Vallisa *et al* treated 13 patients with HCV-associated low-grade B-NHL characterized by an indolent course (i.e., doubling time no less than 1 year, no bulky disease) with pegylated IFN and ribavirin. Hematologic responses were observed in the majority of patients (complete and partial responses, 75%) and were highly significantly associated with clearance or decrease in serum HCV viral load following treatment, strongly providing a role for antiviral treatment in HCV-related, low-grade, B-cell NHL. Hermine *et al* showed that most patients with HCV and splenic lymphoma with villous lymphocytes (SLVL) entered complete remission upon treatment with IFN[71]. The inclusion of a control group integrated by patients with the same LPD but without HCV infection demonstrated that, in contrast with HCV-infected patients, HCV-negative subjects did not respond to IFN therapy, strongly suggesting that the response observed in HCV-positive patients was not merely due to the antiproliferative

effect of IFN. Analogously, regression of clonal proliferation in response to antiviral treatment was shown to be clearly associated with virological response[95,128]. Interestingly, also in HCV patients without evidence of LPD, a close association between the virological response and the loss of B-cell monoclonality with persistence of expanded t(14:18)-positive B-cell clones in non virological responders was shown, indicating that the more effective the antiviral therapy is, the more likely is loss of B-cell clonality[100].

Unfortunately, only some lymphomas may be cured with antiviral therapy. In addition, also in cases of responsive SLVL, the rearrangement of the monoclonal immunoglobulin genes observed at diagnosis is still detectable in the blood even after a complete hematological response has been achieved[72]. These data suggest that the multi-step lymphomagenetic cascade is complicated by points of no return, making LPD more and more independent of HCV infection. Although antiviral therapy appears to be an attractive therapeutic tool for low-grade HCV-positive NHL, in intermediate and high-grade NHL, chemotherapy is expected to be necessary and antiviral treatment may be suggested as maintenance therapy after chemotherapy completion[137]. Further studies are needed to better standardize the antiviral therapy for HCV-related NHL patients.

The use of rituximab in HCV-associated NHL, in monotherapy or in combination with antiviral treatment and/or chemotherapy, appears very promising, especially in the setting of low-grade NHL, where rituximab monotherapy has been proposed as first-line treatment[138]. Interestingly, Hainsworth *et al* showed that the use of rituximab in low-grade NHL with scheduled maintenance at 6-mo intervals produced high overall and complete response rates and a longer progression-free survival than has been reported with a standard 4-wk treatment[138].

Few data are presently available specifically concerning patients with HCV-associated NHL. For example, Somer *et al* observed that after rituximab treatment a patient with Sjogren's syndrome (SS) and lymphoma showed improvement of parotidomegaly, ocular tests and salivary flow rate[139]. In addition, Ramos-Casals *et al* reported two patients with HCV-related SS who developed B-cell lymphoma and who responded successfully to treatment with rituximab[140].

In synthesis, in spite of the limited number of described cases, it appears reasonable to consider rituximab as a safe and effective therapy for HCV-related indolent B-cell lymphoma.

REFERENCES

1 **Zignego AL**, Bréchot C. Extrahepatic manifestations of HCV infection: facts and controversies. *J Hepatol* 1999; **31**: 369-376
2 **Cacoub P**, Poynard T, Ghillani P, Charlotte F, Olivi M, Piette JC, Opolon P. Extrahepatic manifestations of chronic hepatitis C. MULTIVIRC Group. Multidepartment Virus C. *Arthritis Rheum* 1999; **42**: 2204-2212
3 **Zignego AL**, Ferri C, Pileri SA, Caini P, Bianchi FB. Extrahepatic manifestations of Hepatitis C Virus infection: a general overview and guidelines for a clinical approach. *Dig Liver Dis* 2007; **39**: 2-17
4 **Misiani R**, Bellavita P, Fenili D, Borelli G, Marchesi D, Massazza M, Vendramin G, Comotti B, Tanzi E, Scudeller G. Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. *Ann Intern Med* 1992; **117**: 573-577
5 **Ferri C**, Greco F, Longombardo G, Palla P, Moretti A, Marzo E, Mazzoni A, Pasero G, Bombardieri S, Highfield P. Association between hepatitis C virus and mixed cryoglobulinemia [see comment]. *Clin Exp Rheumatol* 1991; **9**: 621-624
6 **Zignego AL**, Ferri C, Giannini C, La Civita L, Careccia G, Longombardo G, Bellesi G, Caracciolo F, Thiers V, Gentilini P. Hepatitis C virus infection in mixed cryoglobulinemia and B-cell non-Hodgkin's lymphoma: evidence for a pathogenetic role. *Arch Virol* 1997; **142**: 545-555
7 **Agnello V**, Chung RT, Kaplan LM. A role for hepatitis C virus infection in type II cryoglobulinemia. *N Engl J Med* 1992; **327**: 1490-1495
8 **Brouet JC**, Clauvel JP, Danon F, Klein M, Seligmann M. Biologic and clinical significance of cryoglobulins. A report of 86 cases. *Am J Med* 1974; **57**: 775-788
9 **Agnello V**. Hepatitis C virus infection and type II cryoglobulinemia: an immunological perspective. *Hepatology* 1997; **26**: 1375-1379
10 **Lunel F**, Musset L, Cacoub P, Frangeul L, Cresta P, Perrin M, Grippon P, Hoang C, Valla D, Piette JC. Cryoglobulinemia in chronic liver diseases: role of hepatitis C virus and liver damage. *Gastroenterology* 1994; **106**: 1291-1300
11 **Wong VS**, Egner W, Elsey T, Brown D, Alexander GJ. Incidence, character and clinical relevance of mixed cryoglobulinaemia in patients with chronic hepatitis C virus infection. *Clin Exp Immunol* 1996; **104**: 25-31
12 **Pawlotsky JM**, Roudot-Thoraval F, Simmonds P, Mellor J, Ben Yahia MB, André C, Voisin MC, Intrator L, Zafrani ES, Duval J, Dhumeaux D. Extrahepatic immunologic manifestations in chronic hepatitis C and hepatitis C virus serotypes. *Ann Intern Med* 1995; **122**: 169-173
13 **Ferri C**, Monti M, La Civita L, Longombardo G, Greco F, Pasero G, Gentilini P, Bombardieri S, Zignego AL. Infection of peripheral blood mononuclear cells by hepatitis C virus in mixed cryoglobulinemia. *Blood* 1993; **82**: 3701-3704
14 **Ferri C**, Sebastiani M, Giuggioli D, Cazzato M, Longombardo G, Antonelli A, Puccini R, Michelassi C, Zignego AL. Mixed cryoglobulinemia: demographic, clinical, and serologic features and survival in 231 patients. *Semin Arthritis Rheum* 2004; **33**: 355-374
15 **Ferri C**, La Civita L, Cirafisi C, Siciliano G, Longombardo G, Bombardieri S, Rossi B. Peripheral neuropathy in mixed cryoglobulinemia: clinical and electrophysiologic investigations. *J Rheumatol* 1992; **19**: 889-895
16 **Tembl JI**, Ferrer JM, Sevilla MT, Lago A, Mayordomo F, Vilchez JJ. Neurologic complications associated with hepatitis C virus infection. *Neurology* 1999; **53**: 861-864
17 **Lidove O**, Cacoub P, Maisonobe T, Servan J, Thibault V, Piette JC, Léger JM. Hepatitis C virus infection with peripheral neuropathy is not always associated with cryoglobulinaemia. *Ann Rheum Dis* 2001; **60**: 290-292
18 **Johnson RJ**, Willson R, Yamabe H, Couser W, Alpers CE, Wener MH, Davis C, Gretch DR. Renal manifestations of hepatitis C virus infection. *Kidney Int* 1994; **46**: 1255-1263
19 **Daghestani L**, Pomeroy C. Renal manifestations of hepatitis C infection. *Am J Med* 1999; **106**: 347-354
20 **D'Amico G**. Renal involvement in hepatitis C infection: cryoglobulinemic glomerulonephritis. *Kidney Int* 1998; **54**: 650-671
21 **Tarantino A**, Campise M, Banfi G, Confalonieri R, Bucci A, Montoli A, Colasanti G, Damilano I, D'Amico G, Minetti L. Long-term predictors of survival in essential mixed cryoglobulinemic glomerulonephritis. *Kidney Int* 1995; **47**: 618-623
22 **Ferri C**, Marzo E, Longombardo G, Lombardini F, La Civita L, Vanacore R, Liberati AM, Gerli R, Greco F, Moretti A , Monti M, Gentilini P, Bombardieri S, Zignego AL. Interferon-alpha in mixed cryoglobulinemia patients: a randomized, crossover-controlled trial. *Blood* 1993; **81**: 1132-1136
23 **Cacoub P**, Fabiani FL, Musset L, Perrin M, Frangeul L, Leger

JM, Huraux JM, Piette JC, Godeau P. Mixed cryoglobulinemia and hepatitis C virus. *Am J Med* 1994; **96**: 124-132
24 **Kayali Z**, Buckwold VE, Zimmerman B, Schmidt WN. Hepatitis C, cryoglobulinemia, and cirrhosis: a meta-analysis. *Hepatology* 2002; **36**: 978-985
25 **Saadoun D**, Asselah T, Resche-Rigon M, Charlotte F, Bedossa P, Valla D, Piette JC, Marcellin P, Cacoub P. Cryoglobulinemia is associated with steatosis and fibrosis in chronic hepatitis C. *Hepatology* 2006; **43**: 1337-1345
26 **Ferri C**, Zignego AL, Pileri SA. Cryoglobulins. *J Clin Pathol* 2002; **55**: 4-13
27 **Musset L**, Diemert MC, Taibi F, Thi Huong Du L, Cacoub P, Leger JM, Boissy G, Gaillard O, Galli J. Characterization of cryoglobulins by immunoblotting. *Clin Chem* 1992; **38**: 798-802
28 **Sansonno D**, De Vita S, Iacobelli AR, Cornacchiulo V, Boiocchi M, Dammacco F. Clonal analysis of intrahepatic B cells from HCV-infected patients with and without mixed cryoglobulinemia. *J Immunol* 1998; **160**: 3594-3601
29 **Magalini AR**, Facchetti F, Salvi L, Fontana L, Puoti M, Scarpa A. Clonality of B-cells in portal lymphoid infiltrates of HCV-infected livers. *J Pathol* 1998; **185**: 86-90
30 **Racanelli V**, Sansonno D, Piccoli C, D'Amore FP, Tucci FA, Dammacco F. Molecular characterization of B cell clonal expansions in the liver of chronically hepatitis C virus-infected patients. *J Immunol* 2001; **167**: 21-29
31 **Vallat L**, Benhamou Y, Gutierrez M, Ghillani P, Hercher C, Thibault V, Charlotte F, Piette JC, Poynard T, Merle-Béral H, Davi F, Cacoub P. Clonal B cell populations in the blood and liver of patients with chronic hepatitis C virus infection. *Arthritis Rheum* 2004; **50**: 3668-3678
32 **Monteverde A**, Pileri S. Lymphoproliferative diseases of uncertain classification. *Ann Ital Med Int* 1991; **6**: 162-170
33 **Ferri C**, Pileri S, Zignego AL. Hepatitis C virus infection and non-Hodgkin's lymphoma. In: Geodert J, (NIH) NCI, eds. Infectious causes of cancer Targets for intervention. Totowa, New Jersey: The Human Press inc., 2000: 349-368
34 **Ferri C**, Zignego AL, Pileri SA. Cryoglobulinemia. Philadelphia (PA): Elsevier, 2006
35 **Santini GF**, Crovatto M, Modolo ML, Martelli P, Silvia C, Mazzi G, Franzin F, Moretti M, Tulissi P, Pozzato G. Waldenström macroglobulinemia: a role of HCV infection? *Blood* 1993; **82**: 2932
36 **Pozzato G**, Mazzaro C, Crovatto M, Modolo ML, Ceselli S, Mazzi G, Sulfaro S, Franzin F, Tulissi P, Moretti M. Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. *Blood* 1994; **84**: 3047-3053
37 **Mussini C**, Ghini M, Mascia MT, Giovanardi P, Zanni G, Lattuada I, Moreali S, Longo G, Ferrari MG, Torelli G. Monoclonal gammopathies and hepatitis C virus infection. *Blood* 1995; **85**: 1144-1145
38 **Mangia A**, Clemente R, Musto P, Cascavilla I, La Floresta P, Sanpaolo G, Gentile R, Viglotti ML, Facciousso D, Carotenuto M, Rizzetto M, Andriulli A. Hepatitis C virus infection and monoclonal gammopathies not associated with cryoglobulinemia. *Leukemia* 1996; **10**: 1209-1213
39 **Musto P**, Dell'Olio M, Carotenuto M, Mangia A, Andriulli A. Hepatitis C virus infection: a new bridge between hematologists and gastroenterologists? *Blood* 1996; **88**: 752-754
40 **Silvestri F**, Barillari G, Fanin R, Salmaso F, Pipan C, Falasca E, Puglisi F, Mariuzzi L, Zaja F, Infanti L, Patriarca F, Candoni A, Rogato A, Di Loreto C, Botta GA, Baccarani M. Impact of hepatitis C virus infection on clinical features, quality of life and survival of patients with lymphoplasmacytoid lymphoma/immunocytoma. *Ann Oncol* 1998; **9**: 499-504
41 **Ferri C**, Monti M, La Civita L, Careccia G, Mazzaro C, Longombardo G, Lombardini F, Greco F, Pasero G, Bombardieri S. Hepatitis C virus infection in non-Hodgkin's B-cell lymphoma complicating mixed cryoglobulinaemia. *Eur J Clin Invest* 1994; **24**: 781-784
42 **Monti G**, Pioltelli P, Saccardo F, Campanini M, Candela M, Cavallero G, De Vita S, Ferri C, Mazzaro C, Migliaresi S, Ossi E, Pietrogrande M, Gabrielli A, Galli M, Invernizzi F. Incidence and characteristics of non-Hodgkin lymphomas in a multicenter case file of patients with hepatitis C virus-related symptomatic mixed cryoglobulinemias. *Arch Intern Med* 2005; **165**: 101-105
43 **Ferri C**, Caracciolo F, La Civita L, Monti M, Longombardo G, Greco F, Zignego AL. Hepatitis C virus infection and B-cell lymphomas. *Eur J Cancer* 1994; **30A**: 1591-1592
44 **Ferri C**, La Civita L, Caracciolo F, Zignego AL. Non-Hodgkin's lymphoma: possible role of hepatitis C virus. *JAMA* 1994; **272**: 355-356
45 **Ferri C**, Caracciolo F, Zignego AL, La Civita L, Monti M, Longombardo G, Lombardini F, Greco F, Capochiani E, Mazzoni A. Hepatitis C virus infection in patients with non-Hodgkin's lymphoma. *Br J Haematol* 1994; **88**: 392-394
46 **Mazzaro C**, Zagonel V, Monfardini S, Tulissi P, Pussini E, Fanni M, Sorio R, Bortolus R, Crovatto M, Santini G, Tiribelli C, Sasso F, Masutti R, Pozzato G. Hepatitis C virus and non-Hodgkin's lymphomas. *Br J Haematol* 1996; **94**: 544-550
47 **Luppi M**, Grazia Ferrari M, Bonaccorsi G, Longo G, Narni F, Barozzi P, Marasca R, Mussini C, Torelli G. Hepatitis C virus infection in subsets of neoplastic lymphoproliferations not associated with cryoglobulinemia. *Leukemia* 1996; **10**: 351-355
48 **Pioltelli P**, Zehender G, Monti G, Monteverde A, Galli M. HCV and non-Hodgkin lymphoma. *Lancet* 1996; **347**: 624-625
49 **Silvestri F**, Pipan C, Barillari G, Zaja F, Fanin R, Infanti L, Russo D, Falasca E, Botta GA, Baccarani M. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. *Blood* 1996; **87**: 4296-4301
50 **Zuckerman E**, Zuckerman T, Levine AM, Douer D, Gutekunst K, Mizokami M, Qian DG, Velankar M, Nathwani BN, Fong TL. Hepatitis C virus infection in patients with B-cell non-Hodgkin lymphoma. *Ann Intern Med* 1997; **127**: 423-428
51 **Silvestri F**, Barillari G, Fanin R, Pipan C, Falasca E, Salmaso F, Zaja F, Infanti L, Patriarca F, Botta GA, Baccarani M. Hepatitis C virus infection among cryoglobulinemic and non-cryoglobulinemic B-cell non-Hodgkin's lymphomas. *Haematologica* 1997; **82**: 314-317
52 **Izumi T**, Sasaki R, Tsunoda S, Akutsu M, Okamoto H, Miura Y. B cell malignancy and hepatitis C virus infection. *Leukemia* 1997; **11** Suppl 3: 516-518
53 **Mele A**, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, Iannitto E, De Renzo A, Martino B, Liso V, Andrizzi C, Pusterla S, Dore F, Maresca M, Rapicetta M, Marcucci F, Mandelli F, Franceschi S. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. *Blood* 2003; **102**: 996-999
54 **Matsuo K**, Kusano A, Sugumar A, Nakamura S, Tajima K, Mueller NE. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies. *Cancer Sci* 2004; **95**: 745-752
55 **Brind AM**, Watson JP, Burt A, Kestevan P, Wallis J, Proctor SJ, Bassendine MF. Non-Hodgkin's lymphoma and hepatitis C virus infection. *Leuk Lymphoma* 1996; **21**: 127-130
56 **Hanley J**, Jarvis L, Simmonds P, Parker A, Ludlam C. HCV and non-Hodgkin lymphoma. *Lancet* 1996; **347**: 1339
57 **McColl MD**, Singer IO, Tait RC, McNeil IR, Cumming RL, Hogg RB. The role of hepatitis C virus in the aetiology of non-Hodgkins lymphoma--a regional association? *Leuk Lymphoma* 1997; **26**: 127-130
58 **Ellenrieder V**, Weidenbach H, Frickhofen N, Michel D, Prümmer O, Klatt S, Bernas O, Mertens T, Adler G, Beckh K. HCV and HGV in B-cell non-Hodgkin's lymphoma. *J Hepatol* 1998; **28**: 34-39
59 **Collier JD**, Zanke B, Moore M, Kessler G, Krajden M, Shepherd F, Heathcote J. No association between hepatitis C and B-cell lymphoma. *Hepatology* 1999; **29**: 1259-1261
60 **Hausfater P**, Cacoub P, Rosenthal E, Bernard N, Loustaud-Ratti V, Le Lostec Z, Laurichesse H, Turpin F, Ouzan D, Grasset D, Perrone C, Cabrol MP, Piette JC. Hepatitis C virus infection and lymphoproliferative diseases in France: a national study. The GERMIVIC Group. *Am J Hematol* 2000; **64**: 107-111
61 **Silvestri F**, Baccarani M. Hepatitis C virus-related lymphomas. *Br J Haematol* 1997; **99**: 475-480

62 **De Vita S**, Sacco C, Sansonno D, Gloghini A, Dammacco F, Crovatto M, Santini G, Dolcetti R, Boiocchi M, Carbone A, Zagonel V. Characterization of overt B-cell lymphomas in patients with hepatitis C virus infection. *Blood* 1997; **90**: 776-782

63 **Ascoli V**, Lo Coco F, Artini M, Levrero M, Martelli M, Negro F. Extranodal lymphomas associated with hepatitis C virus infection. *Am J Clin Pathol* 1998; **109**: 600-609

64 **Luppi M**, Longo G, Ferrari MG, Barozzi P, Marasca R, Morselli M, Valenti C, Mascia T, Vandelli L, Vallisa D, Cavanna L, Torelli G. Clinico-pathological characterization of hepatitis C virus-related B-cell non-Hodgkin's lymphomas without symptomatic cryoglobulinemia. *Ann Oncol* 1998; **9**: 495-498

65 **Trépo C**, Berthillon P, Vitvitski L. HCV and lymphoproliferative diseases. *Ann Oncol* 1998; **9**: 469-470

66 **Talamini R**, Montella M, Crovatto M, Dal Maso L, Crispo A, Negri E, Spina M, Pinto A, Carbone A, Franceschi S. Non-Hodgkin's lymphoma and hepatitis C virus: a case-control study from northern and southern Italy. *Int J Cancer* 2004; **110**: 380-385

67 **Harris NL**, Jaffe ES, Stein H, Banks PM, Chan JK, Cleary ML, Delsol G, De Wolf-Peeters C, Falini B, Gatter KC. A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group. *Blood* 1994; **84**: 1361-1392

68 **Jaffe ES HN**, Stein H, Vardiman JW. Tumours of haematopoietic and lymphoid tissues. World Health Organization classification of tumours. Lyon: IARC Press, 2001

69 **De Vita S**, Sansonno D, Dolcetti R, Ferraccioli G, Carbone A, Cornacchiulo V, Santini G, Crovatto M, Gloghini A, Dammacco F, Boiocchi M. Hepatitis C virus within a malignant lymphoma lesion in the course of type II mixed cryoglobulinemia. *Blood* 1995; **86**: 1887-1892

70 **Luppi M**, Longo G, Ferrari MG, Ferrara L, Marasca R, Barozzi P, Morselli M, Emilia G, Torelli G. Additional neoplasms and HCV infection in low-grade lymphoma of MALT type. *Br J Haematol* 1996; **94**: 373-375

71 **Hermine O**, Lefrère F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, Delmas B, Valensi F, Cacoub P, Brechot C, Varet B, Troussard X. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. *N Engl J Med* 2002; **347**: 89-94

72 **Saadoun D**, Suarez F, Lefrere F, Valensi F, Mariette X, Aouba A, Besson C, Varet B, Troussard X, Cacoub P, Hermine O. Splenic lymphoma with villous lymphocytes, associated with type II cryoglobulinemia and HCV infection: a new entity? *Blood* 2005; **105**: 74-76

73 **Andreone P**, Zignego AL, Cursaro C, Gramenzi A, Gherlinzoni F, Fiorino S, Giannini C, Boni P, Sabattini E, Pileri S, Tura S, Bernardi M. Prevalence of monoclonal gammopathies in patients with hepatitis C virus infection. *Ann Intern Med* 1998; **129**: 294-298

74 **Bartl R**, Frisch B, Fateh-Moghadam A, Kettner G, Jaeger K, Sommerfeld W. Histologic classification and staging of multiple myeloma. A retrospective and prospective study of 674 cases. *Am J Clin Pathol* 1987; **87**: 342-355

75 **Pileri S**, Poggi S, Baglioni P, Montanari M, Sabattini E, Galieni P, Tazzari PL, Gobbi M, Cavo M, Falini B. Histology and immunohistology of bone marrow biopsy in multiple myeloma. *Eur J Haematol Suppl* 1989; **51**: 52-59

76 **Zignego AL**, Ferri C, Monti M, LaCivita L, Giannini C, Careccia G, Giannelli F, Pasero G, Bombardieri S, Gentilini P. Hepatitis C virus as a lymphotropic agent: evidence and pathogenetic implications. *Clin Exp Rheumatol* 1995; **13** Suppl 13: S33-S37

77 **Zignego AL**, Macchia D, Monti M, Thiers V, Mazzetti M, Foschi M, Maggi E, Romagnani S, Gentilini P, Bréchot C. Infection of peripheral mononuclear blood cells by hepatitis C virus. *J Hepatol* 1992; **15**: 382-386

78 **Zignego AL**, De Carli M, Monti M, Careccia G, La Villa G, Giannini C, D'Elios MM, Del Prete G, Gentilini P. Hepatitis C virus infection of mononuclear cells from peripheral blood and liver infiltrates in chronically infected patients. *J Med Virol* 1995; **47**: 58-64

79 **Shimizu YK**, Igarashi H, Kanematu T, Fujiwara K, Wong DC, Purcell RH, Yoshikura H. Sequence analysis of the hepatitis C virus genome recovered from serum, liver, and peripheral blood mononuclear cells of infected chimpanzees. *J Virol* 1997; **71**: 5769-5773

80 **Bronowicki JP**, Loriot MA, Thiers V, Grignon Y, Zignego AL, Bréchot C. Hepatitis C virus persistence in human hematopoietic cells injected into SCID mice. *Hepatology* 1998; **28**: 211-218

81 **Lerat H**, Rumin S, Habersetzer F, Berby F, Trabaud MA, Trépo C, Inchauspé G. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* 1998; **91**: 3841-3849

82 **Roque Afonso AM**, Jiang J, Penin F, Tareau C, Samuel D, Petit MA, Bismuth H, Dussaix E, Féray C. Nonrandom distribution of hepatitis C virus quasispecies in plasma and peripheral blood mononuclear cell subsets. *J Virol* 1999; **73**: 9213-9221

83 **Sansonno D**, Lotesoriere C, Cornacchiulo V, Fanelli M, Gatti P, Iodice G, Racanelli V, Dammacco F. Hepatitis C virus infection involves CD34(+) hematopoietic progenitor cells in hepatitis C virus chronic carriers. *Blood* 1998; **92**: 3328-3337

84 **Roque-Afonso AM**, Ducoulombier D, Di Liberto G, Kara R, Gigou M, Dussaix E, Samuel D, Féray C. Compartmentalization of hepatitis C virus genotypes between plasma and peripheral blood mononuclear cells. *J Virol* 2005; **79**: 6349-6357

85 **Galli M**, Zehender G, Monti G, Ballaré M, Saccardo F, Piconi S, De Maddalena C, Bertoncelli MC, Rinaldi G, Invernizzi F. Hepatitis C virus RNA in the bone marrow of patients with mixed cryoglobulinemia and in subjects with noncryoglobulinemic chronic hepatitis type C. *J Infect Dis* 1995; **171**: 672-675

86 **Pal S**, Sullivan DG, Kim S, Lai KK, Kae J, Cotler SJ, Carithers RL, Wood BL, Perkins JD, Gretch DR. Productive replication of hepatitis C virus in perihepatic lymph nodes in vivo: implications of HCV lymphotropism. *Gastroenterology* 2006; **130**: 1107-1116

87 **Ivanovski M**, Silvestri F, Pozzato G, Anand S, Mazzaro C, Burrone OR, Efremov DG. Somatic hypermutation, clonal diversity, and preferential expression of the VH 51p1/VL kv325 immunoglobulin gene combination in hepatitis C virus-associated immunocytomas. *Blood* 1998; **91**: 2433-2442

88 **De Re V**, De Vita S, Marzotto A, Rupolo M, Gloghini A, Pivetta B, Gasparotto D, Carbone A, Boiocchi M. Sequence analysis of the immunoglobulin antigen receptor of hepatitis C virus-associated non-Hodgkin lymphomas suggests that the malignant cells are derived from the rheumatoid factor-producing cells that occur mainly in type II cryoglobulinemia. *Blood* 2000; **96**: 3578-3584

89 **Sansonno D**, Lauletta G, De Re V, Tucci FA, Gatti P, Racanelli V, Boiocchi M, Dammacco F. Intrahepatic B cell clonal expansions and extrahepatic manifestations of chronic HCV infection. *Eur J Immunol* 2004; **34**: 126-136

90 **Mariette X**. Lymphomas complicating Sjögren's syndrome and hepatitis C virus infection may share a common pathogenesis: chronic stimulation of rheumatoid factor B cells. *Ann Rheum Dis* 2001; **60**: 1007-1010

91 **Pileri P**, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. *Science* 1998; **282**: 938-941

92 **Machida K**, Cheng KT, Sung VM, Shimodaira S, Lindsay KL, Levine AM, Lai MY, Lai MM. Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. *Proc Natl Acad Sci USA* 2004; **101**: 4262-4267

93 **Zignego AL**, Giannelli F, Marrocchi ME, Giannini C, Gentilini P, Innocenti F, Ferri C. Frequency of bcl-2 rearrangement in patients with mixed cryoglobulinemia and HCV-positive liver diseases. *Clin Exp Rheumatol* 1997; **15**: 711-712

94 **Zignego AL**, Giannelli F, Marrocchi ME, Mazzocca A, Ferri C, Giannini C, Monti M, Caini P, Villa GL, Laffi G, Gentilini P. T(14;18) translocation in chronic hepatitis C virus infection. *Hepatology* 2000; **31**: 474-479

95 **Zignego AL**, Ferri C, Giannelli F, Giannini C, Caini P, Monti M, Marrocchi ME, Di Pietro E, La Villa G, Laffi G, Gentilini P. Prevalence of bcl-2 rearrangement in patients with hepatitis C virus-related mixed cryoglobulinemia with or without B-cell lymphomas. *Ann Intern Med* 2002; **137**: 571-580

96 **Kitay-Cohen Y**, Amiel A, Hilzenrat N, Buskila D, Ashur Y, Fejgin M, Gaber E, Safadi R, Tur-Kaspa R, Lishner M. Bcl-2 rearrangement in patients with chronic hepatitis C associated with essential mixed cryoglobulinemia type II. *Blood* 2000; **96**: 2910-2912

97 **Zuckerman E**, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, Yeshurun D, Rowe J. bcl-2 and immunoglobulin gene rearrangement in patients with hepatitis C virus infection. *Br J Haematol* 2001; **112**: 364-369

98 **Sasso EH**, Martinez M, Yarfitz SL, Ghillani P, Musset L, Piette JC, Cacoub P. Frequent joining of Bcl-2 to a JH6 gene in hepatitis C virus-associated t(14;18). *J Immunol* 2004; **173**: 3549-3556

99 **Libra M**, Gloghini A, Navolanic PM, Zignego AL, De Re V, Carbone A. JH6 gene usage among HCV-associated MALT lymphomas harboring t(14;18) translocation. *J Immunol* 2005; **174**: 3839; author reply 3839

100 **Giannelli F**, Moscarella S, Giannini C, Caini P, Monti M, Gragnani L, Romanelli RG, Solazzo V, Laffi G, La Villa G, Gentilini P, Zignego AL. Effect of antiviral treatment in patients with chronic HCV infection and t(14;18) translocation. *Blood* 2003; **102**: 1196-1201

101 **Mazzaro C**, Franzin F, Tulissi P, Pussini E, Crovatto M, Carniello GS, Efremov DG, Burrone O, Santini G, Pozzato G. Regression of monoclonal B-cell expansion in patients affected by mixed cryoglobulinemia responsive to alpha-interferon therapy. *Cancer* 1996; **77**: 2604-2613

102 **Giannini C**, Giannelli F, Zignego AL. Association between mixed cryoglobulinemia, translocation (14;18), and persistence of occult HCV lymphoid infection after treatment. *Hepatology* 2006; **43**: 1166-1167; author reply 1167-1168

103 **Fornasieri A**, Bernasconi P, Ribero ML, Sinico RA, Fasola M, Zhou J, Portera G, Tagger A, Gibelli A, D'amico G. Hepatitis C virus (HCV) in lymphocyte subsets and in B lymphocytes expressing rheumatoid factor cross-reacting idiotype in type II mixed cryoglobulinaemia. *Clin Exp Immunol* 2000; **122**: 400-403

104 **Bonomo L**, Casato M, Afeltra A, Caccavo D. Treatment of idiopathic mixed cryoglobulinemia with alpha interferon. *Am J Med* 1987; **83**: 726-730

105 **Casato M**, Laganà B, Antonelli G, Dianzani F, Bonomo L. Long-term results of therapy with interferon-alpha for type II essential mixed cryoglobulinemia. *Blood* 1991; **78**: 3142-3147

106 **Ferri C**, Zignego AL, Longombardo G, Monti M, La Civita L, Lombardini F, Greco F, Mazzoni A, Pasero G, Gentilini P. Effect of alpha-interferon on hepatitis C virus chronic infection in mixed cryoglobulinemia patients. *Infection* 1993; **21**: 93-97

107 **Marcellin P**, Descamps V, Martinot-Peignoux M, Larzul D, Xu L, Boyer N, Pham BN, Crickx B, Guillevin L, Belaich S. Cryoglobulinemia with vasculitis associated with hepatitis C virus infection. *Gastroenterology* 1993; **104**: 272-277

108 **Johnson RJ**, Gretch DR, Yamabe H, Hart J, Bacchi CE, Hartwell P, Couser WG, Corey L, Wener MH, Alpers CE. Membranoproliferative glomerulonephritis associated with hepatitis C virus infection. *N Engl J Med* 1993; **328**: 465-470

109 **Misiani R**, Bellavita P, Fenili D, Vicari O, Marchesi D, Sironi PL, Zilio P, Vernocchi A, Massazza M, Vendramin G. Interferon alfa-2a therapy in cryoglobulinemia associated with hepatitis C virus. *N Engl J Med* 1994; **330**: 751-756

110 **Dammacco F**, Sansonno D, Han JH, Shyamala V, Cornacchiulo V, Iacobelli AR, Lauletta G, Rizzi R. Natural interferon-alpha versus its combination with 6-methyl-prednisolone in the therapy of type II mixed cryoglobulinemia: a long-term, randomized, controlled study. *Blood* 1994; **84**: 3336-3343

111 **Johnson RJ**, Gretch DR, Couser WG, Alpers CE, Wilson J, Chung M, Hart J, Willson R. Hepatitis C virus-associated glomerulonephritis. Effect of alpha-interferon therapy. *Kidney Int* 1994; **46**: 1700-1704

112 **Mazzaro C**, Pozzato G, Moretti M, Crovatto M, Modolo ML, Mazzi G, Santini G. Long-term effects of alpha-interferon therapy for type II mixed cryoglobulinemia. *Haematologica* 1994; **79**: 342-349

113 **Casaril M**, Capra F, Gabrielli GB, Bassi A, Squarzoni S, Dagradi R, De Maria E, Corrocher R. Cryoglobulinemia in hepatitis C virus chronic active hepatitis: effects of interferon-alpha therapy. *J Interferon Cytokine Res* 1996; **16**: 585-588

114 **Cohen P**, Nguyen QT, Dény P, Ferrière F, Roulot D, Lortholary O, Jarrousse B, Danon F, Barrier JH, Ceccaldi J, Constans J, Crickx B, Fiessinger JN, Hachulla E, Jaccard A, Seligmann M, Kazatchkine M, Laroche L, Subra JF, Turlure P, Guillevin L. Treatment of mixed cryoglobulinemia with recombinant interferon alpha and adjuvant therapies. A prospective study on 20 patients. *Ann Med Interne* (Paris) 1996; **147**: 81-86

115 **Akriviadis EA**, Xanthakis I, Navrozidou C, Papadopoulos A. Prevalence of cryoglobulinemia in chronic hepatitis C virus infection and response to treatment with interferon-alpha. *J Clin Gastroenterol* 1997; **25**: 612-618

116 **Casato M**, Agnello V, Pucillo LP, Knight GB, Leoni M, Del Vecchio S, Mazzilli C, Antonelli G, Bonomo L. Predictors of long-term response to high-dose interferon therapy in type II cryoglobulinemia associated with hepatitis C virus infection. *Blood* 1997; **90**: 3865-3873

117 **Calleja JL**, Albillos A, Moreno-Otero R, Rossi I, Cacho G, Domper F, Yebra M, Escartín P. Sustained response to interferon-alpha or to interferon-alpha plus ribavirin in hepatitis C virus-associated symptomatic mixed cryoglobulinaemia. *Aliment Pharmacol Ther* 1999; **13**: 1179-1186

118 **Zuckerman E**, Keren D, Slobodin G, Rosner I, Rozenbaum M, Toubi E, Sabo E, Tsykounov I, Naschitz JE, Yeshurun D. Treatment of refractory, symptomatic, hepatitis C virus related mixed cryoglobulinemia with ribavirin and interferon-alpha. *J Rheumatol* 2000; **27**: 2172-2178

119 **Cacoub P**, Lidove O, Maisonobe T, Duhaut P, Thibault V, Ghillani P, Myers RP, Leger JM, Servan J, Piette JC. Interferon-alpha and ribavirin treatment in patients with hepatitis C virus-related systemic vasculitis. *Arthritis Rheum* 2002; **46**: 3317-3326

120 **Mazzaro C**, Zorat F, Comar C, Nascimben F, Bianchini D, Baracetti S, Donada C, Donadon V, Pozzato G. Interferon plus ribavirin in patients with hepatitis C virus positive mixed cryoglobulinemia resistant to interferon. *J Rheumatol* 2003; **30**: 1775-1781

121 **Heagy W**, Crumpacker C, Lopez PA, Finberg RW. Inhibition of immune functions by antiviral drugs. *J Clin Invest* 1991; **87**: 1916-1924

122 **Durand JM**, Cacoub P, Lunel-Fabiani F, Cosserat J, Cretel E, Kaplanski G, Frances C, Bletry O, Soubeyrand J, Godeau P. Ribavirin in hepatitis C related cryoglobulinemia. *J Rheumatol* 1998; **25**: 1115-1117

123 **Jaccard A**, Loustaud V, Turlure P, Rogez S, Bordessoule D. Ribavirin and immune thrombocytopenic purpura. *Lancet* 1998; **351**: 1660-1661

124 **Alric L**, Plaisier E, Thébault S, Péron JM, Rostaing L, Pourrat J, Ronco P, Piette JC, Cacoub P. Influence of antiviral therapy in hepatitis C virus-associated cryoglobulinemic MPGN. *Am J Kidney Dis* 2004; **43**: 617-623

125 **Cacoub P**, Saadoun D, Limal N, Sene D, Lidove O, Piette JC. PEGylated interferon alfa-2b and ribavirin treatment in patients with hepatitis C virus-related systemic vasculitis. *Arthritis Rheum* 2005; **52**: 911-915

126 **Mazzaro C**, Zorat F, Caizzi M, Donada C, Di Gennaro G, Maso LD, Carniello G, Virgolini L, Tirelli U, Pozzato G. Treatment with peg-interferon alfa-2b and ribavirin of hepatitis C virus-associated mixed cryoglobulinemia: a pilot study. *J Hepatol* 2005; **42**: 632-638

127 **Levine JW**, Gota C, Fessler BJ, Calabrese LH, Cooper SM. Persistent cryoglobulinemic vasculitis following successful treatment of hepatitis C virus. *J Rheumatol* 2005; **32**: 1164-1167

128 **Zuckerman E**, Zuckerman T, Sahar D, Streichman S, Attias D, Sabo E, Yeshurun D, Rowe JM. The effect of antiviral therapy on t(14;18) translocation and immunoglobulin gene rearrangement in patients with chronic hepatitis C virus

infection. *Blood* 2001; **97**: 1555-1559
129 **Ferri C**, Giuggioli D, Cazzato M, Sebastiani M, Mascia MT, Zignego AL. HCV-related cryoglobulinemic vasculitis: an update on its etiopathogenesis and therapeutic strategies. *Clin Exp Rheumatol* 2003; **21**: S78-S84
130 **Ballarè M**, Bobbio F, Poggi S, Bordin G, Bertoncelli MC, Catania E, Monteverde A. A pilot study on the effectiveness of cyclosporine in type II mixed cryoglobulinemia. *Clin Exp Rheumatol* 1995; **13** Suppl 13: S201-S203
131 **Zaja F**, De Vita S, Mazzaro C, Sacco S, Damiani D, De Marchi G, Michelutti A, Baccarani M, Fanin R, Ferraccioli G. Efficacy and safety of rituximab in type II mixed cryoglobulinemia. *Blood* 2003; **101**: 3827-3834
132 **Sansonno D**, De Re V, Lauletta G, Tucci FA, Boiocchi M, Dammacco F. Monoclonal antibody treatment of mixed cryoglobulinemia resistant to interferon alpha with an anti-CD20. *Blood* 2003; **101**: 3818-3826
133 **Zaja F**, Russo D, Fuga G, Patriarca F, Ermacora A, Baccarani M. Rituximab for the treatment of type II mixed cryoglobulinemia. *Haematologica* 1999; **84**: 1157-1158
134 **Zaja F**, De Vita S, Russo D, Michelutti A, Fanin R, Ferraccioli G, Baccarani M. Rituximab for the treatment of type II mixed cryoglobulinemia. *Arthritis Rheum* 2002; **46**: 2252-2254; author reply 2254-2255
135 **Vallisa D**, Bernuzzi P, Arcaini L, Sacchi S, Callea V, Marasca R, Lazzaro A, Trabacchi E, Anselmi E, Arcari AL, Moroni C, Bertè R, Lazzarino M, Cavanna L. Role of anti-hepatitis C virus (HCV) treatment in HCV-related, low-grade, B-cell, non-Hodgkin's lymphoma: a multicenter Italian experience. *J Clin Oncol* 2005; **23**: 468-473
136 **Kelaidi C**, Rollot F, Park S, Tulliez M, Christoforov B, Calmus Y, Podevin P, Bouscary D, Sogni P, Blanche P, Dreyfus F. Response to antiviral treatment in hepatitis C virus-associated marginal zone lymphomas. *Leukemia* 2004; **18**: 1711-1716
137 **Gisbert JP**, García-Buey L, Pajares JM, Moreno-Otero R. Systematic review: regression of lymphoproliferative disorders after treatment for hepatitis C infection. *Aliment Pharmacol Ther* 2005; **21**: 653-662
138 **Hainsworth JD**, Litchy S, Burris HA, Scullin DC, Corso SW, Yardley DA, Morrissey L, Greco FA. Rituximab as first-line and maintenance therapy for patients with indolent non-hodgkin's lymphoma. *J Clin Oncol* 2002; **20**: 4261-4267
139 **Somer BG**, Tsai DE, Downs L, Weinstein B, Schuster SJ. Improvement in Sjögren's syndrome following therapy with rituximab for marginal zone lymphoma. *Arthritis Rheum* 2003; **49**: 394-398
140 **Ramos-Casals M**, López-Guillermo A, Brito-Zerón P, Cervera R, Font J. Treatment of B-cell lymphoma with rituximab in two patients with Sjögren's syndrome associated with hepatitis C virus infection. *Lupus* 2004; **13**: 969-971
141 **Prati D**, Capelli C, Zanella A, Mozzi F, Bosoni P, Pappalettera M, Zanuso F, Vianello L, Locatelli E, de Fazio C, Ronchi G, del Ninno E, Colombo M, Sirchia G. Influence of different hepatitis C virus genotypes on the course of asymptomatic hepatitis C virus infection. *Gastroenterology* 1996; **110**: 178-183
142 **Tighe H**, Warnatz K, Brinson D, Corr M, Weigle WO, Baird SM, Carson DA. Peripheral deletion of rheumatoid factor B cells after abortive activation by IgG. *Proc Natl Acad Sci USA* 1997; **94**: 646-651

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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Immunotherapy of hepatoma with a monoclonal antibody against murine endoglin

Guang-Hong Tan, Feng-Ying Huang, Hua Wang, Yong-Hao Huang, Ying-Ying Lin, Yue-Nan Li

Guang-Hong Tan, Feng-Ying Huang, Hua Wang, Yong-Hao Huang, Ying-Ying Lin, Yue-Nan Li, Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, Hainan Province, China
Guang-Hong Tan, Feng-Ying Huang, Pediatric Department of Affiliated Hospital, Hainan Medical College, Haikou 570102, Hainan Province, China
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Correspondence to: Dr. Guang-Hong Tan, Hainan Provincial Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, Hainan Province, China. tanhoho@163.com
Telephone: +86-898-66893335 Fax: +86-898-66893335
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Abstract

AIM: To explore the capability of a monoclonal antibody (mAb) against murine endoglin to inhibit tumor angiogenesis and suppression of hepatoma growth in murine models.

METHODS: A monoclonal antibody against murine endoglin was purified by affinity chromatography and passively transfused through tail veins in two murine hepatoma models. Tumor volume and survival time were observed at three-day intervals for 48 d. Microvessels in tumor tissues were detected by immunohistochemistry against CD31, and angiogenesis *in vivo* was determined by alginate encapsulated assay. In addition, tumor cell apoptosis was detected by TUNEL assay.

RESULTS: Passive immunotherapy with anti-endoglin mAb could effectively suppress tumor growth, and prolonged the survival time of hepatoma-bearing mice. Angiogenesis was apparently inhibited within the tumor tissues, and the vascularization of alginate beads was also reduced in the mice passively transfused with anti-endoglin mAb. In addition, increased apoptotic cells were observed within the tumor tissues from the mice passively transfused with anti-endoglin mAb.

CONCLUSION: Passive immunotherapy with anti-endoglin mAb effectively inhibits tumor growth *via* inhibiting tumor angiogenesis and increasing tumor cell apoptosis, which may be highly correlated with the blockage of endoglin-related signal pathway induced by anti-endoglin mAb.

Key words: Monoclonal antibody; Endoglin; Passive immunotherapy; Tumor angiogenesis; Apoptosis

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INTRODUCTION

Liver cancer is the 5th leading cancer type in the world[1,2]. Despite aggressive therapeutic approaches made in the past decades, the prognosis of liver cancer remains poor, 5-year mortality exceeds 95%[1-3]. It is thus necessary to seek other more rational approaches for treatment of liver cancer.

Endoglin is a homodimeric transmembrane glycoprotein, which was initially identified as a human leukemia-associated homodimer cell-membrane antigen[4]. Studies performed in different laboratories using various antibodies to endoglin have revealed endoglin expression and up-regulation in a wide range of tumor endothelia, but seldom found in the endothelia of normal tissues, suggesting that endoglin is highly related to tumor angiogenesis[5,6]. In addition, another study demonstrated that blockage of the endoglin pathway in human umbilical vein endothelial cells resulted in marked a inhibition of *in vitro* angiogenesis in combination with TGF-β, indicating that endoglin is a pro-angiogenic component in the endothelial cells[7]. Therefore, therapeutic approach targeting endoglin may potentially have the capability of avoiding system side-effects. It is thus conceivable to consider that passive immunotherapy with anti-endoglin mAb may potentially have the capability of inhibiting tumor growth and/or tumor metastasis through interference of the endoglin-related angiogenesis pathway. In our previous study, we have established a hybridoma cell line secreted monoclonal antibody (mAb) against murine endoglin, which has been demonstrated to have the capability of specifically staining with recombinant murine endoglin and tumor microvessel density by Western blot and immunohistochemistry[8]. In

the current study, we produced and purified the mAb, and then passively transfused with the mAb in two murine hepatoma models to observe the therapeutic effects against hepatoma.

MATERIALS AND METHODS

Materials

Hybridoma cell line (mEDG) secreted mAb against murine endoglin was established by us[8]. Nude mice were purchased from the Animal Center of Central South University (Hunan, China). Hepa1-6 and H22 hepatoma cell lines were presented by State Key Laboratory of Biotherapy (Sichuan University, Chengdu, China) and stored by us. BALB/c and C57BL/6 mice were purchased from the Animal Center of Hainan province, China. EMAM and RPMI-1640 culture media were purchased from Gibco (USA). CM Affi-Gel blue gel kit was purchased from Bio-Rad (USA). mAb against CD31 and labeled streptavidin biotin reagents were purchased from Dako (USA). Alginate and FITC–dextran solution were purchased from Sigma (USA). *In Situ* Cell Death Detection kit (AP) was purchased from Roche (USA).

Production and purification of antibodies

The production and purification of the mAb against murine endoglin were carried out as previously described[8]. Briefly, the EMAM hybridoma cell line mEDG was cultured in complete EMAM medium supplemented with 100 mL/L fetal calf serum at 37℃ in a humidified atmosphere containing 50 mL/L CO_2 in air. Hybridoma cells grown at log-phase were collected and 5×10^6 cells (in 2 mL suspension) were intraperitoneally injected into nude mice. About one to two weeks later, the ascetic fluid was harvested, purified by affinity chromatography (CM Affi-Gel blue gel kit) following the manufacturer's instructions. In addition, for control observation, antibodies were also purified from the sera derived from the normal mice at 12 wk of age by affinity chromatography as previously performed by us[9].

Passive immunotherapy of hepatoma with anti-endoglin mAb

For the investigation of the therapeutic efficacy of the anti-endoglin mAb in anti-tumor activity *in vivo*, mice at 6 to 8 wk of age were firstly inoculated with 2×10^6 live tumor cells into the right flank and left untreated until palpable tumors of distinct size (about 4-6 mm in diameter) appeared in the mice. Then, the mice were randomly divided into three groups of ten mice each. Group 1 (anti-endoglin mAb therapy, anti-mAb) mice were administered intravenously with the purified anti-endoglin mAb (50 mg/kg) *via* tail vein, and then treated twice per week for 4 consecutive weeks. Group 2 (control therapy with the purified antibodies from normal mice at 12 wk of age, cont-Ab) mice were administered intravenously with the purified antibodies (50 mg/kg) from normal mice at 12 wk of age *via* tail vein, and then treated twice per week for 4 consecutive weeks, as did in group 1. Group 3 (untreated control, cont-NS) mice were injected with equal volume of normal saline (NS) without containing any antibodies. The Hepa1-6 hepatoma (Hepal-6) model was established in C57BL/6 mice, and the H22 hepatoma (H22) model was in BALB/c mice. All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee. The tumor size and survival rate were monitored at a three-day interval, and the tumor volume was calculated by the following formula: Tumor volume = $0.52 \times \text{length} \times \text{width}^2$.

Immunohistochemical detection of tumor microvessel density

To determine microvessel density (MVD), frozen sections of tumor tissues were prepared, and subjected to immunohistochemical staining for CD31 as previously described[9-12]. Briefly, frozen sections were fixed in acetone, incubated, and stained with an antibody reactive to CD31. Sections were then stained with labeled streptavidin biotin reagents. Any endothelial cell or endothelial cluster positive for CD31 (purple staining) was considered to be a single countable microvessel.

Alginate encapsulation assay

Alginate-encapsulated tumor cell assays were performed as previously described[13]. Briefly, Hepal-6 or H22 hepatoma cells were resuspended in a 15 g/L sodium alginate solution and added dropwise into a swirling 37℃ solution of 250 mmol/L calcium chloride. Alginate beads were formed containing approximately 1×10^5 tumor cells per bead. Mice were anesthetized, and four beads were subcutaneously implanted into an incision made on the dorsal side. Incisions were closed with surgical clamps. Thereafter, mice were grouped and treated with antibodies or NS as aforementioned. After 14 d, mice were injected intravenously with 100 μL of 100 mg/kg FITC-dextran solution. Beads were surgically removed and FITC-dextran was quantified against a standard curve of FITC-dextran as previously described[9-12].

Detection of tumor cell apoptosis in situ

To detect *in situ* tumor cell apoptosis, sections of tumor tissues from both Hepal-6 and H22 models were fixed with 10 g/L paraformaldehyde in PBS, and apoptotic cells were detected using TUNEL assay according to the manufacturer's instructions (*In Situ* Cell Death Detection kit, Roche, USA). Apoptosis rate was quantified in a blind fashion by two independent reviewers by determining the percentage of positively stained cell nuclei in 20 randomly chosen fields/section at 200 × magnification.

Statistical analysis

For comparison of individual time points, ANOVA and an unpaired Student's *t*-test were used. Kaplan-Meier method was used to plot survival curves and the statistical significance was determined by the log-rank test. $P < 0.05$ was considered statistically significant. Error bars represent the SD (standard deviation) unless otherwise indicated.

RESULTS

Inhibition of tumor growth

The tumor volume was monitored at a three-day interval.

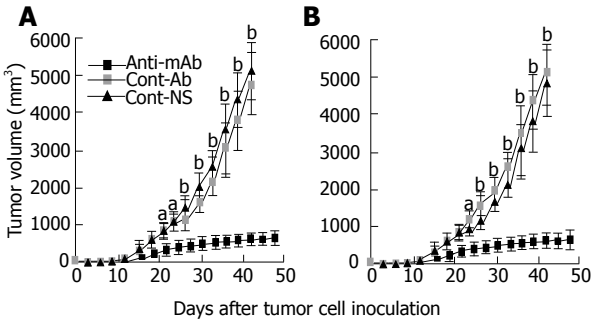

Figure 1 Tumor volumes at different time-points in Hepal-6 (**A**) and H22 (**B**) models. Cont-Ab: mice transfused with control antibodies; Cont-NS: mice transfused with NS; anti-mAb: mice transfused with anti-endoglin mAb. $^{a}P < 0.05$, $^{b}P < 0.01$. Data are shown as mean ± SD, $n = 10$ in each group.

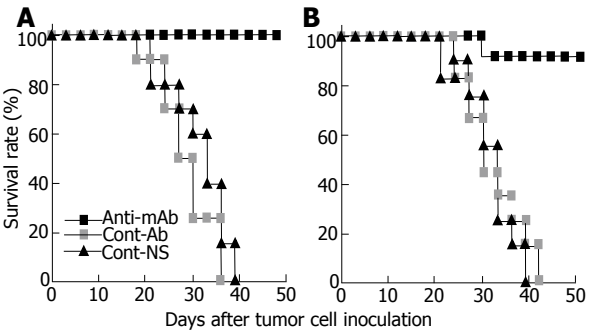

Figure 2 The survival rates at different time-points in Hepal-6 (**A**) and H22 (**B**) models. Log-rank test, $P < 0.01$, $n = 10$ in each group.

Tumors in both Hepal-6 (Figure 1A) and H22 (Figure 1B) models grew progressively in two control group mice treated with normal antibodies or NS, but a significant inhibition of tumor growth was observed in the mice passively transfused with anti-endoglin mAb (Figure 1). Compared with the control groups, tumor volume in the mice passively transfused with anti-endoglin mAb significantly decreased ($P < 0.05$) from d 21 or 24 after tumor cell injection (Figure 1).

Increased survival rate of tumor-bearing mice

The survival rate of the tumor-bearing mice was also monitored at a three-day interval. Compared with the control group mice treated with normal antibodies or NS, the survival rate of the mice passively transfused with anti-endoglin mAb significantly increased in both Hepal-6 (Figure 2A) and H22 (Figure 2B) models ($P < 0.01$ by log-rank test). Thus, significant increase in survival rate and inhibition of tumor growth in the two hepatoma models suggested that passive immunotherapy with an anti-endoglin mAb has significant therapeutic effects against murine hepatoma.

Inhibition of tumor angiogenesis in tumor tissues

Significantly more microvessels (purple color staining) were seen in the tumor tissue sections from the control mice (Figures 3B and C), while almost no such stained microvessels were seen in the tumor tissue sections from the mice passively transfused with anti-endoglin mAb (Figure

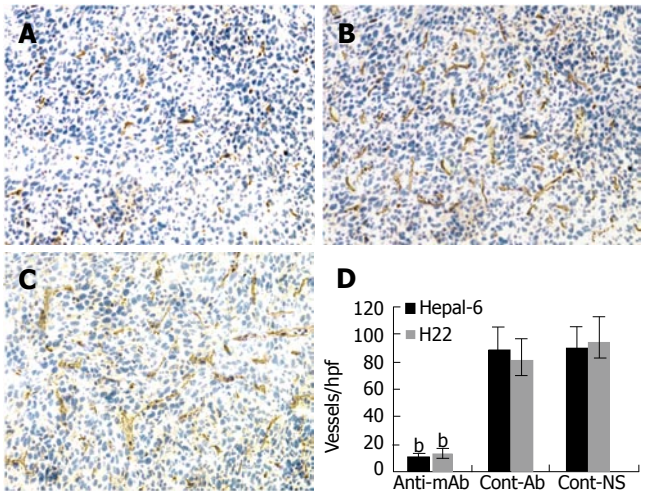

Figure 3 Significant decrease in microvessel density (stained as purple color) in the tumor tissues from the mice transfused with anti-endoglin mAb (**A**) compared to the mice transfused with control antibodies (**B**) and the mice transfused with NS (**C**). Quantitative analysis of microvessels in Hepal-6 and H22 models (**D**), the bars represent mean ± SD, $^{b}P < 0.001$.

3A). The average number of microvessels per high-power field in both Hepal-6 and H22 hepatoma models (10.32 ± 3.51 and 12.85 ± 4.27, respectively) in the mice passively transfused with anti-endoglin mAb were significantly lower than those in the mice transfused with control antibodies (89.69 ± 15.06 and 80.56 ± 15.38, respectively) and the mice transfused with NS (89.12 ± 16.31 and 94.35 ± 17.89, respectively) (Figure 3D; $P < 0.001$).

Inhibition of angiogenesis in alginate encapsulation beads in vivo

Inhibition of angiogenesis was further confirmed by using alginate encapsulation assay. Alginate implanted angiogenesis was quantified by measuring the uptake of FITC-dextran into beads. Compared with the two control groups (Figures 4B and C), a significant inhibition of angiogenesis was found on the beads from the mice passively transfused with anti-endoglin mAb (Figure 4A). FITC-dextran uptake (μg) was significantly decreased in both Hepal-6 and H22 hepatoma models (0.42 ± 0.07 and 0.51 ± 0.09, respectively) in the mice passively transfused with anti-endoglin mAb than that in the mice transfused with control antibodies (4.38 ± 0.94 and 4.79 ± 1.28, respectively) and the mice transfused with NS (4.56 ± 1.02 and 4.93 ± 1.15) (Figure 4D; $P < 0.001$).

Increase in tumor cell apoptosis

To further investigate the anti-tumor mechanism induced by the passive transfusion with anti-endoglin mAb, a TUNEL assay was performed to observe the tumor cell apoptosis *in situ*. TUNEL assay revealed a marked increase in apoptosis in tumor tissues from both Hepal-6 and H22 hepatoma models (43.68% ± 7.32% and 39.54% ± 5.93%, respectively) in the mice passively transfused with anti-endoglin mAb than that in the mice transfused with control antibodies (8.43% ± 2.37% and 7.18% ± 1.96%, respectively) and the mice transfused with NS (9.02% ± 3.02% and 6.95% ± 2.39%, respectively) (Figure 5; $P < 0.001$).

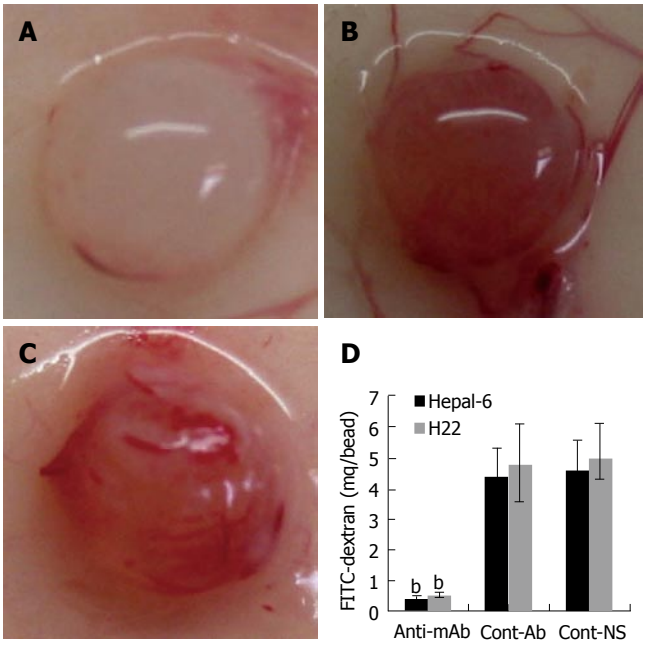

Figure 4 Significant inhibition of angiogenesis in the tumor cell-encapsulated beads from the mice transfused with anti-endoglin mAb (**A**) than that in the tumor tissues from the mice transfused with control antibodies (**B**) and the mice transfused with NS (**C**). Quantitative determination of FITC-dextran uptake in Hepal-6 and H22 models (**D**); the bars represent mean ± SD, $^{b}P < 0.001$.

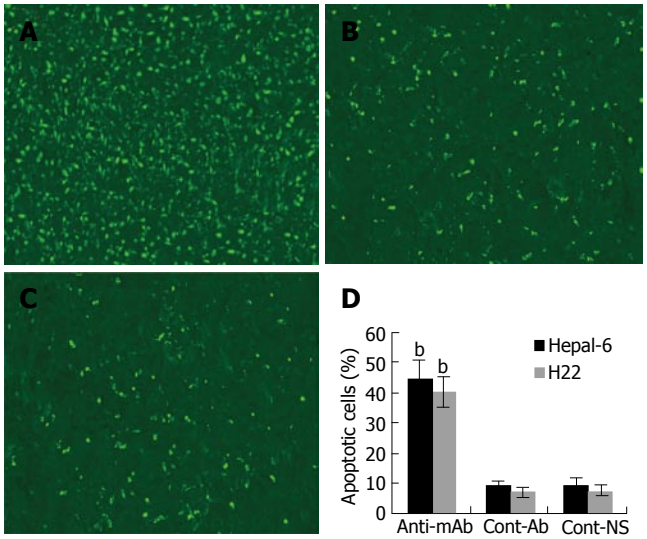

Figure 5 Significantly increased apoptosis in the tumor tissues from the mice transfused with anti-endoglin mAb (**A**) compared to the mice transfused with control antibodies (**B**) and the mice transfused with NS (**C**). Quantitative analysis of apoptotic cells in Hepal-6 and H22 models (**D**), the bars represent mean ± SD, $^{b}P < 0.001$.

DISCUSSION

The generation of new blood vessels, or angiogenesis, is a complex multi-step process. Evidence suggests that the growth and persistence of solid tumors and their metastases are angiogenesis-dependent[14–17]. To date, a number of molecules that stimulate angiogenesis as well as those that inhibit this process have been elucidated[17,18]. In this regard, as a strategy for cancer therapy, anti-angiogenic therapy, which targets genetically stable endothelial cells, has several advantages over conventional tumor cell targeting in the therapy of solid tumors: (1) This approach may have the potential to circumvent the problem of acquired drug resistance[19,20]. The reasonable explanation is that drug resistant mutants are easily generated from tumor cells due to the genetic instability of tumor cells. On the contrary, genetically stable normal cells, such as vascular endothelial cells, would be far less adept at generating such mutants; (2) anti-angiogenic therapy may have the capability of overcoming the problem of tumor heterogeneity[21]. At present, tumor heterogeneity has become a major problem with the tumor cell-targeting therapy; (3) physiological barriers for the high-molecular weight drugs (such as antibodies and immunoconjugates) to penetrate into solid tumors will be also circumvented by targeting a tumor's vasculature rather than tumor cells themselves[22,23]. The possible reason is that, unlike tumor cells in the solid tumors, the vascular endothelial cells are directly accessible to circulating high-molecular weight drugs; (4) many thousands of dependent tumor cells will die of nutrient and/or oxygen deprivation if a capillary or a sector of the capillary bed fails[24,25]. Therefore, killing of only a minority of vascular endothelial cells of tumors may be sufficient to eradicate most malignant cells in tumor tissues; and (5) a single agent developed for anti-angiogenic therapy could be applied to various types of solid tumors and other angiogenesis-associated diseases.

A few lines of evidence have demonstrated that endoglin is one of the marker molecules of tumor angiogenesis, and is specifically expressed and up-regulated in tumor-associated angiogenic vasculatures, specially in tumor microvessels[1-3]. The level of endoglin in serum and tumor tissues detected by ELISA or immunohistochemistry correlated well with the prognosis of solid tumors[2,3,26]. Thus, a specific mAb against endoglin may be a good tool for diagnosis and treatment of solid tumors, including hepatoma.

In the current study, we used an anti-endoglin mAb established by us in our previous study to observe the anti-tumor activities by passive immunotherapy in two hepatoma models. We found that passive immunotherapy with anti-endoglin mAb could effectively suppress tumor growth, and prolong the survival rate of hepatoma-bearing mice. Angiogenesis was apparently inhibited within the tumor tissues, and the vascularization of alginate beads was also reduced in the mice passively transfused with anti-endoglin mAb. In addition, there were increased apoptotic cells within the tumor tissues from the mice passively transfused with anti-endoglin mAb. Based on the aforementioned findings, we may thus conclude the possibility that the therapeutic activities against hepatoma by passive immunotherapy with anti-endoglin mAb may result from the inhibition of tumor angiogenesis and the increase of tumor cell apoptosis, which may highly correlate with the blockage of endoglin-related signal pathway induced by anti-endoglin mAb.

The endothelium in normal vasculatures is considered the most quiescent because the turnover of the endothelial cells is very low[27-30]. However, the endothelial cells in the tumor tissues can undergo rapid proliferation which promote spurts of tumor angiogenesis due to hypoxia and

secretion of various molecules that stimulate angiogenesis by the endothelial cells themselves and tumor cells. Therefore, a proliferation-associated antigen on endothelial cells may be a good candidate for anti-angiogenic therapy. Endoglin has been proven to be a proliferation-associated antigen on endothelial cells[7,31,32]. Previous studies demonstrated that anti-endoglin mAbs showed a highly restricted reactivity to proliferating endothelial cells, immature acute lymphoblastic leukemia (ALL) cells and myeloid/monocytic leukemia cells[4,33,34]. In the current study, we further found that passive immunotherapy with anti-endoglin mAb reached effectively anti-hepatoma activities in murine models. Thus, this study also demonstrated that passive immunotherapy with anti-endoglin mAb may be a potential approach for treatment of hepatoma and other solid tumors.

REFERENCES

1 **Pisani P**, Parkin DM, Bray F, Ferlay J. Estimates of the worldwide mortality from 25 cancers in 1990. *Int J Cancer* 1999; **83**: 18-29
2 **Parkin DM**. Global cancer statistics in the year 2000. *Lancet Oncol* 2001; **2**: 533-543
3 **Okuda K**. Hepatocellular carcinoma. *J Hepatol* 2000; **32**: 225-237
4 **Gougos A**, Letarte M. Identification of a human endothelial cell antigen with monoclonal antibody 44G4 produced against a pre-B leukemic cell line. *J Immunol* 1988; **141**: 1925-1933
5 **Fonsatti E**, Sigalotti L, Arslan P, Altomonte M, Maio M. Emerging role of endoglin (CD105) as a marker of angiogenesis with clinical potential in human malignancies. *Curr Cancer Drug Targets* 2003; **3**: 427-432
6 **Duff SE**, Li C, Garland JM, Kumar S. CD105 is important for angiogenesis: evidence and potential applications. *FASEB J* 2003; **17**: 984-992
7 **She X**, Matsuno F, Harada N, Tsai H, Seon BK. Synergy between anti-endoglin (CD105) monoclonal antibodies and TGF-beta in suppression of growth of human endothelial cells. *Int J Cancer* 2004; **108**: 251-257
8 **Li YN**, Tan GH, Jiao JG, Huang FY, Jiang J. Establishment of a monoclonal antibody against extracellular domain of murine endoglin. *Zhongguo Xiandai Yixue* 2007; **17**: 1-4
9 **Tan GH**, Wei YQ, Tian L, Zhao X, Yang L, Li J, He QM, Wu Y, Wen YJ, Yi T, Ding ZY, Kan B, Mao YQ, Deng HX, Li HL, Zhou CH, Fu CH, Xiao F, Zhang XW. Active immunotherapy of tumors with a recombinant xenogeneic endoglin as a model antigen. *Eur J Immunol* 2004; **34**: 2012-2021
10 **Wei YQ**, Huang MJ, Yang L, Zhao X, Tian L, Lu Y, Shu JM, Lu CJ, Niu T, Kang B, Mao YQ, Liu F, Wen YJ, Lei S, Luo F, Zhou LQ, Peng F, Jiang Y, Liu JY, Zhou H, Wang QR, He QM, Xiao F, Lou YY, Xie XJ, Li Q, Wu Y, Ding ZY, Hu B, Hu M, Zhang W. Immunogene therapy of tumors with vaccine based on Xenopus homologous vascular endothelial growth factor as a model antigen. *Proc Natl Acad Sci USA* 2001; **98**: 11545-11550
11 **He QM**, Wei YQ, Tian L, Zhao X, Su JM, Yang L, Lu Y, Kan B, Lou YY, Huang MJ, Xiao F, Liu JY, Hu B, Luo F, Jiang Y, Wen YJ, Deng HX, Li J, Niu T, Yang JL. Inhibition of tumor growth with a vaccine based on xenogeneic homologous fibroblast growth factor receptor-1 in mice. *J Biol Chem* 2003; **278**: 21831-21836
12 **Jiao JG**, Li YN, Wang H, Liu Q, Cao JX, Bai RZ, Huang FY. A plasmid DNA vaccine encoding the extracellular domain of porcine endoglin induces anti-tumour immune response against self-endoglin-related angiogenesis in two liver cancer models. *Dig Liver Dis* 2006; **38**: 578-587
13 **Hoffmann J**, Schirner M, Menrad A, Schneider MR. A highly sensitive model for quantification of in vivo tumor angiogenesis induced by alginate-encapsulated tumor cells. *Cancer Res* 1997; **57**: 3847-3851
14 **Folkman J**. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 1990; **82**: 4-6
15 **Risau W**. Mechanisms of angiogenesis. *Nature* 1997; **386**: 671-674
16 **Wickham TJ**, Haskard D, Segal D, Kovesdi I. Targeting endothelium for gene therapy via receptors up-regulated during angiogenesis and inflammation. *Cancer Immunol Immunother* 1997; **45**: 149-151
17 **Gupta MK**, Qin RY. Mechanism and its regulation of tumor-induced angiogenesis. *World J Gastroenterol* 2003; **9**: 1144-1155
18 **Paleolog EM**. Angiogenesis: a critical process in the pathogenesis of RA-a role for VEGF? *Br J Rheumatol* 1996; **35**: 917-919
19 **Kerbel RS**. Inhibition of tumor angiogenesis as a strategy to circumvent acquired resistance to anti-cancer therapeutic agents. *Bioessays* 1991; **13**: 31-36
20 **Boehm T**, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 1997; **390**: 404-407
21 **Matsuno F**, Haruta Y, Kondo M, Tsai H, Barcos M, Seon BK. Induction of lasting complete regression of preformed distinct solid tumors by targeting the tumor vasculature using two new anti-endoglin monoclonal antibodies. *Clin Cancer Res* 1999; **5**: 371-382
22 **Jain RK**. Barriers to drug delivery in solid tumors. *Sci Am* 1994; **271**: 58-65
23 **Dvorak HF**, Nagy JA, Dvorak AM. Structure of solid tumors and their vasculature: implications for therapy with monoclonal antibodies. *Cancer Cells* 1991; **3**: 77-85
24 **Denekamp J**. Review article: angiogenesis, neovascular proliferation and vascular pathophysiology as targets for cancer therapy. *Br J Radiol* 1993; **66**: 181-196
25 **Thorpe PE**, Burrows FJ. Antibody-directed targeting of the vasculature of solid tumors. *Breast Cancer Res Treat* 1995; **36**: 237-251
26 **Thorpe PE**. Vascular targeting agents as cancer therapeutics. *Clin Cancer Res* 2004; **10**: 415-427
27 **Folkman J**, Shing Y. Angiogenesis. *J Biol Chem* 1992; **267**: 10931-10934
28 **Auerbach W**, Auerbach R. Angiogenesis inhibition: a review. *Pharmacol Ther* 1994; **63**: 265-311
29 **Engerman RL**, Pfaffenbach D, Davis MD. Cell turnover of capillaries. *Lab Invest* 1967; **17**: 738-743
30 **Risau W**. Differentiation of endothelium. *FASEB J* 1995; **9**: 926-933
31 **Burrows FJ**, Derbyshire EJ, Tazzari PL, Amlot P, Gazdar AF, King SW, Letarte M, Vitetta ES, Thorpe PE. Up-regulation of endoglin on vascular endothelial cells in human solid tumors: implications for diagnosis and therapy. *Clin Cancer Res* 1995; **1**: 1623-1634
32 **Seon BK**, Matsuno F, Haruta Y, Kondo M, Barcos M. Long-lasting complete inhibition of human solid tumors in SCID mice by targeting endothelial cells of tumor vasculature with antihuman endoglin immunotoxin. *Clin Cancer Res* 1997; **3**: 1031-1044
33 **Haruta Y**, Seon BK. Distinct human leukemia-associated cell surface glycoprotein GP160 defined by monoclonal antibody SN6. *Proc Natl Acad Sci USA* 1986; **83**: 7898-7902
34 **Rokhlin OW**, Cohen MB, Kubagawa H, Letarte M, Cooper MD. Differential expression of endoglin on fetal and adult hematopoietic cells in human bone marrow. *J Immunol* 1995; **154**: 4456-4465

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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COLORECTAL CANCER

Synergistic anti-tumor effect of recombinant chicken fibroblast growth factor receptor-1-mediated anti-angiogenesis and low-dose gemcitabine in a mouse colon adenocarcinoma model

Shao-Jiang Zheng, Shao-Ping Zheng, Feng-Ying Huang, Chang-Liang Jiao, Ren-Liang Wu

Shao-Jiang Zheng, Ren-Liang Wu, Department of Pathology, Tongji Medical College, Huazhong University of Science and Technology; Pulmonary Disease Laboratory, The Ministry of Health of China, Wuhan 430030, Hubei Province, China
Shao-Jiang Zheng, Chang-Liang Jiao, Department of Pathology, Hainan Medical College and The Affiliated Hospital, Haikou 571101, Hainan Province, China
Shao-Ping Zheng, Department of Ultrasonography, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China
Shao-Ping Zheng, Feng-Ying Huang, Hainan Province Key Laboratory of Tropical Medicine, Hainan Medical College, Haikou 571101, Hainan Province, China
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Co-first-authors: Shao-Jiang Zheng and Shao-Ping Zheng
Correspondence to: Ren-Liang Wu, Department of Pathology, Tongji Medical College, Huazhong University of Science and Technology; Pulmonary Disease Laboratory, The Ministry of Health of China,Wuhan 430030, Hubei Province, China. renliang_wu@yahoo.com.cn
Telephone: +86-27-83691894 Fax: +86-27-83691894
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Abstract

AIM: To evaluate whether the combination of recombinant chicken fibroblast growth factor receptor -1 (FGFR-1) protein vaccine (cFR-1) combined with low-dose gemcitabine would improve anti-tumor efficacy in a mouse CT26 colon adenocarcinoma (CT26) model.

METHODS: The CT26 model was established in BABL/c mice. Seven days after tumor cell injection, mice were randomly divided into four groups: combination therapy, cFR-1 alone, gemcitabine alone, and normal saline groups. Tumor growth, survival rate of tumor-bearing mice, and systemic toxicity were observed. The presence of anti-tumor auto-antibodies was detected by Western blot analysis and enzyme-linked immunospot assay, microvessel density (MVD) of the tumors and tumor cell proliferation were detected by Immunohistochemistry staining, and tumor cell apoptosis was detected by TdT-mediated biotinylated-dUTP nick end label staining.

RESULTS: The combination therapy results in apparent decreases in tumor volume, microvessel density and tumor cell proliferation, and an increase in apoptosis without obvious side-effects as compared with either therapy alone or normal control groups. Also, both auto-antibodies and the antibody-producing B cells against mouse FGFR-1 were detected in mice immunized with cFR-1 vaccine alone or with combination therapy, but not in non-immunized mice. In addition, the deposition of auto-antibodies on endothelial cells from mice immunized with cFR-1 was observed by immunofluorescent staining, but not on endothelial cells from control groups. Synergistic indexes of tumor volume, MVD, cell apoptosis and proliferation in the combination therapy group were 1.71 *vs* 1.15 *vs* 1.11 and 1.04, respectively, 31 d after tumor cell injection.

CONCLUSION: The combination of cFR-1-mediated anti-angiogenesis and low-dose gemcitabine synergistically enhances the anti-tumor activity without overt toxicity in mice.

Key words: Fibroblast growth factor receptor-1; Gemcitabine; Anti-angiogenesis; Vaccine; Combination therapy

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INTRODUCTION

Colorectal cancer is the third most common malignancy in the world and the fourth killer among all tumors in China[1,2]. The prognosis of advanced colorectal cancer remains poor, estimated 5-year survival rates less than 10%[1-3]. Thus, it is essential to seek multidisciplinary approaches for the treatment of colorectal cancer.

It is generally believed that the growth and metastases of a tumor are angiogenesis-dependent and thus that anti-angiogenic therapy, which targets genetically stable

endothelial cells as a strategy for cancer therapy, is highly warranted[4-6]. At present, basic fibroblast growth factor (bFGF) has been shown to be one of the most important angiogenic growth factors for tumor angiogenesis. bFGF serves its biological function through interaction with its high-affinity receptor, fibroblast growth factor receptor-1 (FGFR-1), which is markedly expressed both in active endothelial cell and in many different forms of tumor and plays an important role in tumor angiogenesis and tumor growth[7-10]. Accumulating evidence indicates that an FGFR-1-mediated anti-angiogenesis target for tumor immunotherapy could suppress angiogenesis and further inhibit tumor growth[11-13]. Our data indicate that vaccination with the cFR-1 protein can induce auto-antibodies against FGFR-1 in mice[14].

Chemotherapy remains one of the major systemic therapies for cancer, but acquired drug resistance is one of the major hindrances to chemotherapy. Recently, evidence has confirmed that low dosages of conventional chemotherapeutic drugs can damage or kill the endothelial cells of tumor neovasculature through various direct or indirect mechanisms, and delay acquired resistance to these chemotherapeutic drugs[15-17]. Other findings have also demonstrated that anti-angiogenic therapy combined with various chemotherapy drugs could more effectively inhibit tumor growth without overt toxicity relative to either therapy alone[16-20]. Gemcitabine is a new deoxycytidine analog that inhibits DNA synthesis and has shown cytotoxicity against a wide range of cancer cell lines *in vitro* and applied widely in clinical anti-tumor therapy[20-22]. Moreover, studies have also confirmed that the combination of an anti-angiogenic biotherapy with a low-dose gemcitabine strategy can effectively suppress tumor angiogenesis without increased overt toxicity relative to either therapy alone[20]. Thus, in this study, we primarily evaluated the anti-tumor activities of the recombinant cFR-1 protein vaccine in combination with low-dose gemcitabine in a mouse tumor model.

MATERIALS AND METHODS

Vaccine preparation

The lyophilized recombinant proteins of cFR-1 and mouse FGFR-1 (mFR-1) were dissolved in NS and mixed with an equal volume of aluminum hydroxide adjuvant at 4 mg/mL for 60 min before use in vaccination[23].

Design of animal experiments

The CT26 tumor model was established in BALB/c mice to evaluate whether the combination of cFR-1 vaccine and low-dose gemcitabine would improve the anti-tumor efficacy. Six to eight-week-old female mice were transplanted with 1×10^6 live tumor cells. After tumors had grown for 7 d, the mice were randomly divided into the following four groups of 10 mice each. Group 1 mice, treated with a combination of cFR-1 vaccine and low-dose gemcitabine (C + G), received cFR-1 vaccine plus low-dose gemcitabine as follows: after d 0 (7 d after tumor cell injection), cFR-1 vaccine was injected subcutaneously (s.c.) once a week for 4 wk with a dose of 10 μg per mouse. At d 7 (14 d after tumor cell injection), 20 mg/kg of gemcitabine was injected intraperitoneal (i.p.) at an interval of every 3 d for a total of 4 doses. Group 2 mice, treated with cFR-1 vaccine alone (cFR), received cFR-1 vaccine in a similar scheme as that in group 1, except that it lacked gemcitabine. Group 3 mice, treated with low-dose gemcitabine alone (G), received the same dose of gemcitabine as group 1, but they did not receive the cFR-1 vaccine. Group 4 mice, the untreated group (NS), received sterile NS s.c. as the scheme of vaccination or were treated i.p. as in group 1, respectively. Tumor growth was evaluated every 3 d and tumor volume was estimated using the formula for an ellipsoid (0.5 × length × width × height). At the end of the experiment, the tumor tissues, major organs and blood samples of the mice were collected for subsequent histologic and immunologic investigations. All studies involving mice were approved by the Institute's Animal Care and Use Committee.

Western blot analysis

Western blot analysis was performed as described previously[14]. Briefly, the recombinant proteins were separated by 12 % SDS-PAGE. Gels were transblotted with the Mini Polyacrylamide Gel System (Bio-Rad, USA) onto a polyvinylidene difluoride membrane. Membrane blots were blocked at 4℃ in 5% nonfat dry milk, washed and probed with mouse sera at a 1:500 dilution. Blots were then washed and incubated with goat anti-mouse IgG HRP-labeled secondary antibody and then stained with the Vectastain ABC kit (Vector, Burlingame, USA).

Enzyme-linked immunospot (ELISPOT) assay

The ELISPOT assay for the enumeration of antibody-producing B cells (APBCs) has been described[14]. Briefly, PVDF-bottomed, 96-well filtration plates (Millipore, Bedford, USA) were coated with 30 μg/mL of recombinant FGFR-1 protein. Mononuclear cells prepared from spleen were incubated on the plates at 37℃ for 4 h. IgG bound to the membrane was revealed as spots with alkaline phosphatase-conjugated anti-mouse IgG antibodies.

Immunohistochemistry

For microvessel density (MVD) and cell proliferation analyses, frozen sections were fixed in acetone, incubated and stained with antibodies reactive to either CD31 or proliferating cell nuclear antigen (PCNA) (BD Pharmingen, USA), respectively. The MVD was determined by counting the number of microvessels and the proliferation index was calculated as the ratio of the proliferation cell number to the total cell number per high-power field in tumor sections as described[24].

To identify the endothelial deposition of auto-antibody by immunofluorescent staining, frozen sections were fixed in acetone, washed with PBS, and incubated with FITC-conjugated antibody against mouse IgG, IgA, or IgM (Sigma, St. Louis, USA). Moreover, sections of tissue were fixed with 1% paraformaldehyde in PBS and stained for apoptosis analysis by using the TdT-mediated biotinylated-dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions (In Situ Cell Death Detection Kit; Roche, UK). These slides were imaged using a fluorescence microscope and the apoptosis index was

Table 1 Synergistic indexes of combination therapy relative fraction[1]

Day[2]	FGFR-1	Gemcitabine	Combination therapy		Index[4]
			Expected[3]	Observed	
Tumor volume index	0.48	0.60	0.29	0.17	1.71
MVD index	0.35	0.66	0.23	0.20	1.15
Apoptosis index	0.48	0.43	0.21	0.19	1.11
Proliferation index	0.49	0.46	0.22	0.21	1.04

[1]Relative Fraction (RF) = mean tumor volume (or MVD, apoptosis and proliferation index) experimental/mean tumor volume (or MVD, apoptosis and proliferation index) untreated on d 31, respectively. –[2]Day after tumor cell transplantation. –[3]RF of gemcitabine × RF of cFR-1 vaccine. –[4]Obtained by dividing the expected RF by the observed RF.

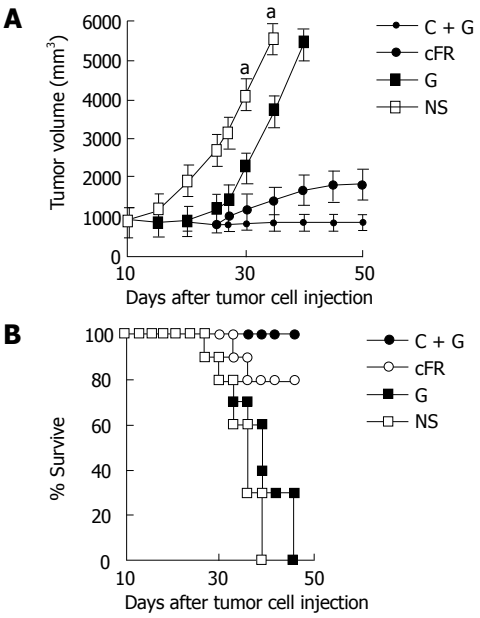

Figure 1 The combination therapy inhibited tumor growth (**A**) and improved the survival of tumor-bearing mice (**B**) significantly. [a]Significant difference compared to untreated group, [a]$P < 0.05$.

calculated as the ratio of the apoptotic cell number to the total cell number in each high-power field.

Calculation of synergistic indexes: Mean values of tumor volume, MVD, cell apoptosis and proliferation were used for calculation of the correspondent synergistic indexes using the methods described before[24]. Briefly, the mean tumor volume, MVD, cell apoptosis index or proliferation index in each treatment group was obtained by dividing the mean value by that in the untreated control group. The expected relative ratio of the combination treatment group was obtained by timing the observed relative ratio of the xenogeneic FGFR-1 vaccine treatment group to that of the low-dose gemcitabine treatment group. Then, the corresponding synergistic index of tumor volume, MVD or proliferation (compared to the untreated control group, the resultant value was decreased) was obtained by dividing the expected relative ratio by the observed relative ratio, whereas the synergistic index of apoptosis (compared to the untreated control group, the resultant value was increased) was obtained by dividing the observed relative ratio by the expected relative ratio. The

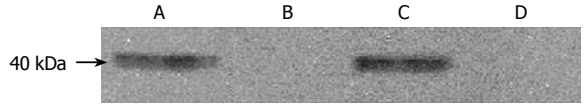

Figure 2 Induction of auto-antibodies by cFR-1 vaccine alone or combination therapy as assessed by Western blot analysis.

synergistic indexes of tumor volume, MVD, cell apoptosis and proliferation are further detailed in Table 1. An index of greater than 1 indicates a synergistic effect, whereas an index of less than 1 indicates a less than additive effect.

Statistical analysis

Results were presented as mean ± SD. All statistical analyses were carried out using SPSS 12.0 for Windows statistical software (SPSS Inc, USA). For comparison of individual time points, ANOVA and an unpaired Student's *t* were used. Survival curves were constructed according to the Kaplan-Meier method. Statistical significance was determined by the log-rank test. $P < 0.05$ was considered statistically significant.

RESULTS

The effect of the combined therapy on tumor growth and survival

In the first part, cFR-1 vaccine or low-dose gemcitabine treatment resulted in the inhibition of tumor growth, to a certain extent, compared with the untreated control group. Remarkably, the combination therapy resulted in more significant anti-tumor activity (Figure 1A). The relative ratio of tumor volume in the combination group showed a synergistic relationship about 31 d after tumor cell transplantation in the tumor model (Table 1). In addition, the survival of tumor-bearing mice had similar results to that of tumor growth (Figure 1B).

Induction of anti-tumor autoimmunity by the combined therapy

The possibility that the cFR-1 vaccine alone or the combination treatment induces production of anti-tumor autoimmunity in the mouse model was examined by using Western blot analysis, ELISPOT assay and immunofluorescent staining.

In Western blot analysis, sera from these cFR-1-immunized mice recognized a protein as indicated by the positive staining of an about 40 kDa band (Figure 2A and C) that was not stained by sera from the untreated control or low-dose gemcitabine treatment groups (Figure 2B and D).

The endothelial deposition of auto-antibodies was found within tumor tissues from cFR-1-immunized mice, as detected by immunofluorescent staining (Figure 3A and 3B); however this deposition was not detected in the non-immunized control groups. In addition, detectable deposition of auto-antibodies was not found within the major organs of immunized and non-immunized mice.

The number of APBCs, which were detected by ELISPOT assay, was significantly elevated in the spleen of mice immunized with cFR-1 vaccine, both in the vaccine alone and in the combination treatment groups, as

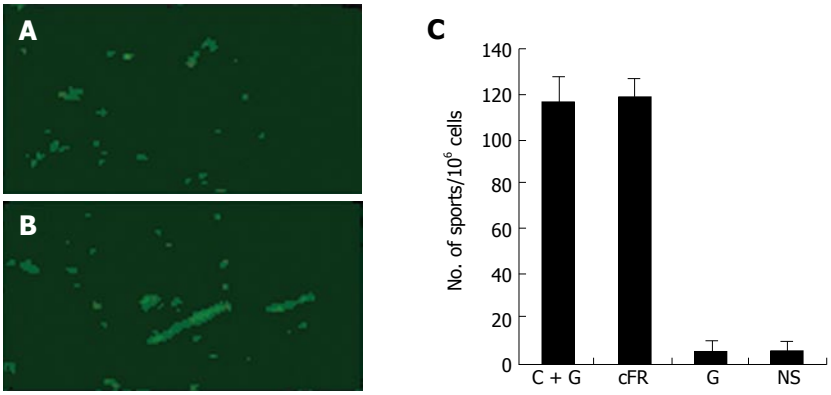

Figure 3 The deposition of auto-antibodies on the endothelial cells by immunofluorescent staining (**A-B**) and the numbers of APBCs in spleens of mice by ELISPOT assay (**C**) in cFR-1 vaccine alone or combination therapy.

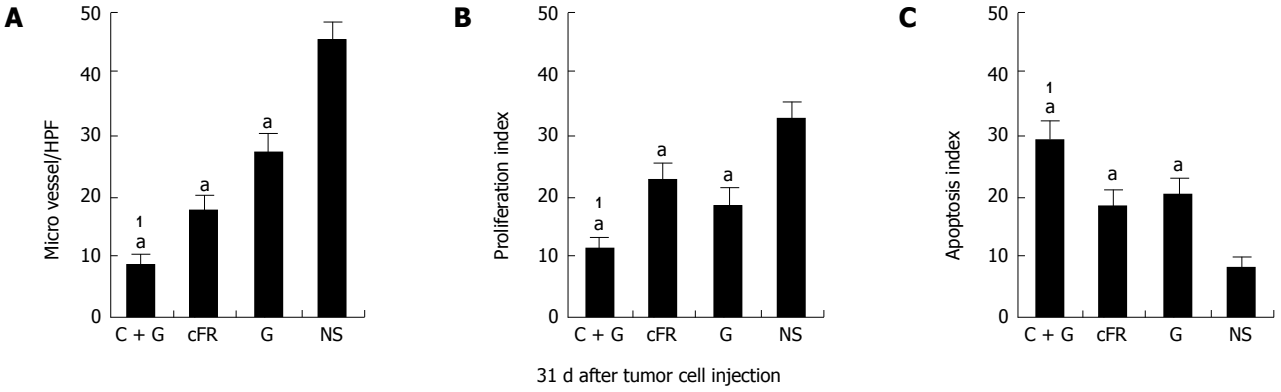

Figure 4 The combination therapy synergistic inhibition of angiogenesis (**A**) and proliferation (**B**), and induction apoptosis (**C**) at d 31 after tumor cell injection. [1]Synergistic relationship in the combination therapy (synergistic index > 1). [a]$P < 0.05$ *vs* untreated group.

compared with those in the non-immunized groups (Figure 3C). Figure 3C shows that the number of APBCs was not different between the vaccine alone and combination treatment groups, which suggested that the low-dose gemcitabine scheme did not inhibit the immune response to cFR-1 immunization.

Synergistic effects of the combined therapy on microvessel density, apoptosis and cell proliferation

In this study, the combination treatment resulted in more significant synergistic suppression of tumor growth than treatment with the single agents individually. MVD was determined in tumor tissue sections stained with antibody reactive to CD31, cell apoptosis was evaluated by TUNEL, and cell proliferation was assayed by the presence of PCNA (Figure 4). About 31 d after tumor cell transplantation, the synergistic indexes of tumor volume, MVD, cell apoptosis and proliferation in the combination therapy group were 1.71 *vs* 1.15 *vs* 1.11 and 1.04, respectively (Table 1).

Tolerability and side effects of the combined therapy

In this study, potential toxicity in gross measures was not observed in either the combination or single agent groups. The gross measures include such things as ruffling of fur behavior, body weight and life span. It should be noted that there was less feeding in mice treated with gemcitabine, which was both minor and transient, compared to those that did not receive chemotherapy. In addition, no pathologic changes in liver, lung, kidney or heart tissue sections, which were stained with hematoxylin and eosin (HE), were observed by microscopic examination (Figure 5). Furthermore, no detectable toxicity of bone marrow, kidney or liver was found by complete blood count and enzyme analyses (data not shown).

DISCUSSION

Angiogenesis is critical to the growth and metastasis of a tumor. Due to the genetic stability and accessibility to systemically delivered therapeutic agents, endothelial cells that line tumor blood vessels are attractive targets for anti-tumor therapy[5]. Since FGFR-1 is an important molecule for angiogenesis in solid tumors, as described previously, it is conceivable that breaking immune tolerance against FGFR-1-involved angiogenesis in solid tumors may be used as a useful and new approach for cancer therapy with active immunity. Some recent data have confirmed that xenogeneic homologous molecules can induce a cross-immunity reaction against self-homologous molecules that is responsible for anti-tumor activity[14,25,26]. Although anti-angiogenic therapy has proven to be effective at stopping tumor growth in many preclinical studies, it remains uncertain whether it is tumoricidal. Many studies have also concluded that this therapeutic limitation may be overcome by using a combination of angiogenic inhibitors with various chemotherapeutic drugs, such as cisplatin, gemcitabine, oxaliplatin, *etc*[16-20]. Thus, the strategy of combining anti-angiogenic biotherapy with chemotherapeutic drugs shows potential and promise for

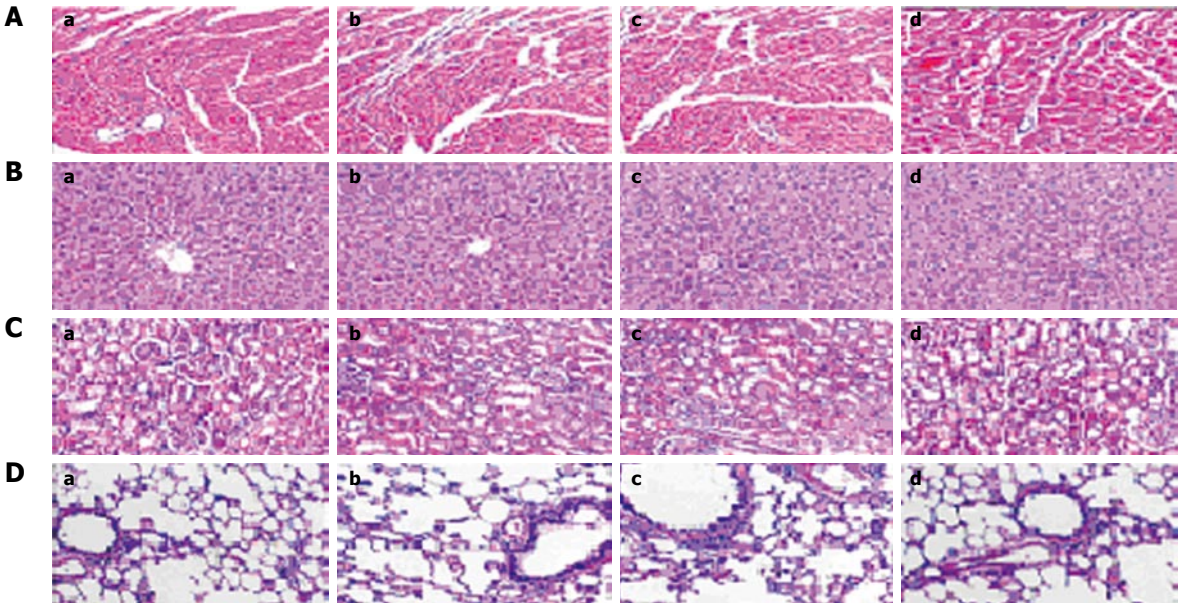

Figure 5 HE staining of heart (**A**), liver (**B**), kidney (**C**) and lung (**D**) in recipient mice. No organic hemorrhage appeared in the combination therapy group and no differences were found among of C + G (**a**), cFR (**b**), G (**c**), and NS groups (**d**).

anti-tumor therapy.

Both acquired drug resistance and considerable systemic toxicities are major reasons for the limited advances made in cancer chemotherapy and have resulted in the failure of treatments. Gemcitabine is a new deoxycytidine analog that has been widely applied in clinical anti-tumor therapy. Many studies have also confirmed that the combination of anti-angiogenic biotherapy with low-dose gemcitabine can suppress tumor growth more effectively than conventional chemotherapy or anti-angiogenic biotherapy alone, including reversal of acquired drug resistance and minimization or elimination of systemic toxicity[17,20]. The purpose of our study was to evaluate the anti-tumor efficacy of cFR-1 protein vaccine combined with low-dose gemcitabine and the potential toxicity of the treatments in a mice colorectal cancer model.

Our present studies demonstrate that the combination strategy resulted in more effective inhibition of tumor growth, not only by induction of more effective anti-angiogenesis, but also by promotion of apoptosis and up-regulation of the suppression of cell proliferation in tumor tissues as compared with either therapy alone or with untreated groups, without obvious side-effects. The mechanism responsible for the interaction between cFR-1 vaccine and low-dose gemcitabine therapy may involve a synergistic anti-angiogenic effect and synergistic apoptosis and proliferation of tumor cells. On the one hand, the immunotherapy with cFR-1 vaccine could induce a special anti-tumor immunity reaction through induction of the production of auto-antibodies against FGFR-1, which could block bFGF/FGFR-1 signal transduction and further inhibit tumor growth by anti-angiogenesis. On the other hand, low-dose gemcitabine therapy could interfere with DNA synthesis and induce DNA breakage, thus resulting in tumor cell apoptosis[20-22]. Without acquisition of necessary oxygen and nutrients, there would be an increase in tumor cell apoptosis coupled with less proliferation and tumor angiogenesis. Moreover, the low-dose gemcitabine did not inhibit the host cross-immune response, but it potentiated anti-tumor effects as was demonstrated in the synergistic indexes of tumor volume, MVD, apoptosis and proliferation, and the presence of both antibodies and APBCs in the cFR-1-immunized mice which indicate gemcitabine has an anti-tumor effect. Therefore, the combination therapy strategy showed effective and synergistic anti-tumor activity.

In conclusion, our findings demonstrated that the combination therapy strategy of cFR-1 vaccine combined with low-dose metronomic gemcitabine effectively and synergistically suppressed tumor growth *via* inhibition of tumor angiogenesis without systemic toxicity in mice.

REFERENCES

1 **Hawk ET**, Levin B. Colorectal cancer prevention. *J Clin Oncol* 2005; **23**: 378-391
2 **Li M**, Gu J. Changing patterns of colorectal cancer in China over a period of 20 years. *World J Gastroenterol* 2005; **11**: 4685-4688
3 **Coluccia AM**, Benati D, Dekhil H, De Filippo A, Lan C, Gambacorti-Passerini C. SKI-606 decreases growth and motility of colorectal cancer cells by preventing pp60(c-Src)-dependent tyrosine phosphorylation of beta-catenin and its nuclear signaling. *Cancer Res* 2006; **66**: 2279-2286
4 **Folkman J**. Angiogenesis-dependent diseases. *Semin Oncol* 2001; **28**: 536-542
5 **Fan TP**, Jaggar R, Bicknell R. Controlling the vasculature: angiogenesis, anti-angiogenesis and vascular targeting of gene therapy. *Trends Pharmacol Sci* 1995; **16**: 57-66
6 **He Y**, Zhou J, Wu JS, Dou KF. Inhibitory effects of EGFR antisense oligodeox ynucleotide in human colorectal cancer cell line. *World J Gastroenterol* 2000; **6**: 747-749
7 **Powers CJ**, McLeskey SW, Wellstein A. Fibroblast growth factors, their receptors and signaling. *Endocr Relat Cancer* 2000; **7**: 165-197
8 **Chaudhuri MM**, Moscatelli D, Basilico C. Involvement of the conserved acidic amino acid domain of FGF receptor 1 in ligand-receptor interaction. *J Cell Physiol* 1993; **157**: 209-216
9 **Cross MJ**, Lu L, Magnusson P, Nyqvist D, Holmqvist K,

Welsh M, Claesson-Welsh L. The Shb adaptor protein binds to tyrosine 766 in the FGFR-1 and regulates the Ras/MEK/MAPK pathway via FRS2 phosphorylation in endothelial cells. *Mol Biol Cell* 2002; **13**: 2881-2893

10 **Udayakumar TS**, Klein RD, Maliner MS, Nagle RB, Bowden GT. Aberrant expression of fibroblast growth factor receptor-1 in prostate epithelial cells allows induction of promatrilysin expression by fibroblast growth factors. *Int J Cancer* 2001; **91**: 187-192

11 **Hori A**, Sasada R, Matsutani E, Naito K, Sakura Y, Fujita T, Kozai Y. Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Res* 1991; **51**: 6180-6184

12 **Valesky M**, Spang AJ, Fisher GW, Farkas DL, Becker D. Noninvasive dynamic fluorescence imaging of human melanomas reveals that targeted inhibition of bFGF or FGFR-1 in melanoma cells blocks tumor growth by apoptosis. *Mol Med* 2002; **8**: 103-112

13 **Wang F**, McKeehan K, Yu C, McKeehan WL. Fibroblast growth factor receptor 1 phosphotyrosine 766: molecular target for prevention of progression of prostate tumors to malignancy. *Cancer Res* 2002; **62**: 1898-1903

14 **Zheng S**, Huang F, Zheng S, Wang W, Yin H, Wu R. Vaccination with a recombinant chicken FGFR-1 bypasses immunological tolerance against self-FGFR-1 in mice. *J Huazhong Univ Sci Technolog Med Sci* 2006; **26**: 389-391

15 **Boehm T**, Folkman J, Browder T, O'Reilly MS. Antiangiogenic therapy of experimental cancer does not induce acquired drug resistance. *Nature* 1997; **390**: 404-407

16 **Klement G**, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 2000; **105**: R15-R24

17 **Bocci G**, Nicolaou KC, Kerbel RS. Protracted low-dose effects on human endothelial cell proliferation and survival in vitro reveal a selective antiangiogenic window for various chemotherapeutic drugs. *Cancer Res* 2002; **62**: 6938-6943

18 **Browder T**, Butterfield CE, Kräling BM, Shi B, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 2000; **60**: 1878-1886

19 **Sweeney CJ**, Miller KD, Sissons SE, Nozaki S, Heilman DK, Shen J, Sledge GW. The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. *Cancer Res* 2001; **61**: 3369-3372

20 **Bruns CJ**, Shrader M, Harbison MT, Portera C, Solorzano CC, Jauch KW, Hicklin DJ, Radinsky R, Ellis LM. Effect of the vascular endothelial growth factor receptor-2 antibody DC101 plus gemcitabine on growth, metastasis and angiogenesis of human pancreatic cancer growing orthotopically in nude mice. *Int J Cancer* 2002; **102**: 101-108

21 **Hertel LW**, Boder GB, Kroin JS, Rinzel SM, Poore GA, Todd GC, Grindey GB. Evaluation of the antitumor activity of gemcitabine (2',2'-difluoro-2'-deoxycytidine). *Cancer Res* 1990; **50**: 4417-4422

22 **Jiang PH**, Motoo Y, Sawabu N, Minamoto T. Effect of gemcitabine on the expression of apoptosis-related genes in human pancreatic cancer cells. *World J Gastroenterol* 2006; **12**: 1597-1602

23 **Zheng SJ**, Huang FY, Zheng SP, Wang W, Wu RL. Construction and identification of recombinant plasmid encoding extracellular domain of FGFR-1 in chicken. *Huazhong Kejidaxue Xuebao Yixueban* 2006, **35**: 433-435

24 **Wild R**, Ramakrishnan S, Sedgewick J, Griffioen AW. Quantitative assessment of angiogenesis and tumor vessel architecture by computer-assisted digital image analysis: effects of VEGF-toxin conjugate on tumor microvessel density. *Microvasc Res* 2000; **59**: 368-376

25 **Gregor PD**, Wolchok JD, Turaga V, Latouche JB, Sadelain M, Bacich D, Heston WD, Houghton AN, Scher HI. Induction of autoantibodies to syngeneic prostate-specific membrane antigen by xenogeneic vaccination. *Int J Cancer* 2005; **116**: 415-421

26 **Srinivasan R**, Wolchok JD. Tumor antigens for cancer immunotherapy: therapeutic potential of xenogeneic DNA vaccines. *J Transl Med* 2004; **2**: 12

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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VIRAL HEPATITIS

High expression of hepatitis B virus based vector with reporter gene in hepatitis B virus infection system

Shi-Hong Li, Wen-Ge Huang, Bing Huang, Xi-Gu Chen

Shi-Hong Li, Wen-Ge Huang, Xi-Gu Chen, Center of Experimental Animals, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China
Bing Huang, State KeyLaboratory of Ophthalmology, ZhongShan Ophthalmic Center, Sun Yat-sen University, Guangzhou 510060, Guangdong Province, China
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Correspondence to: Xi-Gu Chen, Center of Experimental Animals, Sun Yat-sen University, 58 Zhongshan 2 Road, Guangzhou, Guangdong 510080, China. xiguchen@163.com
Telephone: +86-20-87331393 Fax: +86-20-87331230
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Abstract

AIM: To construct a hepatitis B virus (HBV)-based vector with a reporter gene and to establish an HBV infection system to evaluate the availability of the vector.

METHODS: The HBV-based vectors with green fluorescence protein (GFP) were packaged into the liver of immunodeficient mice through transfer and helper plasmid using hydrodynamic technology. Wild type HBV (wt HBV) was provided by plasmid MC2009. Primary human hepatocytes (PHH) were isolated and infected with recombinant HBV (rHBV) or wt HBV. GFP expression was monitored by confocal and flow cytometry. HBV DNA and HBV surface antigen (HBSAg) were analyzed by PCR and ELISA.

RESULTS: 3×10^7 wt HBV copies/mL and 5×10^6 rHBV copies/mL were collected from mice serum. In the wt HBV infected group, HBV progeny was 2×10^7 copies/mL and HBSAg was 770 ng/mL. In the rHBV infected group, GFP fluorescence was detected on d 3 post-infection and over 85% of the parenchymal cells expressed green fluorescence on d 12 post-infection. Compared with wt HBV in the PHH infection system, no rHBV DNA or HBSAg were detected in PHH culture media.

CONCLUSION: An effective HBV based vector was developed, which proved to be a useful HBV infection system. This vector and infection system can be applied to develop a therapeutic vector and study the HBV life cycle and viral pathogenesis.

Key words: Hepatitis B virus; Primary human hepatocyte; Transfer plasmid; Helper plasmid; Infection system

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INTRODUCTION

The hepatitis B virus (HBV) belongs to the family Hepadnaviridae and is the smallest DNA virus known[1]. The HBV genome consists of a partially double-stranded, relaxed circular DNA, which has a compact organization. It employs widely overlapping open reading frames and regulatory sequences[2].

Despite the availability of a safe and efficient HBV vaccine, HBV is still a major cause of infectious liver disease throughout the world. While the majority of acutely infected adults recover from this disease, chronic HBV infections remains a major public health problem, affecting more than 350 million people worldwide[3]. Systemic administration of interferon has been the best available therapy for chronic HBV infections for the past 2 decades; however, a sustained response is achieved in only one-third of affected patients[4]. Currently, nucleoside analogues, such as lamivudine and adefovir, are new treatment options that result in a rapid decrease in serum HBV DNA, as well as an improvement of liver histology. Unfortunately, these agents are associated with a rapid rate of relapse and selection of resistant viral variants[5,6].

As such, novel therapeutic alternatives are continually being explored. One of the more attractive therapeutic approaches involves the development of methods that permit the selective delivery of genes into parenchymal cells of the liver. While hydrodynamic injection can result in a high level of transgene expression in the liver, the combined effect of a large injection volume and high injection speed can result in irregular hearty function[7]. Further, few of the established viral vectors can specifically target hepatocytes[8]. Because HBV is capable of specifically targeting hepatocytes after inoculation into the bloodstream and efficiently infects quiescent hepatocytes, human HBV as liver-directed gene transfer has been pursued for more than one decade. Recently, the ability of recombinant HBV (rHBV) and duck HBV to serve as vectors for a hepatocyte-specific gene transfer has

been demonstrated[9,10].

An appropriate and effective *in vitro* infection system that permits evaluation of antiviral drugs and the study of the kinetics of the hepadenaviral infection, such as replication and viral resistance, is very important yet unavailable to date. This stems from the inability to grow HBV in culture and the absence of a convenient and reliable *in vitro* system to efficiently study different mutations in the various stages of HBV replication and their influence on viral pathogenesis.

Hepadnaviruses infect only the well-differentiated primary hepatocytes of their specific hosts in cell culture[11]. Several cell modes based on HBV-related hepadnaviruses, such as Pekin duck and Tupaia primary hepatocytes, are presently available to assess antiviral drugs, and to provide information about factors involved in the establishment of HBV infection. Duck HBV (DHBV) is a well characterized model system of hepadnaviral infection, and cultures of primary duck hepatocytes (PDHs) can be readily established and efficiently infected[12]. There are, however, significant differences between duck and human hepatitis viruses. Most importantly perhaps is the fact that, human HBV envelope polypeptides, the likely mediators of entry, are N-glycosylated, whereas DHBV envelope polypeptides are not. Thus, the degree to which information from DHBV applies to human HBV infection may be limited[13].

Primary tupaia hepatocytes are susceptible to infection with serum-derived HBV. The HBV infection of primary tupaia hepatocytes develops slowly, and the virus life cycle is not the same as HBV in human hepatocytes[14,15]. Hepatoma cell lines such as Huh7 and HepG2.2.15, and the HepaRG cell line are known to be non-susceptible to HBV infection but permit viral replication after artificial import of the viral genome (e.g. transfection of cloned HBV DNA)[16-18]. Drawbacks to these transient transfections, inefficient transfection and thereby produce low virus yields that unavoidably vary from experiment to experiment, since only a fraction of the cells are transfected. This leaves a high background of non-producer cells, and virus expression ceases within a few days[19]. Although they are difficult to maintain in culture and become non-permissive for HBV very quickly after plating, primary human hepatocytes (PHH) are susceptible to infection with HBV[20].

In light of the above-described challenges and clinical importance of studying HBV, the purpose of this study was to establish an effective HBV infection system including standard virus origin, and to construct the HBV based vector with a reporter gene capable of targeting hepatocytes naturally with high expression without expressing viral DNA and protein. Ultimately, this study will lay the foundation for the construction of a therapeutic HBV vector and will permit the intensive investigation of the HBV life cycle and viral pathogenesis.

MATERIALS AND METHODS

Animals

NOD/SCID mice were purchased from the Animal center of Sun Yat-Sen University (GuangZhou, China). All animals were maintained under specific pathogen-free conditions on alternating 12 h light/dark cycles. Food and water were available ad libitum.

Isolation and culture of primary human hepatocytes (PHH)

PHH were isolated from fresh surgical specimens of patients undergoing partial hepatectomy, with the informed consent of each patient and the local ethics committee. In all cases, patients were negative for HBV, hepatitis C virus serologic markers and specific HBV-DNA sequences (determined by PCR). Healthy human liver tissue samples (3 cm × 3 cm × 2 cm) were placed on ice in Dulbecco's modified Eagle's medium (DMEM) solution with 1% Gentamycin (Sigma). Immediately after collection, the liver tissue was treated by a two-step collagenase perfusion procedure[21]. Briefly, perfusion was initiated with the preperfusion solution of D-Hanks balanced salt solution (Sigma) containing 0.5 mmol/L ethylene-glycoltetraacetic acid (EGTA, Sigma) and 50 mmol/L HEPES heated to 37℃ for 15 to 25 min at a flow rate of 20-40 mL/min, then, continued with perfusion solution of F12 medium-DMEM (Invitrogen) containing 0.05% collagenase type Ⅳ (Gibco) and 5 mmol/L $CaCl_2$ for 15-25 min at a flow rate of 25-50 mL/min. After gentle mechanical dissociation, the PHH were washed and separated by three successive centrifugations at 50 × *g*, for 5 min each. The PHH were subsequently resuspended in DMEM medium and viability was assessed by trypan blue exclusion. After isolation, 1.0×10^5 viable cells/cm^2 were seeded onto collagen-coated culture plates (collagen type I, sigma) in medium containing 8% fetal calf serum (FCS) and maintained at 37℃ with 5% CO_2. Cell culture media were supplemented with L-glutamine (5 mmol/L), HEPES (23 mmol/L, pH 7.4), gentamycin (50 mg/mL), penicillin (50 IU/mL, sigma), streptomycin (50 mg/mL, sigma), hydrocortisone (4.8 mg/mL, sigma) and insulin (1 mg/mL, sigma). Culture medium was changed at 3 h, then 12 to 16 h after seeding. Medium was changed every 24 to 48 h thereafter, but without the addition of serum[20].

Plasmid constructs

The DNA vector MC2009 contains 2 tandem copies of the HBV genome by head to tail connection (Figure 1B), This vector will produce wild-type (wt) HBV virus capable of infection after injection into mouse liver, or delivered to cultures of human hepatocytes. This vector can be used to generate serum with a high titer of HBV to serve as a standard for this project.

The DNA vector MC2018, the helper plasmid (Figure 1C), is capable of expressing all HBV proteins, including the large, middle, and small envelope proteins, the core protein, and the polymerase. The DNA sequence is the same as that in MC2009, except that it contains only one copy of the HBV genome with a deletion of the encapsidation signal ε and direct repeats necessary for reverse transcription and be derived by an ubiquitin promoter (UB). Thus, the DNA vector MC2018 can only provide HBV proteins needed for packaging in trans, but is not able to generate a complete HBV virus[22].

DNA vector MC2013, the transfer plasmid (Figure 1D), was obtained by replacing a DNA fragment containing the small envelope gene (HBV position 1446 to 2347)

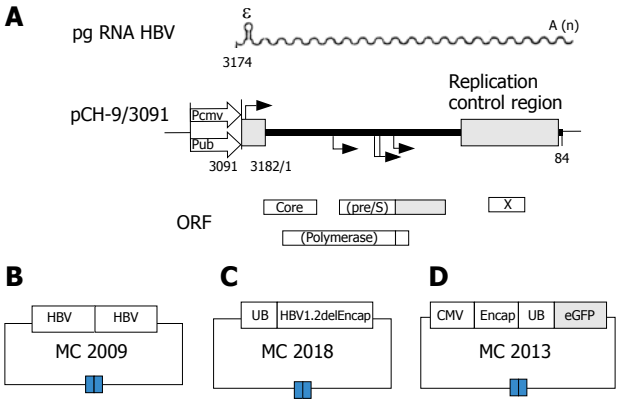

Figure 1 Schematic representation of plasmid constructs. **A**: Plasmid constructs used for the production of recombinant hepatitis B virus (rHBV)[10], (pgRNA HBV = sinusoidal line; ε = encapsidation signal; A (n) = poly A tail; CMV = cytomegalovirus; UB= ubiquitin); **B**: The vector MC2009 contains 2 tandem copies of HBV genome; **C**: The transfer plasmid DNA vector MC2013 obtained by replacing a DNA fragment containing the small envelope gene (HBV position 1446 to 2347) with a PCR fragment encoding a fluorescence-enhanced green fluorescence protein (eGFP), meanwhile, a DNA fragment encompassing the HBV preS2/S promoter was substituted by a PCR fragment encoding UB promoter which could drive transgene expression. A CMV promoter was introduced to drive the expression of the UB.eGFP reporter expression cassette with the HBV encapsidation signal, and premature stop codons were introduced into all remaining HBV open reading frames; **D**: The helper plasmid MC2018 contains only one copy of the HBV genome with a deletion of the encapsidation signal ε or direct repeats necessary for reverse transcription. The UB promoter was introduced to drive expression of HBV 1.2delEncap.

with a PCR fragment encoding a fluorescence-enhanced green fluorescence protein (eGFP)[9]. The DNA fragment encompassing the HBV preS2/S promoter was substituted by a PCR fragment encoding a ubiquitin promoter to drive transgene expression[23]. A cytomegalovirus (CMV) promoter was introduced to drive the expression of UB, eGFP reporter expression cassette with the HBV encapsidation signal and premature stop codons were introduced into all remaining HBV open reading frames[10].

Generation of HBV virions

Five μg of plasmid MC2009 was dissolved in a volume of saline equivalent to 8% of the body mass of the target mouse (i.e. 1.6 mL for a 20 g mouse) to obtain a working solution. It was then injected into the tail vein of 6- to 9-wk-old NOD/SCID mice within 5-8 s[24]. After 1 d, blood (and ultimately serum) was collected from the mice by cutting the mouse tail. The HBV DNA copies in the mouse serum were determined by quantitative PCR assay, Mouse serum containing wild-type HBV virus was maintained at -20℃ until use.

Preparation of recombinant HBV virions

Five μg of plasmids MC2013 and MC2018 (each) was combined and diluted with saline to a volume equivalent to 8% of the body mass of the mouse. This was then injected into the tail vein of 6- to 9-wk-old NOD/SCID mice within 5-8 s[24], The number of recombinant HBV (rHBV) DNA copes was determined by quantitative PCR. The rHBV was then purified from the mouse blood and measured by a quantitative PCR assay on d 3 to 6 after injection, and stored until use.

Infection of PHH with HBV and rHBV

On d 2 post-seeding, PHH cultures were incubated with wt HBV or rHBV. After infecting cultures overnight, the PHH cells were washed three times, every third day, and the culture medium was exchanged and collected for virus product analysis to test the HBV infection of PHH *in vitro*, To detect the susceptibility of PHH to HBV, PHH cultures were treated on d 12 post-seeding (using the same method).

Detection of transgene expression

GFP expression in PHH incubated with rHBV or wt HBV were detected by confocal microscopy (Leica, Germany) using a standard fluorescein isothiocyanate (FITC) filter set with excitation by blue light (488 nm) on d 3, 6 and 12 post-infection.

Evaluation of the efficiency of transgene expression

To determine the efficiency of transgene delivery, PHH cultures were analyzed by flow cytometry (BD FACSAria™ US) on d 12 after seeding. Liver parenchymal cells with transgene expression were identified by GFP, Liver sinusoidal endothelial cells were identified by acetylated low-density lipoprotein (Alexa Fluor®594 AcLDL, Invitrogen), and kupffer cells were identified by Dextran conjugates (Carboxy-Q-Rhodamine, Invitrogen).

Analysis of viral DNA and proteins

HBV DNA in the culture medium was quantified using a quantitative PCR assay (HBV-DNA detection kit, Da'an company, China) according to the manufacturer's instructions. HBsAg in the supernatant of wt HBV-infected or rHBV-infected PHH was determined using an ELISA kit and the AxSYM system (Abbott Diagnostics, Chicago, USA) according to the manufacturer's instructions.

RESULTS

Isolation and culture of PHH

PHH were isolated from fresh surgical specimens of liver tissue. Mean cell yield was 5×10^5 cells/g liver tissue with 80% mean viability. Morphology of typical PHH cultures on d 30 post-seeding is shown in Figure 2A. PHH growth was inhibited following the addition of DMSO, but continued to grow when the DMSO was withdraw (data not shown).

Production of Recombinant HBV and Wild type HBV

MC2009 DNA vector was delivered to NOD/SCID mice using the hydrodynamic technique. On d 1 through 3 post-injection, no HBV DNA could be detected, whereas after d 3 post-injection, HBV DNA levels were 3×10^7 copies/mL.

MC2018 and MC2013 were co-injected into NOD/SCID mouse liver using hydrodynamic technology. It was subsequently determined that rHBV DNA could be detected only on d 3 to d 6 post-injection. The average amount of rHBV DNA detected was 5×10^6 copies/mL.

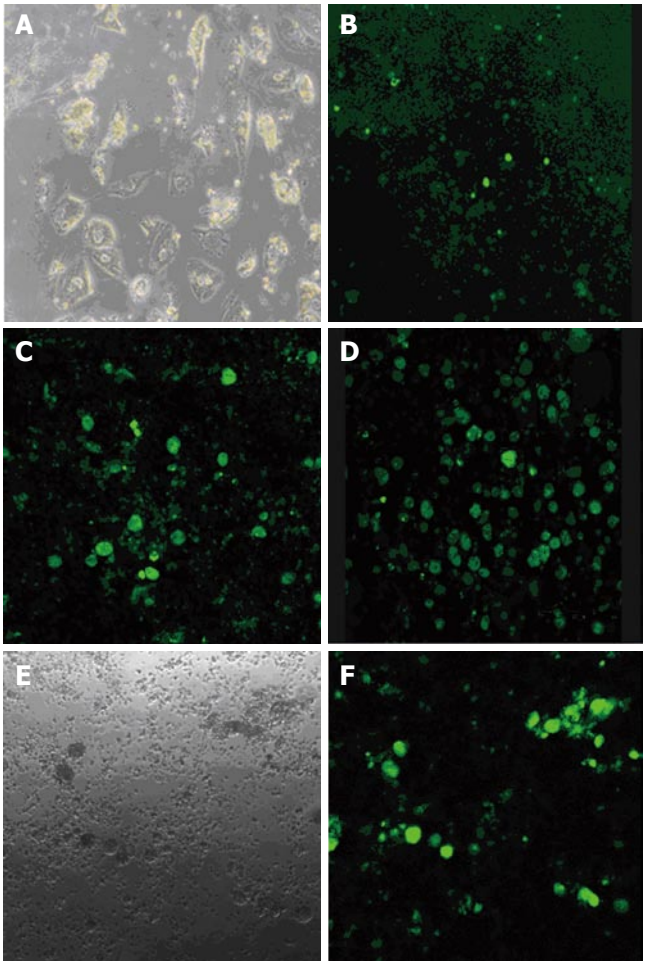

Figure 2 Transduction of primary human hepatocytes (PHH) by the HBV-based vector. PHH were infected with rHBV-GFP, a recombinant HBV that carries a GFP gene under control of the UB promoter (described previously in Figure 1). **A**: Cell morphology of PHH on d 30; **B**: GFP expression on d 3 post-infection; **C**: GFP expression on d 6 post-infection; **D**: on d 12 post-infection; **E**: Transduction of PHH demonstrated by an overlay of the fluorescence; **F**: Phase contrast of the same field.

Gene transfer in PHH by rHBV vector

Infectivity of the rHBV particles was demonstrated by incubating primary human hepatocytes with rHBV at a multiplicity of infection (moi) of 10 copies/cell for 24 h on d 2 after plating. GFP fluorescence was clearly detected on d 3 post-infection (Figure 2B), and cells expressing GFP reached its maximum on d 12 post-infection (Figure 2D). This finding indicates that the HBV-based vector that was generated can target liver cells and deliver the genetic information in a 'natural' way.

The efficiency of transgene expression

In PHH cultures, 75.2% of the cells were GFP positive (Figure 3B), 2% of the cells were identified as Kupffer cells (Figure 3C), and 10.4% of the cells were liver sinusoidal endothelial cells (Figure 3D). In addition, cultures contained small amounts of red cells and debris. Thus, nonparenchymal liver cells, which cannot be infected by HBV, accounted for only 12.4% of the total number of cells up to d 12 after seeding. Of the 75.2% GFP-expressing cells, over 85% of parenchymal liver cells were infected by the rHBV, and stable GFP expression was

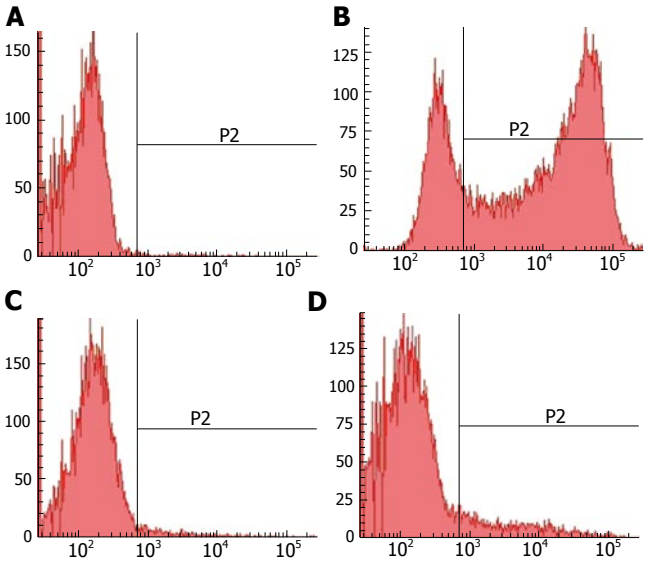

Figure 3 PHH were detected by FACS. The purity of PHH cultures was analyzed by identification of nonparenchymal and parenchymal liver cells on d 6 after seeding. **A**: Control; **B**: FACS analysis of parenchymal liver cells with GFP; **C**: FACS analysis of kupffer cells with Dextran conjugates; **D**: FACS analysis of liver sinusoidal endothelial cells (LSEC) with acetylated low-density lipoprotein. The population consisted of 10.4% AcLDL positive cells, 2% Carboxy-Q-Rhodamine positive cells and 75.2% GFP positive cells, meaning that total parenchymal cells is about 87.6%, approximately 85% of it were infected by HBV based vector with GFP.

observed for up to 4 wk. These results strongly suggest that the HBV-based vector has high infectivity to PHH and a high efficiency of transgene expression.

Analysis the kinetics of HBV or rHBV infection in PHH

PHH cultures were incubated with mouse serum containing wt HBV or rHBV at a moi of 10 copies/cell on d 2 after plating. The cell culture media was collected every 3 d post-infection. In the wt HBV infected group, wt HBV DNA copies were 1×10^3 to 2×10^7 copies/mL (Figure 4A) and HBV surface antigen (HBSAg) was 175 to 770 ng/mL (Figure 4B). In contrast, rHBV DNA copies were 1×10^1 to 0 (Figure 4A) and HBSAg was undetectable at all times (Figure 4B). Collectively, these results suggest that the PHH system can be infected by wt HBV (with a time constraint), and that the rHBV vector cannot express HBV products in PHH.

DISCUSSION

Although PHH are difficult to maintain in culture for HBV infection, PHH are undoubtedly the ideal tool for studying HBV infection and hepatotropic vectors. In the present report, it was demonstrated that: (1) PHH cultures, maintained under optimized PHH culture conditions, can serve as a useful infection system for HBV *in vitro*; (2) HBV from MC2009 are useful for HBV infection studies; and (3) the constructed HBV-based vectors described herein is highly efficient and safe.

The fundamental prerequisites for the viral life-cycle include the ability to replicate the genome, and to produce infectious progeny virions. At present, the major limitation in studying HBV entry is the lack of an *in vitro* infection

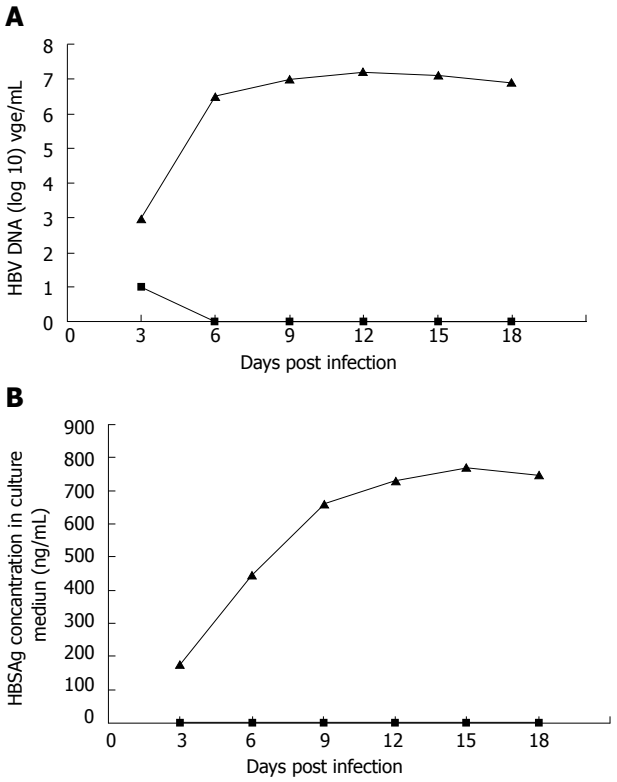

Figure 4 The efficiency of PHH infected with wt HBV or rHBV. PHH were infected at a multiplicity of infection of 10 DNA-containing HBV particles/cell on d 1 post-seeding, HBV DNA (**A**), and HBSAg (**B**) secreted into cell culture medium were determined every 3 d after infection. The triangles denote PHH cells infected with wt HBV, and squares denote PHH cells infected with rHBV. In the supernatant from PHH infected with rHBV, no HBV DNA and HBsAg can be detected, meaning that all viral gene were knocked out.

system capable of supporting the entire life cycle of HBV. Several systems derived from hepatoma cells have previously been used to study HBV infections *in vitro*[16-18]. Unfortunately, none of the described systems have proven ideal. HPP cultures derived from liver explants have shown to be susceptible to HBV infection, but infection occurs in only 10% to 20% of the cells[25]. Further, infection of PHH cultures is only for a limited amount of time following explantation[11,13].

In this study, PHH were isolated from small pieces of healthy liver tissue from patients and maintained under optimized culture conditions. The PHH cultures survived up to 10 wk. wt HBV DNA reached the level of 2×10^7 copies/mL, and HBSAg reached 770 ng/mL in PHH cultures infected with wt HBV. Over 85% of parenchymal liver cells were infected by rHBV. In previous PHH systems and some hepatoma cell systems[18,20], high dose DMSO (1.5%-2%) was used to enhance HBV infection *in vitro*. In the present study, it was found that growth of PHH cultures was seriously inhibited with addition of DMSO, but continued to grow once the DMSO was withdrawn. In addition, cell survival in the culture was reduced by the addition of complement inactivated serum. In our PHH system, cells lost their susceptibility to HBV on d 12 after seeding. This suggests that the PHH system has a time constraint and is consistent with prior reports[11].

In previously described HBV infection systems[22,26], HBV virion was primarily prepared from the serum of chronic HBV patients, because this serum always carried variant virus, HBV proteins and cccDNA[27]. Since these products could falsify study results, the serum of chronic HBV patients was unsuitable for research on the HBV life cycle. The construct MC2009 could solve this problem by providing standard wild type HBV without accessory viral products.

The goal of this study was to generate hepatocyte-specific viral vectors, which only expressed the transgene at a high level of expression. In addition, we wished to completely eliminate HBV viral gene expression, and to insert foreign promoter and functional transgenes of at least 800 base pairs. In the transfer plasmid MC2013, all viral genes were knocked out and endogenous HBV preS2/S promoter was substituted by a UB promoter to drive reporter gene eGFP expression. The expression of UB. eGFP reporter expression cassette with the HBV encapsidation signal was driven by a CMV promoter. This helper plasmid can provide HBV proteins needed for packaging in trans so that the mRNA is packed into a recombinant HBV virion.

In PHH incubated with this rHBV virion, nearly 85% of parenchyma cells expressed GFP on d 12 post-infection. The data from this study demonstrated that the HBV based vector can specifically infect liver cells, and provides direct evidence that gene transfer into PHH by a HBV vector is possible. The data presented here go much beyond earlier studies reporting the efficacy of transgene expression: the use of the UB promoter leads to higher GFP expression than the original preS2/S-and CMV-promoters[9,10]. In the vector created in this study, a greater than 800 bp eGFP gene was inserted, This gene can potentially be replaced with useful effector genes coding, for example, specific antisense-RNAs or RNAi, or a large number of immunomodulatory cytokines. This may result in novel means of treating HBV infections in the future. Given the high expression efficacy and larger inserts, this study lays the foundation for construction of a therapeutic HBV vector.

If rHBV is to be used as a gene transfer vector, the presence of wt HBV by homologous recombination is a major safety issue. In the HBV system created here, safety did not appear to be of concern: no HBSAg was detected, only 1×10^1 HBV DNA were detected on d 3 post-infection, and no HBV DNA was detected on or after d 6 post-infection. The identification of 1×10^1 HBV DNA on d 3 post-infection might simply be residual from the sample that was added. In the HBV system, homologous recombination was possible either between transfer and helper plasmids or in between the redundant HBV sequences of the helper plasmid[10,22]. To avoid this, all viral ORFs were knocked out in the transfer, and the terminal redundant sequences were deleted to minimize the possibility of transfer-helper recombination and to abolish helper-helper recombination.

Despite the fact that the early steps of HBV infection determine the virus-related pathogenesis, the molecular basis of the steps remains poorly understood. Infection begins with cell attachment, followed by entry and delivery of the viral genetic information to the host cell's nucleus[13]. In the absence of ideal cell and/or animal models for

studying HBV infection, data concerning the early, post-attachment steps in hepadnaviral entry are largely based on studies performed with DHBV in primary duck liver hepatocytes[28]. In the infection system developed by this research group, PHH can be infected naturally by HBV, and the HBV-based vector with reporter gene GFP can also easily enter the cell. As a result, this infection system may provide a novel means of elucidating the molecular basis of hepadnavirus infection.

In conclusion, we have established an HBV infection system using PHH cultures, and constructed a HBV-based vector with reporter gene. This HBV-based vector and HBV infection system may lay the foundation for developing a therapeutic vector and an effective tool for studying HBV life cycle and viral pathogenesis.

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REFERENCES

1 **Tiollais P**, Pourcel C, Dejean A. The hepatitis B virus. *Nature* 1985; **317**: 489-495
2 **Büscher M**, Reiser W, Will H, Schaller H. Transcripts and the putative RNA pregenome of duck hepatitis B virus: implications for reverse transcription. *Cell* 1985; **40**: 717-724
3 **Lok AS**. Chronic hepatitis B. *N Engl J Med* 2002; **346**: 1682-1683
4 **Tillmann HL**. Antiviral therapy and resistance with hepatitis B virus infection. *World J Gastroenterol* 2007; **13**: 125-140
5 **Marcellin P**. Advances in therapy for chronic hepatitis B. *Semin Liver Dis* 2002; **22** Suppl 1: 33-36
6 **Schildgen O**, Sirma H, Funk A, Olotu C, Wend UC, Hartmann H, Helm M, Rockstroh JK, Willems WR, Will H, Gerlich WH. Variant of hepatitis B virus with primary resistance to adefovir. *N Engl J Med* 2006; **354**: 1807-1812
7 **Zhang G**, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, Dean DA, Liu D. Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther* 2004; **11**: 675-682
8 **Nash KL**, Jamil B, Maguire AJ, Alexander GJ, Lever AM. Hepatocyte-specific gene expression from integrated lentiviral vectors. *J Gene Med* 2004; **6**: 974-983
9 **Protzer U**, Nassal M, Chiang PW, Kirschfink M, Schaller H. Interferon gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus infection. *Proc Natl Acad Sci USA* 1999; **96**: 10818-10823
10 **Untergasser A**, Protzer U. Hepatitis B virus-based vectors allow the elimination of viral gene expression and the insertion of foreign promoters. *Hum Gene Ther* 2004; **15**: 203-210
11 **Gripon P**, Diot C, Guguen-Guillouzo C. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. *Virology* 1993; **192**: 534-540
12 **Tuttleman JS**, Pugh JC, Summers JW. *In vitro* experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. *J Virol* 1986; **58**: 17-25
13 **Lu X**, Block T. Study of the early steps of the Hepatitis B Virus life cycle. *Int J Med Sci* 2004; **1**: 21-33
14 **Luscombe C**, Pedersen J, Uren E, Locarnini S. Long-term ganciclovir chemotherapy for congenital duck hepatitis B virus infection *in vivo*: effect on intrahepatic-viral DNA, RNA, and protein expression. *Hepatology* 1996; **24**: 766-773
15 **Köck J**, Baumert TF, Delaney WE, Blum HE, von Weizsäcker F. Inhibitory effect of adefovir and lamivudine on the initiation of hepatitis B virus infection in primary tupaia hepatocytes. *Hepatology* 2003; **38**: 1410-1418
16 **Rabe B**, Glebe D, Kann M. Lipid-mediated introduction of hepatitis B virus capsids into nonsusceptible cells allows highly efficient replication and facilitates the study of early infection events. *J Virol* 2006; **80**: 5465-5473
17 **Guo Y**, Guo H, Zhang L, Xie H, Zhao X, Wang F, Li Z, Wang Y, Ma S, Tao J, Wang W, Zhou Y, Yang W, Cheng J. Genomic analysis of anti-hepatitis B virus (HBV) activity by small interfering RNA and lamivudine in stable HBV-producing cells. *J Virol* 2005; **79**: 14392-14403
18 **Gripon P**, Rumin S, Urban S, Le Seyec J, Glaise D, Cannie I, Guyomard C, Lucas J, Trepo C, Guguen-Guillouzo C. Infection of a human hepatoma cell line by hepatitis B virus. *Proc Natl Acad Sci USA* 2002; **99**: 15655-15660
19 **Nassal M**, Dallmeier K, Schultz U, Sun D. Phenotyping hepatitis B virus variants: from transfection towards a small animal in vivo infection model. *J Clin Virol* 2005; **34** Suppl 1: S89-S95
20 **Schulze-Bergkamen H**, Untergasser A, Dax A, Vogel H, Büchler P, Klar E, Lehnert T, Friess H, Büchler MW, Kirschfink M, Stremmel W, Krammer PH, Müller M, Protzer U. Primary human hepatocytes--a valuable tool for investigation of apoptosis and hepatitis B virus infection. *J Hepatol* 2003; **38**: 736-744
21 **Mahieu-Caputo D**, Allain JE, Branger J, Coulomb A, Delgado JP, Andreoletti M, Mainot S, Frydman R, Leboulch P, Di Santo JP, Capron F, Weber A. Repopulation of athymic mouse liver by cryopreserved early human fetal hepatoblasts. *Hum Gene Ther* 2004; **15**: 1219-1228
22 **Klöcker U**, Oberwinkler H, Kürschner T, Protzer U. Presence of replicating virus in recombinant hepadnavirus stocks results from recombination and can be eliminated by the use of a packaging cell line. *J Virol* 2003; **77**: 2873-2881
23 **Udagawa T**, Puder M, Wood M, Schaefer BC, D'Amato RJ. Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB J* 2006; **20**: 95-102
24 **Tada M**, Hatano E, Taura K, Nitta T, Koizumi N, Ikai I, Shimahara Y. High volume hydrodynamic injection of plasmid DNA via the hepatic artery results in a high level of gene expression in rat hepatocellular carcinoma induced by diethylnitrosamine. *J Gene Med* 2006; **8**: 1018-1026
25 **Walter E**, Keist R, Niederöst B, Pult I, Blum HE. Hepatitis B virus infection of tupaia hepatocytes *in vitro* and *in vivo*. *Hepatology* 1996; **24**: 1-5
26 **Glebe D**, Aliakbari M, Krass P, Knoop EV, Valerius KP, Gerlich WH. Pre-s1 antigen-dependent infection of Tupaia hepatocyte cultures with human hepatitis B virus. *J Virol* 2003; **77**: 9511-9521
27 **Wong DK**, Yuen MF, Yuan H, Sum SS, Hui CK, Hall J, Lai CL. Quantitation of covalently closed circular hepatitis B virus DNA in chronic hepatitis B patients. *Hepatology* 2004; **40**: 727-737
28 **Stoeckl L**, Funk A, Kopitzki A, Brandenburg B, Oess S, Will H, Sirma H, Hildt E. Identification of a structural motif crucial for infectivity of hepatitis B viruses. *Proc Natl Acad Sci USA* 2006; **103**: 6730-6734

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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BASIC RESEARCH

Relationship of quantitative structure and pharmacokinetics in fluoroquinolone antibacterials

Die Cheng, Wei-Ren Xu, Chang-Xiao Liu

Die Cheng, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China
Die Cheng, Wei-Ren Xu, Chang-Xiao Liu, National Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research, Tianjin 300193, China
Supported by the National Basic Research Program of China, No. 2004BC518902
Correspondence to: Chang-Xiao Liu, National Key Laboratory of Pharmacokinetics and Pharmacodynamics, Tianjin Institute of Pharmaceutical Research, 308 An-Shan West Road, Tianjin 300193, China. liuchangxiao@163.com
Telephone: +86-22-23006863 Fax: +86-22-23006863
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Abstract

AIM: To study the relationship between quantitative structure and pharmacokinetics (QSPkR) of fluoroquinolone antibacterials.

METHODS: The pharmacokinetic (PK) parameters of oral fluoroquinolones were collected from the literature. These pharmacokinetic data were averaged, 19 compounds were used as the training set, and 3 served as the test set. Genetic function approximation (GFA) module of Cerius^2 software was used in QSPkR analysis.

RESULTS: A small volume and large polarizability and surface area of substituents at C-7 contribute to a large area under the curve (AUC) for fluoroquinolones. Large polarizability and small volume of substituents at N-1 contribute to a long half life elimination.

CONCLUSION: QSPkR models can contribute to some fluoroquinolones antibacterials with excellent pharmacokinetic properties.

Key words: Quantitative structure pharmacokinetic relationship; Genetic function approximation; Fluoroquinolones; Elimination half life

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INTRODUCTION

H pylori is generally considered to be the most important cause of peptic ulcer diseases, gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach[1]. The widespread use of antibacterial therapy is suggested to be the cause for the decline in the prevalence of *H pylori* infection[2]. Among the different types of antibacterial agents, the effects of fluoroquinolones are better and have attracted much attention. Unfortunately, complete eradication of *H pylori* is still in the initial stage, especially in South East Asia and Southern Europe, where resistance to antibiotics has become more prevalent[3]. It is therefore important to search for better antibacterial agents against resistant *H pylori* strains[4].

Successful drugs must have suitable properties in toxicity, bioavailability and pharmacokinetic parameters. Screening of a large number of compounds with excellent absorption, distribution, metabolism, and excretion (ADME) properties is time-consuming and expensive[5]. So the extension of the idea of quantitative structure-activity relationship to the pharmacokinetics has led to the emergence of a new tool called the quantitative structure pharmacokinetic relationship (QSPkR) studies. QSPkR studies can be utilized at early stages of drug design. Both one- and two-dimensional topological indices have been used extensively to numerically relate molecular structure with activity[6]. These descriptors rely only on the molecular graph for their calculation. In contrast, three-dimensional descriptors require the absolute conformation of a molecule, and have been successfully used to develop QSPkR analysis[7].

The QSPkR models integrated properties of chemical structures (e.g. LogP) and their pharmacokinetic parameters (total clearance, distribution volume, *etc.*) of fluoroquinolones have been reported[8]. But these existing models cannot demonstrate the influence of the substituents to pharmacokinetic parameters. That is to say, these models can only predict pharmacokinetic parameters of the existing chemicals.

After examining the structures of all marketed fluoroquinolones, we found that their diversities in structures were mainly within R1 and R7 (Figure 1). Considering the connections between the groups (R1 and R7) and matrix were single bonds, the conjugations between groups and matrix were limited, and the groups had relatively independent properties. To simplify the design for high efficiency in practice, the properties of fragments were

applied as the descriptors of calculation. In this study, a two-step process was used to develop QSPkR models clinically using fluoroquinolone antibacterials. The first step was to calculate properties related to chemical structures and their conformation, especially constituent structures. These properties include 2D descriptors representing physical properties (logP), 3D descriptors (volume), and quantum chemical parameters (polarizability). After calculating these properties, the QSPkR models were developed by multivariate linear regression based on genetic algorithms.

Using these QSPkR models, we can illustrate how the changes at N-1 and C-7 of the fluoroquinolones affect their pharmacokinetic parameters. Hopefully, these QSPkR models can contribute to some fluoroquinolones with excellent pharmacokinetic properties.

MATERIALS AND METHODS

Molecules

All 22 compounds used in this study are analogues of the fluoroquinolone antibacterials which are widely used clinically except DW116 (No.5). The matrix of the compounds is shown in Figure 1, and their detailed substituents are listed in Figure 2.

Pharmacokinetic data

The PK parameters of these fluoroquinolones were collected from literature[9-68]. Data were taken from the studies of oral fluoroquinolones. These pharmacokinetic data were averaged after AUC and Cmax data were normalized by 100 mg of drugs (Table 1). $t_{1/2}$ in this paper is elimination half life, it is also known as $t_{1/2(\beta)}$. Nineteen compounds were used as the training set, and the others served as test set.

Molecular descriptors

The 3D structure of each compound was constructed by HyperChem 7.0 (Hypercube Inc., USA) and then optimized with MM+ force field. All molecules were aligned by minimizing the rms distance of their matrix by SYBYL 7.0 (Tripos Inc., 2004). The alignment of molecules is displayed in Figure 3. The descriptors were calculated for substituents R1 and R7 by HyperChem 7.0. The definitions of all descriptors are shown in Table 2.

QSPkR calculation

The logarithmic values of the PK parameters were used as the dependent variables. All the descriptors were scaled by the mean values of data from the training set .

The models related to three dependent variables [ln(AUC), $\ln(t_{1/2})$ and $\ln(C_{max})$] and 14 independent variables were built respectively according to the data of the training set. To obtain a high quality of QSPkR models, genetic algorithms (GA) and partial least squares analysis (PLS) were used in calculation. The calculation was conducted with the QSAR module of Cerius^2 (Accelrys Software Inc.) molecular modeling software.

We selected three and four independent variables to search their best models. QSPkR analysis based on GA began with a population of random models. These models

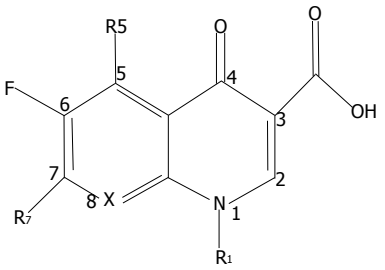


Figure 1 The matrix of all fluoroquinolone antibacterials.

were generated by randomly selecting three or four features from the data file. Product of multiple linear regression coefficient and leave-one-out cross-validation coefficient was used as a fitness function to generate the fitness scores of these models. For this data set 200 populations were used, and the number of elite populations was 100. The genetic operator was applied until the total fitness score of the elite populations could not be improved over a period of 30 crossover operations. The convergence criteria was met after 430 operations for four features and 280 operations for three features.

The parameters like correlation coefficient (R), variance ratio (F), lack of fit (LOF) scores and leave-one-out cross-validation coefficient (S) were also computed for the suitability of fitness.

The data of the left test set were then predicted by these models.

RESULTS

Calculation of descriptors

The descriptors were calculated for substituents R1 and R7 by HyperChem 7.0. And their values are displayed in Tables 3 and 4.

Fitted models

The GA calculation gave 100 models for each pharmacokinetic parameter. The models with the best fitness are listed in Table 5. Results showed that GA was a powerful tool to find the best models. Maximum R^2 of models based on ln(Cmax) was only 0.327. Therefore, these models might not be significant. That is to say these 14 descriptors were not correlated with Cmax.

All the predicted and observed data of ln(AUC) and $\ln(t_{1/2})$ from the training set are displayed in Figure 4.

DISCUSSION

We normalized the data of all the descriptors before model construction, making the coefficient of all the descriptors comparable in the same model. In the model based on AUC, coefficient of V7 was the largest and negative, and that of HE7 was quite small, suggesting that the substituents at position 7 are very significant to AUC, and small volume, large polarizability and large surface area substituents at C-7 are preferred, while hydration energy has little influence on AUC.

In fact, compounds with relatively small volume and large polarizability and surface area of substituents at C-7

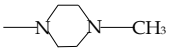
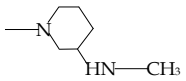
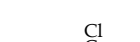
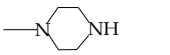
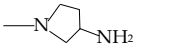
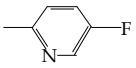
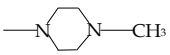
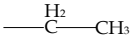
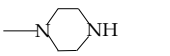
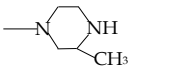
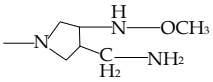
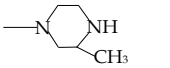
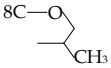
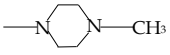
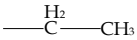
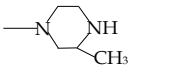
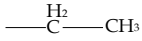
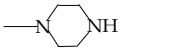
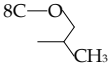
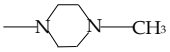
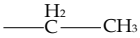
Compounds No.	Name	R1	R5	R7	X
1	Amifloxacin		—H		
2	Balofloxacin		—H		
3	Ciprofloxacin		—H		
4	Clinafloxacin		—H		
5	DW116		—H		
6	Enoxacin		—H		
7	Gatifloxacin		—H		
8	Gemifloxacin		—H		
9	Grepafloxacin		—CH_3		
10	Levofloxacin		—H		
11	Lomefloxacin		—H		
12	Norfloxacin		—H		
13	Ofloxacin		—H		
14	Pefloxacin		—H		
15	Rufloxacin		—H		
16	Sitafloxacin		—H		
17	Sparfloxacin		—NH_2—		
18	Temafloxacin		—H		
19	Trovafloxacin		—H		
20	Difloxacin		—H		
21	Fleroxacin		—H		
22	Tosufloxacin		—H		

Figure 2 The substituents of fluoroquinolone antibacterials.

(Table 4) all had relatively large AUC (Table 1). Although compounds 3, 4, 12 and 22 (Table 4) had substituents at C-7 with very small volume, their AUCs were all small(Table 1) because of extremely small polarizability and surface area (Table 4), suggesting that coefficient of V7 is not the definitive factor to affect AUC. Volume, polarizability and surface area of R7 determined AUC, and small volume, large polarizability and large surface area of substituents at C-7 were of benefit to large AUC. It is coincident with the results of coefficients in the AUC-based model.

In the $t_{1/2}$-based model, coefficient of V1 was the

Table 1 Pharmacokinetic data of fluoroquinolones from human studies

Compounds		PK parameters						References
		[1]$AUC_{0-\infty}$ (μg·h/mL)		[1]$t_{1/2}$ (h)		[1]Cmax (mg/L)		
No.	Name	Range	Average	Range	Average	Range	Average	
				Training set				
1	Amifloxacin	5.5-5.62	5.57	3.58-4.83	4.14	0.9-1.26	1.14	9, 10
2	Balofloxacin	8.55	8.55	7.8	7.8	1.08	1.08	11
3	Ciprofloxacin	2.12-3.53	2.56	3.01-4.7	4.16	0.4-0.69	0.56	12-15
4	Clinafloxacin	4.63-5.93	5.34	5.09-6.13	5.74	0.6-0.84	0.72	16-18
5	DW116	18.54-23.3	21.86	14.53-18.7	15.82	1.1-1.22	1.17	19
6	Enoxacin	2.9-5.47	4.36	2.35-4.98	3.54	0.62-0.81	0.66	20-22
7	Gatifloxacin	6.5-8.92	7.87	6.52-8.6	7.46	0.84-1.03	0.9	23-26
8	Gemifloxacin	2.79-3.43	3.02	5.87-8.2	6.65	0.46-0.73	0.56	27-29
9	Grepafloxacin	2.83-4.05	3.43	9.2-12.7	11.53	0.24-0.41	0.32	11, 12, 30, 31
10	Levofloxacin	8.96-9.5	9.33	6-7.4	6.78	0.16-0.3	0.24	32-34
11	Lomefloxacin	8.05-13.53	9.84	5.5-12.7	7.73	0.95-1.18	1.06	35-37
12	Norfloxacin	1.7-1.85	1.77	3.5-4.02	3.7	0.32-0.36	0.33	38-40
13	Ofloxacin	6.68-11.64	7.67	4.6-6.7	5.32	0.71-1.33	0.87	41-45
14	Pefloxacin	24.4-40.78	29.97	10.9-15.06	14.63	1.03-1.68	1.44	46-48
15	Rufloxacin	35.8-44.03	39.43	28.2-40	34.25	0.68-1.13	0.99	49-52
16	Sitafloxacin	5.62-6.02	5.88	4.6-7	5.4	0.9-0.93	0.92	53-54
17	Sparfloxacin	8.08-11.96	8.35	16.5-25.56	20.06	0.23-0.4	0.34	55-57
18	Temafloxacin	7.42-10.63	8.45	7.8-10.6	8.55	0.61-0.9	0.74	58-60
19	Trovafloxacin	9.75-14.47	11.91	7.8-10.8	9.66	0.97-1.5	1.23	61-63
				Test set				
20	Difloxacin	26.6-28.3	27.8	20.6-28.8	25.7	1.02-1.1	1.04	64
21	Fleroxacin	16.3-20.65	18.13	7.9-13	11.02	1.19-1.58	1.4	32, 65, 66
22	Tosufloxacin	1.49-3.3	2.62	3.6-4.85	4.02	0.21-0.4	0.34	67-69

[1]$AUC_{0-\infty}$ is area under the plasma concentration-time curve from time zero to infinity; $t_{1/2}$ is elimination half life; Cmax is maximum concentration of the drug in plasma.

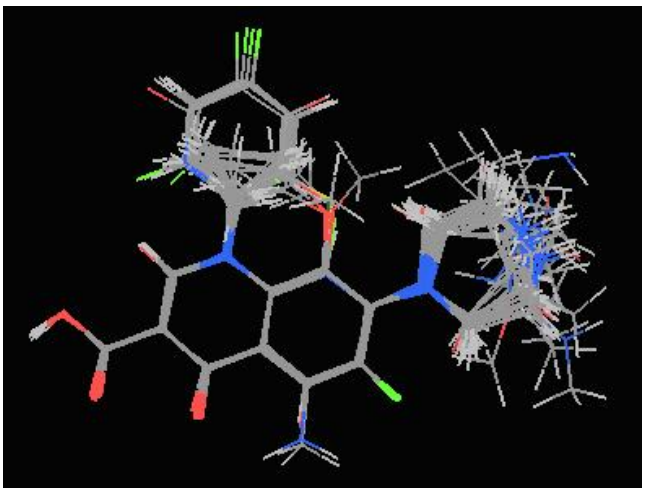


Figure 3 The alignment of fluoroquinolone molecules.

Table 2 Descriptors used in this paper

Descriptors	Physicochemical meaning
SA7	Surface area (grid) of R7
V7	Volume of R7
HE7	Hydration energy of R7
LP7	Logp of R7
RF7	Refractivity of R7
P7	Polarizability of R7
MW7	Molecular weight of R7
SA1	Surface area (grid) of R1
V1	Volume of R1
HE1	Hydration energy of R1
LP1	Logp of R1
RF1	Refractivity of R1
P1	Polarizability of R1
MW1	Molecular weight of R1

largest and negative, but that of P7 and HE7 was quite small, suggesting that the substituents at position 1 are significant to $t_{1/2}$, large polarizability and small volume of substituents at N-1 are therefore preferred.

In fact, compounds with relatively small volume and large polarizability of substituents at N-1 (Table 4) all had relatively large $t_{1/2}$ (Table 1). Compounds 1, 6, 11 and 12 with very small volume of substituents at N-1(Table 4) had small $t_{1/2}$ (Table 1) because of extremely small polarizability (Table 4), and compounds 18, 19 and 22 with extremely large polarizability of substituents at N-1 (Table 4) had relatively small $t_{1/2}$ (Table 1) because of too large volumes. Therefore, volume and polarizability of R1 determine $t_{1/2}$ and small volume and large polarizability of substituents are beneficial to large $t_{1/2}$. It is coincident with the coefficients in the $t_{1/2}$-based model.

Predicted data for test set

The AUC and $t_{1/2}$ data of test set (Table 6) were predicted by models displayed in Table 5.

The ln(AUC) values predicted by the model correlated well with the observed ln(AUC) values for the training data set with correlation coefficient (R2) equal to 0.7369 (Figure 4A). In addition, application of the model to an external test data set consisting of 3 compounds demonstrated

Table 3 Descriptors for group R7 of all fluoroquinolones compounds

No.	R7						
	SA7	V7	HE7	LP7	RF7	P7	MW7
1	271.21	398.4	5.48	-0.36	27.44	11.56	99.16
2	299.04	445.86	5.04	-0.15	31.33	13.39	113.18
3	247.54	347.95	1.46	-0.72	22.15	9.72	85.13
4	248.59	344.96	0.9	-1	21.91	9.72	85.13
5	266.41	382.26	5.33	-0.36	27.44	11.56	99.16
6	251.07	350.09	1.58	-0.72	22.15	9.72	85.13
7	265.94	383.08	3.5	-0.31	26.57	11.56	99.16
8	300	442.55	-2.21	-0.28	35.48	14.96	142.18
9	261.99	381	3.58	-0.31	26.57	11.56	99.16
10	264.36	391.96	5.45	-0.36	27.44	11.56	99.16
11	260.22	378.35	3.68	-0.31	26.57	11.56	99.16
12	250.06	350.92	1.37	-0.72	22.15	9.72	85.13
13	269.89	397.69	5.39	-0.36	27.44	11.56	99.16
14	258.05	374.48	5.68	-0.36	27.44	11.56	99.16
15	258.5	378.79	5.73	-0.36	27.44	11.56	99.16
16	281.66	422.58	3	-0.4	28.87	12.62	111.17
17	298.38	442.76	5.55	0.11	30.99	13.39	113.18
18	266.8	385.12	3.43	-0.31	26.57	11.56	99.16
19	244.47	346.87	4.31	-1.24	24.6	10.78	97.14
20	260.91	378.28	5.31	-0.36	27.44	11.56	99.16
21	264	387.29	5.61	-0.36	27.44	11.56	99.16
22	231.53	320.13	2.75	-1	21.91	9.72	85.13

Table 4 Descriptors for group R1 of all fluoroquinolones compounds

No.	R1						
	SA1	V1	HE1	LP1	RF1	P1	MW1
1	162.65	191.65	-4.39	-0.41	6.5	3.64	30.05
2	184.12	230.99	2.6	1.13	10.1	5.41	41.07
3	183.13	229.07	2.59	1.13	10.1	5.41	41.07
4	181.83	225.71	2.61	1.13	10.1	5.41	41.07
5	232.61	320.17	-3.57	1.43	24.71	9.18	96.08
6	171.57	206.82	0.73	1.32	7.29	4.35	29.06
7	192.14	241.82	2.56	1.13	10.1	5.41	41.07
8	180.91	225.56	2.61	1.13	10.1	5.41	41.07
9	185.87	233.6	2.57	1.13	10.1	5.41	41.07
10	209.76	274.58	0.58	2.4	13.05	6.37	58.08
11	173.79	211.56	0.71	1.32	7.29	4.35	29.06
12	172.99	213.28	0.7	1.32	7.29	4.35	29.06
13	213.93	278.93	0.71	2.4	13.05	6.37	58.08
14	172.31	210.04	0.72	1.32	7.29	4.35	29.06
15	187.74	241.74	-1.24	0.8	15.93	7.69	60.11
16	194.84	249.45	2.6	0.82	9.92	5.32	59.06
17	193.09	243.16	2.55	1.13	10.1	5.41	41.07
18	246.11	343.09	-3.4	2.14	26.43	9.8	113.09
19	246.34	343.09	-3.4	2.14	26.43	9.8	113.09
20	239.88	334.78	-2.47	2	26.21	9.89	95.1
21	168.2	205.76	0.8	0.92	7.37	4.26	47.05
22	248.12	343.14	-3.4	2.14	26.43	9.8	113.09

that the model-predicted AUC values were approximate to the observed AUC values (Table 6), indicating that the constructed model is valid for AUC.

The $\ln(t_{1/2})$ values predicted by the model also correlated well with the observed $\ln(t_{1/2})$ values for the training data set with correlation coefficient (R2) equal to 0.7287 (Figure 4B). In addition, the model-predicted $t_{1/2}$ values were approximate to the observed $t_{1/2}$ values (Table 6), indicating that the constructed model is also valid for $t_{1/2}$.

These models may be used to predict the pharmacokinetic parameters (AUC and $t_{1/2}$) of untried fluoroquinolones. But residual values between predicted and observed data of the test set are slightly larger especially for AUC. It is mainly due to non-precise pharmacokinetic data. Although all the pharmacokinetic data obtained from the literature were averaged, they were not precise enough to get excellent models. The other reason is that we only considered diversities within R1 and R7 to simplify the models. These models however, are very useful as in-silicon prefilters of

Table 5 QSPkR models from the training set data

No.	Model	R^2	R	F	S	LOF
AUC	ln(AUC) = 2.27895 + 1.22614 (HE7) + 9.96141 (P7) - 20.5953 (V7) + 9.13637 (SA7)	0.737	0.858	9.801	0.550	0.472
$t_{1/2}$	ln($t_{1/2}$) = 1.49842 + 1.80503 (P7) + 0.492241 (HE7) - 5.26324 (V1) + 3.53476 (P1)	0.729	0.854	9.400	0.555	0.280
Cmax	ln(Cmax) = 2.96161 - 5.92537 (V1) + 2.15698 (MW1) + 0.206369 (P7) + 0.26873 (HE7)	0.327	0.572	1.697	-0.093	0.523

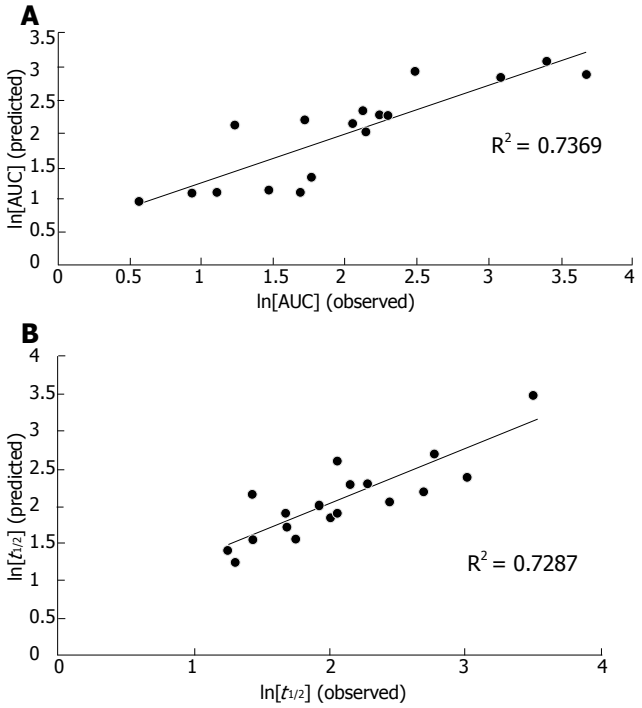


Figure 4 The comparison of the predicted and observed ln(AUC) (**A**) and ln($t_{1/2}$) (**B**).

Table 6 Predicted and observed data of the compounds in the test set

Compounds	Observed		Predicted	
	AUC	$t_{1/2}$	AUC	$t_{1/2}$
20	27.8	25.7	17.431	16.395
21	18.13	11.02	13.269	9.388
22	2.62	4.02	12.034	6.852

fluoroquinolone compounds in virtual high throughput screening. And qualitative analysis of substituents at N-1 and C-7 may contribute to guide design of novel fluoroquinolones with excellent pharmacokinetic properties

In conclusion, this model can contribute to a series of fluoroquinolone antibacterial drugs with excellent pharmacokinetic properties for complete eradication of *H pylori*.

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COMMENTS

Background

Successful drugs must have suitable properties in toxicity, bioavailability and pharmacokinetic parameters. Screening for a large number of compounds with excellent absorption, distribution, metabolism, and excretion (ADME) properties is time-consuming and expensive. So the extension of the idea of quantitative structure-activity relationship (QSAR) to pharmacokinetic data has led to emergence of new tool called quantitative structure pharmacokinetic relationship (QSPkR) study. QSPkR study can be utilized in drug design.

Research frontiers

Both one- and two-dimensional topological indices have been used extensively to numerically relate molecular structure with activity and/or property. (These descriptors rely only on the molecular graph for their calculation. In contrast, three-dimensional descriptors require the absolute conformation of a molecule. They, too, have been successfully used to develop QSPkRs.

Innovations and breakthroughs

In this study the authors have developed and demonstrated novel computational approaches for the efficient and accurate prediction of AUC and $t_{1/2}$ of fluoroquinolones. They constructed simple models which can directly correlate physical and chemical properties to pharmacokinetic data. These models can be used not only to predict pharmacokinetic parameters but also to guide the design of novel fluoroquinolones.

Applications

Using these QSPkR models, the authors can illustrate how the changes at N-1 and C-7 of the fluoroquinolones affect their pharmacokinetic parameters. Such computational models may be useful as in-silico prefilters of fluoroquinolones compounds in a virtual high throughput screening environment and as a research tool for identifying and improving the pharmacokinetic profiles of fluoroquinolones candidates.

Peer review

In the present study, the authors have tried to develop computational approaches for the prediction of the pharmacokinetics of fluoroquinolones. Quantitative structure-pharmacokinetics relationship analysis can be an important tool at the early stage of drug design. The authors demonstrated that small volume and large polarizability of substitutents of R-1 are beneficial to large $t_{1/2}$ and small volume, large polarizability and surface area of substitutents at C-7 are of benefit to large AUC in fluoroquinolones.

REFERENCES

1 **NIH Consensus Conference**. Helicobacter pylori in peptic ulcer disease. NIH Consensus Development Panel on Helicobacter pylori in Peptic Ulcer Disease. *JAMA* 1994; **272**: 65-69

2 **Leung WK**, Hung LC, Kwok CK, Leong RW, Ng DK, Sung JJ. Follow up of serial urea breath test results in patients after consumption of antibiotics for non-gastric infections. *World J Gastroenterol* 2002; **8**: 703-706

3 **Ibrahim M**, Khan AA, Tiwari SK, Habeeb MA, Khaja MN, Habibullah CM. Antimicrobial activity of Sapindus mukorossi and Rheum emodi extracts against H pylori: In vitro and in vivo studies. *World J Gastroenterol* 2006; **12**: 7136-7142

4 **Klopman G**, Fercu D, Renau TE, Jacobs MR. N-1-tert-butyl-substituted quinolones: in vitro anti-Mycobacterium avium

activities and structure-activity relationship studies. *Antimicrob Agents Chemother* 1996; **40**: 2637-2643
5 **Norris DA**, Leesman GD, Sinko PJ, Grass GM. Development of predictive pharmacokinetic simulation models for drug discovery. *J Control Release* 2000; **65**: 55-62
6 **Ghafourian T**, Fooladi S. The effect of structural QSAR parameters on skin penetration. *Int J Pharm* 2001; **217**: 1-11
7 **Feher M**, Sourial E, Schmidt JM. A simple model for the prediction of blood-brain partitioning. *Int J Pharm* 2000; **201**: 239-247
8 **Wajima T**, Fukumura K, Yano Y, Oguma T. Prediction of human pharmacokinetics from animal data and molecular structural parameters using multivariate regression analysis: oral clearance. *J Pharm Sci* 2003; **92**: 2427-2440
9 **Cook JA**, Silverman MH, Schelling DJ, Nix DE, Schentag JJ, Brown RR, Stroshane RM. Multiple-dose pharmacokinetics and safety of oral amifloxacin in healthy volunteers. *Antimicrob Agents Chemother* 1990; **34**: 974-979
10 **Stroshane RM**, Silverman MH, Sauerschell R, Brown RR, Boddy AW, Cook JA. Preliminary study of the pharmacokinetics of oral amifloxacin in elderly subjects. *Antimicrob Agents Chemother* 1990; **34**: 751-754
11 **Kozawa O**, Uematsu T, Matsuno H, Niwa M, Nagashima S, Kanamaru M. Comparative study of pharmacokinetics of two new fluoroquinolones, balofloxacin and grepafloxacin, in elderly subjects. *Antimicrob Agents Chemother* 1996; **40**: 2824-2828
12 **Lubasch A**, Keller I, Borner K, Koeppe P, Lode H. Comparative pharmacokinetics of ciprofloxacin, gatifloxacin, grepafloxacin, levofloxacin, trovafloxacin, and moxifloxacin after single oral administration in healthy volunteers. *Antimicrob Agents Chemother* 2000; **44**: 2600-2603
13 **Boy D**, Well M, Kinzig-Schippers M, Sörgel F, Ankel-Fuchs D, Naber KG. Urinary bactericidal activity, urinary excretion and plasma concentrations of gatifloxacin (400 mg) versus ciprofloxacin (500 mg) in healthy volunteers after a single oral dose. *Int J Antimicrob Agents* 2004; **23** Suppl 1: S6-S16
14 **Zhang J**, Zhang YY, Shi YG, Yu JC, Wang L. Clinical pharmacokinetics study on ciprofloxacin. *Zhongguo Yaolixue Tongbao* 1996; **12**: 430-433
15 **Xiao YH**, Wang QN, Qian YS, Du JZ, Zheng XP, Liu HY. Pharmacokinetics and in vitro antibacterial activity of ciprofloxacin. *Zhongguo Kangshengsu Zazhi* 1992; **17**: 293-296
16 **Randinitis EJ**, Koup JR, Rausch G, Abel R, Bron NJ, Hounslow NJ, Vassos AB, Sedman AJ. Clinafloxacin pharmacokinetics in subjects with various degrees of renal function. *Antimicrob Agents Chemother* 2001; **45**: 2536-2542
17 **Randinitis EJ**, Brodfuehrer JI, Eiseman I, Vassos AB. Pharmacokinetics of clinafloxacin after single and multiple doses. *Antimicrob Agents Chemother* 2001; **45**: 2529-2535
18 **Wise R**, Jones S, Das I, Andrews JM. Pharmacokinetics and inflammatory fluid penetration of clinafloxacin. *Antimicrob Agents Chemother* 1998; **42**: 428-430
19 **Meyerhoff C**, Dilger C, Yoon SJ, Chung YH, Lee DK, Lee CW, Ryu JM, Choi MS, Pabst G, Reh C. Safety, tolerability and pharmacokinetics of the new long-acting quinolone DW-116 after single and multiple dosing in healthy subjects. *J Antimicrob Chemother* 1998; **42**: 349-361
20 **Qian YS**, Wang QN, Xiao YH, Du JZ, Zheng XP, Li CZ, Liu HY, Qiu J, Jiang FY. Pharmacokinetics study of enoxacin in human and in vitro antibactial activity. *Zhongguo Kangshengsu Zazhi* 1992; **17**: 215-218
21 **Zeng JZ**, Liang DR, Qin YP, Liang MZ, Liu DY, Huang Y, Zhang HM, Yu Q, Zou YG., Zhang HL. Study on the pharmacokinetics of oral enoxacin and bioavailabilities of preparations in healthy volunteers. *Zhongguo Kangshengsu Zazhi* 1993; **18**: 341-346
22 **Xiao YH**, Wang QN, Qian YS, Du JZ, He HX. Pharmacokinetics of enoxacin, ciprofloxacin, ofloxacin and pefloxacin. *Chongqing Yikedaxue Xuebao* 1994; **19**: 177-179
23 **Trampuz A**, Laifer G, Wenk M, Rajacic Z, Zimmerli W. Pharmacokinetics and pharmacodynamics of gatifloxacin against Streptococcus pneumoniae and Staphylococcus aureus in a granulocyte-rich exudate. *Antimicrob Agents Chemother* 2002; **46**: 3630-3633
24 **Lober S**, Ziege S, Rau M, Schreiber G, Mignot A, Koeppe P, Lode H. Pharmacokinetics of gatifloxacin and interaction with an antacid containing aluminum and magnesium. *Antimicrob Agents Chemother* 1999; **43**: 1067-1071
25 **Liu XD**, Xie L, Wang J, Liang Y, Li L, Lu L. Comparison of pharmacokinetics of gatifloxacin in rats, dogs and humans. *Asian J drug Metab Pharmacokinet* 2005; **5**: 71-76
26 **Wise R**, Andrews JM, Ashby JP, Marshall J. A study to determine the pharmacokinetics and inflammatory fluid penetration of gatifloxacin following a single oral dose. *J Antimicrob Chemother* 1999; **44**: 701-704
27 **Allen A**, Bygate E, Oliver S, Johnson M, Ward C, Cheon AJ, Choo YS, Kim IC. Pharmacokinetics and tolerability of gemifloxacin (SB-265805) after administration of single oral doses to healthy volunteers. *Antimicrob Agents Chemother* 2000; **44**: 1604-1608
28 **Gee T**, Andrews JM, Ashby JP, Marshall G, Wise R. Pharmacokinetics and tissue penetration of gemifloxacin following a single oral dose. *J Antimicrob Chemother* 2001; **47**: 431-434
29 **Allen A**, Bygate E, Vousden M, Oliver S, Johnson M, Ward C, Cheon A, Choo YS, Kim I. Multiple-dose pharmacokinetics and tolerability of gemifloxacin administered orally to healthy volunteers. *Antimicrob Agents Chemother* 2001; **45**: 540-545
30 **Child J**, Andrews JM, Wise R. Pharmacokinetics and tissue penetration of the new fluoroquinolone grepafloxacin. *Antimicrob Agents Chemother* 1995; **39**: 513-515
31 **Efthymiopoulos C**. Pharmacokinetics of grepafloxacin. *J Antimicrob Chemother* 1997; **40** Suppl A: 35-43
32 **Chien SC**, Rogge MC, Gisclon LG, Curtin C, Wong F, Natarajan J, Williams RR, Fowler CL, Cheung WK, Chow AT. Pharmacokinetic profile of levofloxacin following once-daily 500-milligram oral or intravenous doses. *Antimicrob Agents Chemother* 1997; **41**: 2256-2260
33 **Chien SC**, Chow AT, Natarajan J, Williams RR, Wong FA, Rogge MC, Nayak RK. Absence of age and gender effects on the pharmacokinetics of a single 500-milligram oral dose of levofloxacin in healthy subjects. *Antimicrob Agents Chemother* 1997; **41**: 1562-1565
34 **Stone JW**, Andrews JM, Ashby JP, Griggs D, Wise R. Pharmacokinetics and tissue penetration of orally administered lomefloxacin. *Antimicrob Agents Chemother* 1988; **32**: 1508-1510
35 **Kovarik JM**, Hoepelman AI, Smit JM, Sips PA, Rozenberg-Arska M, Glerum JH, Verhoef J. Steady-state pharmacokinetics and sputum penetration of lomefloxacin in patients with chronic obstructive pulmonary disease and acute respiratory tract infections. *Antimicrob Agents Chemother* 1992; **36**: 2458-2461
36 **Tan L**, Xu DK, Diao Y, Yuan YS. Highperformance liquid chromatographic assay for lomefloxacin in plasma and its pharmacokinetics in healthy volunteers. *Yaoxue Xuebao* 1993; **28**: 286-289
37 **Tang X**, Cai YM. Effects of probenicid on the pharmacokinetics of norfloxacin. *Shenyang Yaoxueyuan Xuebao* 1991; **8**: 1-4
38 **Adhami ZN**, Wise R, Weston D, Crump B. The pharmacokinetics and tissue penetration of norfloxacin. *J Antimicrob Chemother* 1984; **13**: 87-92
39 **Lehto P**, Kivistö KT. Effect of sucralfate on absorption of norfloxacin and ofloxacin. *Antimicrob Agents Chemother* 1994; **38**: 248-251
40 **Yuk JH**, Nightingale CH, Quintiliani R, Sweeney KR. Bioavailability and pharmacokinetics of ofloxacin in healthy volunteers. *Antimicrob Agents Chemother* 1991; **35**: 384-386
41 **Immanuel C**, Hemanthkumar AK, Gurumurthy P, Venkatesan P. Dose related pharmacokinetics of ofloxacin in healthy volunteers. *Int J Tuberc Lung Dis* 2002; **6**: 1017-1022
42 **Hameed A**, Iqbal T, Nawaz M, Batool F, Baig TT, Ali S, Nurjis F. Pharmacokinetics of ofloxacin in male volunteers following oral administration. *Pak J Biol Sci* 2002; **5**: 1098-1100
43 **Israel D**, Gillum JG, Turik M, Harvey K, Ford J, Dalton H, Towle M, Echols R, Heller AH, Polk R. Pharmacokinetics

and serum bactericidal titers of ciprofloxacin and ofloxacin following multiple oral doses in healthy volunteers. *Antimicrob Agents Chemother* 1993; **37**: 2193-2199

44 **Lode H**, Höffken G, Olschewski P, Sievers B, Kirch A, Borner K, Koeppe P. Pharmacokinetics of ofloxacin after parenteral and oral administration. *Antimicrob Agents Chemother* 1987; **31**: 1338-1342

45 **De Lepeleire I**, Van Hecken A, Verbesselt R, Tjandra-Maga TB, De Schepper PJ. Comparative oral pharmacokinetics of fleroxacin and pefloxacin. *J Antimicrob Chemother* 1988; **22**: 197-202

46 **Wang WL**, Lu H, Wang T, Hou F, Li JT. Pharmacokinetics of pefloxacin in normal volunteers. *Zhongguo Linchuang Yaolixue Zazhi* 1990; **6**: 235-241

47 **Xiao YH**, Wang QN, Qian YS, Du JZ, Zheng XP, Liu HY. Pharmacokinetics and in vitro antibacterial activity of pefloxacin. *Zhongguo Kangshengsu Zazhi* 1992; **17**: 302-305

48 **Imbimbo BP**, Broccali G, Cesana M, Crema F, Attardo-Parrinello G. Inter- and intrasubject variabilities in the pharmacokinetics of rufloxacin after single oral administration to healthy volunteers. *Antimicrob Agents Chemother* 1991; **35**: 390-393

49 **Wise R**, Johnson J, O'Sullivan N, Andrews JM, Imbimbo BP. Pharmacokinetics and tissue penetration of rufloxacin, a long acting quinolone antimicrobial agent. *J Antimicrob Chemother* 1991; **28**: 905-909

50 **Perry G**, Mant TG, Morrison PJ, Sacks S, Woodcook J, Wise R, Imbimbo BP. Pharmacokinetics of rufloxacin in patients with impaired renal function. *Antimicrob Agents Chemother* 1993; **37**: 637-641

51 **Kisicki JC**, Griess RS, Ott CL, Cohen GM, McCormack RJ, Troetel WM, Imbimbo BP. Multiple-dose pharmacokinetics and safety of rufloxacin in normal volunteers. *Antimicrob Agents Chemother* 1992; **36**: 1296-1301

52 **O'Grady J**, Briggs A, Atarashi S, Kobayashi H, Smith RL, Ward J, Ward C, Milatovic D. Pharmacokinetics and absolute bioavailability of sitafloxacin, a new fluoroquinolone antibiotic, in healthy male and female Caucasian subjects. *Xenobiotica* 2001; **31**: 811-822

53 **Nakashima M**, Uematsu T, Kosuge K, Umemura K, Hakusui H, Tanaka M. Pharmacokinetics and tolerance of DU-6859a, a new fluoroquinolone, after single and multiple oral doses in healthy volunteers. *Antimicrob Agents Chemother* 1995; **39**: 170-174

54 **Johnson JH**, Cooper MA, Andrews JM, Wise R. Pharmacokinetics and inflammatory fluid penetration of sparfloxacin. *Antimicrob Agents Chemother* 1992; **36**: 2444-2446

55 **Zix JA**, Geerdes-Fenge HF, Rau M, Vöckler J, Borner K, Koeppe P, Lode H. Pharmacokinetics of sparfloxacin and interaction with cisapride and sucralfate. *Antimicrob Agents Chemother* 1997; **41**: 1668-1672

56 **Mody VD**, Pandya KK, Satia MC, Modi IA, Modi RI, Gandhi TP. High performance thin-layer chromatographic method for the determination of sparfloxacin in human plasma and its use in pharmacokinetic studies. *J Pharm Biomed Anal* 1998; **16**: 1289-1294

57 **Matsuoka M**, Banno K, Sato T. Analytical chiral separation of a new quinolone compound in biological fluids by high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 1996; **676**: 117-124

58 **Granneman GR**, Carpentier P, Morrison PJ, Pernet AG. Pharmacokinetics of temafloxacin in humans after single oral doses. *Antimicrob Agents Chemother* 1991; **35**: 436-441

59 **Granneman GR**, Braeckman R, Kraut J, Shupien S, Craft JC. Temafloxacin pharmacokinetics in subjects with normal and impaired renal function. *Antimicrob Agents Chemother* 1991; **35**: 2345-2351

60 **Teng R**, Liston TE, Harris SC. Multiple-dose pharmacokinetics and safety of trovafloxacin in healthy volunteers. *J Antimicrob Chemother* 1996; **37**: 955-963

61 **Vincent J**, Venitz J, Teng R, Baris BA, Willavize SA, Polzer RJ, Friedman HL. Pharmacokinetics and safety of trovafloxacin in healthy male volunteers following administration of single intravenous doses of the prodrug, alatrofloxacin. *J Antimicrob Chemother* 1997; **39** Suppl B: 75-80

62 **Wise R**, Mortiboy D, Child J, Andrews JM. Pharmacokinetics and penetration into inflammatory fluid of trovafloxacin (CP-99,219). *Antimicrob Agents Chemother* 1996; **40**: 47-49

63 **Granneman GR**, Snyder KM, Shu VS. Difloxacin metabolism and pharmacokinetics in humans after single oral doses. *Antimicrob Agents Chemother* 1986; **30**: 689-693

64 **Stass H**, Dalhoff A, Kubitza D, Schühly U. Pharmacokinetics, safety, and tolerability of ascending single doses of moxifloxacin, a new 8-methoxy quinolone, administered to healthy subjects. *Antimicrob Agents Chemother* 1998; **42**: 2060-2065

65 **Siefert HM**, Domdey-Bette A, Henninger K, Hucke F, Kohlsdorfer C, Stass HH. Pharmacokinetics of the 8-methoxyquinolone, moxifloxacin: a comparison in humans and other mammalian species. *J Antimicrob Chemother* 1999; **43** Suppl B: 69-76

66 **Minami R**, Inotsume N, Nakamura C, Nakano M. Stereoselective analysis of the disposition of tosufloxacin enantiomers in man. *Eur J Clin Pharmacol* 1993; **45**: 489-491

67 **Niki Y**. Pharmacokinetics and safety assessment of tosufloxacin tosilate. *J Infect Chemother* 2002; **8**: 1-18

68 **Minami R**, Nakamura C, Inotsume N, Nakano M. Effects of aluminum hydroxide and famotidine on bioavailability of tosufloxacin in healthy volunteers. *Antimicrob Agents Chemother* 1998; **42**: 453-455

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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CASE REPORT

Extrapulmonary sarcoidosis of liver and pancreas: A case report and review of literature

Hermann Harder, Markus W Büchler, Boris Fröhlich, Philipp Ströbel, Frank Bergmann, Wolfgang Neff, Manfred V Singer

Hermann Harder, Manfred V Singer, Department of Medicine II (Gastroenterology, Hepatology and Infectious Diseases), University Hospital of Heidelberg at Mannheim, Germany
Philipp Ströbel, Institute of Pathology, University Hospital of Heidelberg at Mannheim, Germany
Wolfgang Neff, Institute of Radiology, University Hospital of Heidelberg at Mannheim, Germany
Markus W Büchler, Boris Fröhlich, Department of Surgery, University of Heidelberg, Germany
Frank Bergmann, Institute of Pathology, University of Heidelberg, Germany
Correspondence to: Manfred V Singer, MD, Professor of Medicine and Chairman, Department of Medicine II(Gastroenterology, Hepatology and Infectious Diseases), University Hospital of Heidelberg at Mannheim, Theodor-Kutzer-Ufer 1-3, D-68167 Mannheim,
Germany. manfred.v.singer@med.ma.uni-heidelberg.de
Telephone: +49-621-3833284 Fax: +49-621-3833805
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Abstract

Sarcoidosis is a chronic multisystemic granulomatous disease of unknown origin, which can involve nearly all organs. In the case of an infrequent gastrointestinal tract involvement in systemic sarcoidosis, granulomas of the liver are most commonly described while isolated pancreatic sarcoid lesions are rarely seen. We report a case of systemic sarcoidosis with exclusive extrapulmonal involvement of the liver and the pancreas in a 71-year-old white man. The diagnosis of liver involvement was confirmed by biopsy. Pancreatic surgery was needed because preoperative evaluation could not exclude pancreatic cancer and for biliary decompression. An extensive literature review of systemic sarcoidosis, focusing on reported cases with unusual presentation of sarcoidosis in the liver and the pancreas, its diagnosis, treatment, and prognosis was made.

Key words: Systemic; Sarcoidosis; Extrapulmonary; Liver; Pancreas

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INTRODUCTION

Sarcoidosis is a chronic, multisystemic non-caseous granulomatous disease of unknown origin[1]. It can involve almost any organ, but most commonly affects the lung (90%), lymph nodes (75%), eye (25%) and skin (25%)[2]. The prevalence of systemic sarcoidosis is 1-40/100 000, being predominantly observed in Afro-Americans and in persons of Scandinavian descent, affecting mostly young adults between 20 and 40 years of age[3,4].

Involvement of the gastrointestinal tract in patients with systemic sarcoidosis is rare, and in 60%-90% of these cases, liver granulomas can be found in biopsy[2]. Exclusive liver involvement without lung disease is less frequent, documented in only about 13% of patients with systemic sarcoidosis[5]. The diagnosis of liver sarcoidosis is difficult, because symptoms or functional derangement due to involvement of the liver are uncommon in sarcoidosis[6]. If not being asymptomatic, the clinical presentation of liver sarcoidosis may be hepatosplenomegaly, increased liver enzymes, intrahepatic cholestasis and portal hypertension, as a consequence of cirrhosis due to long-standing intrahepatic cholestasis[6].

As compared to liver involvement in sarcoidosis, pancreatic sarcoidosis is rare with a prevalence in autopsy studies of 1%-5% in patients with systemic sarcoidosis[7-11]. However, pancreatic involvement has been reported even less frequently[12-26] and only isolated cases of gastrointestinal sarcoidosis exclusively in the pancreas can be found in the available literature[27-29]. Since the symptoms related to pancreatic sarcoidosis are mainly due to pancreatic tissue infiltration or ductal obstruction, the clinical presentation of pancreatic sarcoidosis resembles more common entities such as pancreatitis or pancreatic cancer[30].

We present a case of exclusive extra-pulmonary gastrointestinal sarcoidosis with hepatic and pancreatic manifestation, but without signs of other organs. In our patient, the single clinical sign for hepatic manifestation of sarcoidosis was an increase in liver enzymes. Pancreatic sarcoidosis presented as a mass in the head of the pancreas with symptoms resembling pancreatic malignancy. Pancreatic surgery was needed because the preoperative

evaluation could not exclude pancreatic cancer and for biliary decompression. We reviewed the literature on hepatic and pancreatic manifestation of sarcoidosis, its diagnosis, treatment, and prognosis.

CASE REPORT

A 71-year-old white man was admitted to the Department of Medicine II, University Hospital of Heidelberg at Mannheim with acute upper abdominal cramps, subfebrile temperature of 37.5℃ and increasing fatigue. He had no history of regular consumption of alcohol or drugs. The patient had been treated with antimycotics (Amphomoronal®) for mycotic stomatitis, which presented as disturbance of taste and a dry mouth for about four months. Physical examination showed a red tongue and a discrete pressure pain in the left upper abdomen. About one year before the admission, the patient had had ambulatory diarrhoea and diffuse abdominal pain and cramps. He had a history of laparoscopic rectosigmoid resection and adhaesiolysis for diverticulitis two years before. Postoperative colonoscopy showed no macroscopic or histological signs for inflammatory bowel disease or specific inflammation. There was only a slight unspecific inflammation and fibrosis at the rectosigmoideal anastomosis. Symptoms could be controlled by medication with mebeverin 200 mg (Duspatal®) and butylscopolaminbromid (Buscopan®) on demand. Laboratory findings, especially pancreatic and liver enzymes were in the normal range.

Pathologic findings of the patient at admission are given in Table 1. All the other tested parameters were in the normal range, including serum amylase, aspartate aminotransferase, alanine cholinesterase and bilirubin levels and electrolytes. Tests for hepatitis A, B, and C virus were all negative. Autoantibody screens (antimitochondrial antibody (AMA), antineutrophil cytolasmic antibody (ANCA), antinuclear antibody (ANA), liver kidney microsome antibody (LKM), smooth-muscle antibody (SMA)) and serum variables of iron and copper metabolism were all in the normal range. Epstein-Barr IgM, cytomegal virus (CMV) IgG and IgM were negative. The tuberculin test was negative. The X-ray of the chest and subsequent computed tomography scan corresponded to age, and no hilar adenopathy was observed and lung capacity was normal. Electrocardiogram and ultrasound of the heart were also normal.

Upper endoscopy was macroscopically normal, and histology showed mild chronic antrum- and corpus gastritis with antral *H pylori* infection. We performed a laparoscopy to take liver biopsy. Macroscopically, there were no signs of liver cirrhosis or portal hypertension. The liver biopsy showed numerous dispersed granulomatous foci at low magnification, which at higher magnification were noncaseating and contained multiple giant cells. Plasma cells and eosinophils were not conspicuous. The reported histopathological diagnosis was "granulomatous hepatitis, compatible with sarcoidosis". Ultrasound of the abdomen showed a slightly increased echo intensity of the liver parenchyma. There was a lipomatosis of the pancreas and in the pancreatic head a hypoechogenic lesion measuring 4.7 cm × 3.2 cm × 2.9 cm was found. MRI with MRCP of the abdomen confirmed the ultrasound finding of the pancreatic head mass and demonstrated intrapancreatic stenosis of the DHC and the pancreatic duct. The former lesion did not infiltrate the surrounding vessels, with signs for lymphadenopathy but without evidence of hepatic metastasis (Figure 1).

Table 1 Laboratory findings of the patient on admission

Pathologic parameters	Values	Normal range
Serum lipase	1799 U/L	114-286 U/L
Alkaline phosphatase (AP)	236 U/L	38–126 U/L
Gamma-glutamyl transpetidase (GGT)	249 U/L	0-85 U/L
Aminotransferase (ALAT)	67 U/L	0-50 U/L
CA 19-9	52 ku/L	0-40 ku/L
CRP	19 mg/L	< 5 mg/L
Albumin	41.5 g/L	60.3-71.4 g/L
Epstein–Barr virus (EBV) IgG	975 U/L	0-400 U/L
Angiotensin Converting Enzyme	1.9 U/L	8-52 U/L

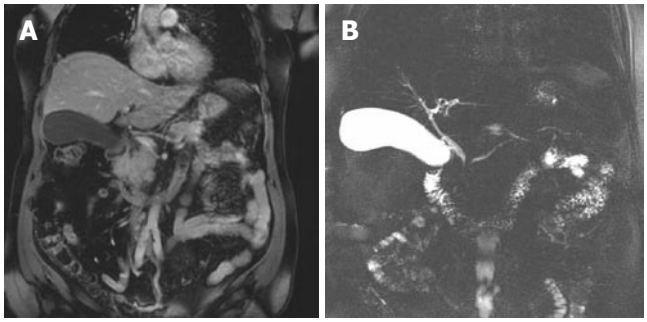

Figure 1 Coronal T1-weighted fat-saturated MR image demonstrates pancreatic head mass measuring 4.7 × 3.2 × 2.9 cm (**A**) and MRCP shows the stenosis of the pancreatic and bile duct (**B**).

Endosonography of the pancreatic head was suspicious for pancreatic cancer. Due to the fact that the main differential diagnosis was a primary pancreatic cancer, an explorative laparotomy and partial pancreaticoduodenectomy was indicated, which was performed in the Department of Surgery, University of Heidelberg.

Operative procedure

Exploration of the abdominal cavity revealed no signs of metastasis. The pancreatic mass was resected, and a pylorus preserving partial pancreaticoduodenectomy was performed with resection of the distal common bile duct. Pathologic examination of the surgical specimen revealed a firm consistency of the pancreatic head, which displayed a lobular architecture with fibrosis on the cut surface (Figure 2). The peripancreatic lymph nodes (n = 28) were firm and enlarged, measuring up to 2 cm. Microscopically, the pancreatic parenchyma presented inter- and intralobular fibrosis, moderate to dense inflammatory cell infiltrates including eosinophils, numerous noncaseating granulomas composed of Langhans´giant cells, epithelioid cells and lymphocytes (Figure 3A-B). Similar granulomas were seen in the peripancreatic lymph nodes (Figure 3C). Atypical cells were not detected. The diagnosis of a noncaseating

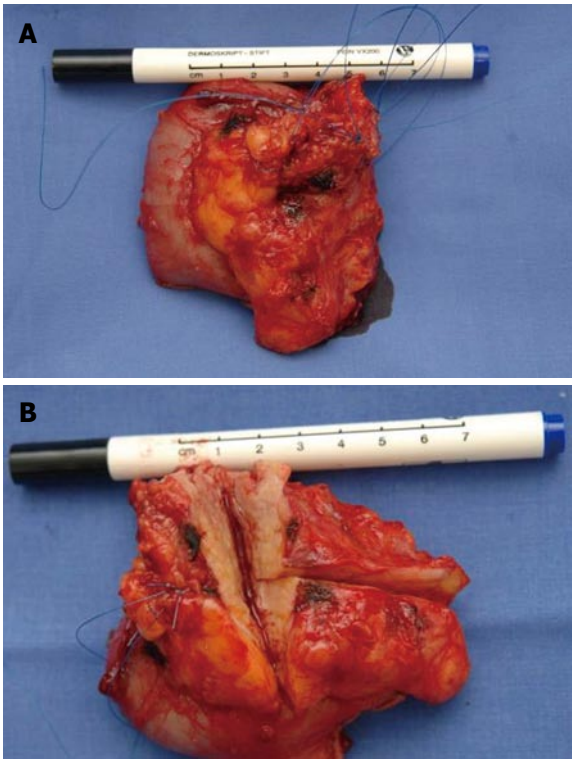

Figure 2 Pathologic examination revealed a firm consistency of the pancreatic head. The peripancreatic lymph nodes (*n* = 28) were firm and enlarged, measuring up to 2 cm.

granulomatous pancreatitis and lymphadenitis, compatible with sarcoidosis, was established. A side to end jejuno-pancreaticostomy, a side to end hepatico-jejunostomy and a duodeno-jejunostomy with a running loop avoiding brown´s anastomosis[31] were performed.

Drains were removed on the third postoperative day when the drained fluid contained no increased amounts of amylase or lipase[32]. The postoperative course was prolonged by delayed gastric empting which was treated by placing a naso-gastric tube and i.v. administration of 250 mg of erythromycin three times a day for two days. After removing the naso-gastric tube, the patient was back to solid food in two days. The wounds were healing and staples were removed eleven days after surgery. The patient was discharged from the surgical department and admitted to the Department of Medicine II, University Hospital of Heidelberg at Mannheim on the 12th postoperative day.

Post-operative course

The postoperative course was uncomplicated. A urinary tract infection was treated with antibiotics, levofloxacine 500 mg (Tavanic®) over 5 d and signs for infection vanished. The patient complained of right upper abdominal and thoracic pain and had a computer tomography (CT) of the chest and the abdomen, which showed a regular postoperative status and ruled out lung embolia. He was discharged after 4 d with discrete elevated alkaline phosphatase (AP) levels (319 U/L; normal, 38-126 U/L) and elevated gamma-glutamyl transpetidase (GGT) levels (423 U/L; normal, -85 U/L) but serum lipase levels and aminotransferase (ALAT) levels were in the normal range.

The physical and general condition of the patient was

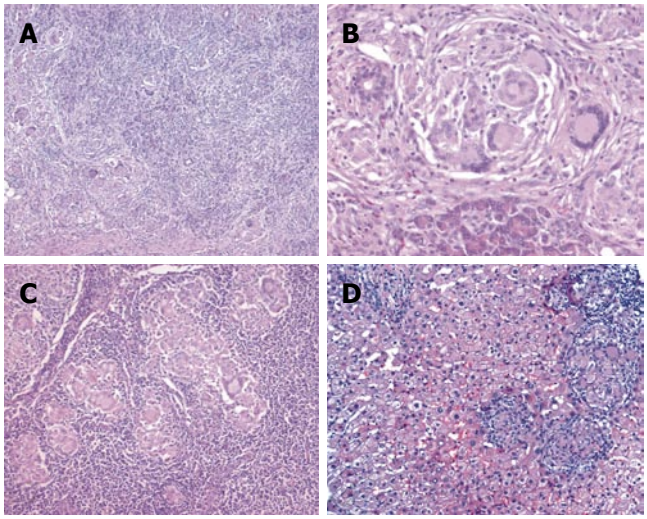

Figure 3 Microscopic findings in the pancreas (**A-B**), peripancreatic lymph nodes (**C**) and the liver (**D**) with numerous noncaseating granulomas composed of giant cells (Langhans type), epitheloid cells, and lymphocytes. Hematoxylin & eosin staining (**A-D**). Original magnifications × 16 (**A**), × 64 (**B**), × 32 (**C**) and × 250 (**D**).

improved. He had no more sub-febrile body temperature, and had decreased alkaline phosphatase (AP) (178 U/L; normal, < 129 U/L) and gamma-glutamyl transpetidase (GGT) (162 U/L; normal, -85 U/L). In the course of observation, AP and GGT levels rose again and the patient felt sleepy so that we started a treatment with corticoids. With 40 mg prednisone daily for 8 wk and then gradually tapering the dose, the patient became asymptomatic again and liver enzymes were all in the normal range. Final diagnosis was extrapulmonary sarcoidosis of the liver and the pancreas.

DISCUSSION

Diagnosis of systemic sarcoidosis, a multisystemic disease with unknown etiology, is based on a compatible clinical presentation involving at least two organ systems, supportive histologic evidence of noncaseating granulomas, and a reasonable exclusion of other granulomatous diseases[33,34]. Since extrapulmonary manifestation of sarcoidosis and in particular exclusive involvement of the liver and pancreas is extremely rare, few data have been published on the diagnosis, treatment and clinical course of this disease.

Liver involvement of sarcoidosis

The frequency of exclusive liver involvement without lung disease in patients with systemic sarcoidosis is infrequent and seen in only 13% of patients with sarcoidosis[5]. Our patient presented with derangement of liver function. It is interesting that, in most of the patients, hepatic involvement with granulomas is asymptomatic and associated with no abnormal liver function or mild derangement[6]. Fever, as an unspecific clinical sign, correlates with hepatic manifestation of the disease. Thus, nearly 60% of patients with hepatic manifestation of sarcoidosis have fever or arthralgia, in contrast to patients without liver manifesta-tion[6]. Therefore in this case, fever constitutes an additional indication for liver biopsy[6]. Although our patient had sub-febrile body temperature, he

was asymptomatic. Since granulomas in the liver are small and preferably located in the portal space, clinical hepatic manifestations are rare. Those may include jaundice and chronic cholestasis[6,35-38], portal hypertension[39,40], or Budd Chiari syndrome[41,42]. Intrahepatic cholestasis can resemble primary biliary cirrhosis[43] or sclerosing cholangitis[44]. Sarcoidosis can also coexist with these two entities. Cirrhosis[5,45,46] and portal hypertension[39,40,45,47,48] are only found in 1% of patients with sarcoidosis. Furthermore, extrahepatic biliary tract obstruction[37] in sarcoidosis from enlarged granulomatous lymph nodes has been described, but this was not the case in our patient. Transthoracic ultrasound, CT and MRI did not raise suspicion for liver sarcoidosis. Since no reason for the derangement of liver function was found and an outpatient CT-scan had raised suspicion for liver cirrhosis, we decided to perform laparoscopic liver biopsy to confirm the diagnosis of liver sarcoidosis. It is interesting that only 20%-40% of patients with sarcoidosis had AP and GGT values increased[49,50]. Slightly increased CA 19-9 levels can be interpreted as an indicator of cholestasis[51]. Angiotensin converting enzyme (ACE), which is increased in 55% of patients with sarcoidosis, with a higher frequency in those with active disease[52,53], was not increased in our patient and serum calcium was within the normal range.

In liver biopsies, the incidence of sarcoidosis varies widely in countries and among ethnic groups. In general, granulomas are found in 4% of all liver biopsy specimens[54-56]. Of these granulomas, only 13.5%-22% are due to sarcoidosis. Histological findings of liver biopsy in our patient are given in Figure 3D. In Europe, sarcoidosis is seen in about 18% of epithelioid granulomas of the liver[54]. Others are primary biliary cirrhosis (55%) and tuberculosis or various infectious diseases[57,58]. Chronic inflammatory bowel disease is a well recognized cause of hepatic dysfunction and granulomas are found in the liver in up to 5% in this disease[59]. Epithelioid granulomas can be found in patients with psoriasis, drug hypersensitivity[60], chronic active hepatitis, extrahepatic biliary obstruction and very rarely in carcinoma[61,62] or Hodgkin disease[63-65].

Pancreatic involvement of sarcoidosis

Pancreatic involvement in systemic sarcoidosis is uncommon with a prevalence in autopsy studies of 1%-5%[7-11] and an even lower prevalence in clinical series[12-26]. Only isolated cases of gastrointestinal sarcoidosis presenting exclusively in the pancreas have been reported[27-29]. Symptomatic sarcoidosis presenting as a pancreatic mass is extremely rare and only 25 cases have been reported in the literature since first described by Curran and Curran in 1950[14]. Out of these cases, 12 presented with a pancreatic mass, that in most cases was located in the head of the pancreas[14]. The remaining cases revealed a diffusely indurated nodular pancreas.

Our patient had a pancreatic mass in the head of the pancreas by ultrasound, MRI and endoscopic ultrasound. Based on imaging procedures alone, it was not possible to exclude pancreatic cancer or pancreatitis. In accordance to the literature, he had abdominal pain which is the most common symptom (66% of cases) in patients with liver sarcoidosis. He had no other frequent complaints such as weight loss (45%), jaundice (29%) and nausea/emesis (20%). Unfortunately, there is no specific test for the preoperative diagnosis of pancreatic sarcoid without the presence of more classic findings of this disease or a previous pathologic diagnosis. History of sarcoidosis before the manifestation that led to the discovery of pancreatic involvement was only present in 4 (16%) of 35 patients.

Symptoms related to the presence of pancreatic sarcoidosis originate secondary to pancreatic tissue infiltration or double duct sign due to bile stock. This is the reason why it clinically presents like pancreatitis or pancreatic cancer[30] (Figure 1). Given the inability to rule out pancreatic cancer and the dilatation of the biliary tree, our patient required surgical intervention for decompression and diagnosis. Furthermore, the usefulness of diagnostic tests in this setting is questionable and no reports exist in the literature of pancreatic sarcoidosis diagnosed by non-surgical means of biopsy, including CT or endoscopic ultrasound. Preoperative and operative findings could not exclude pancreatic cancer and therefore standard pancreaticoduodenectomy was performed. An exception to this procedure would have required a definite previous diagnosis of sarcoidosis but we had to assume that the finding of isolated granulomas found in the preoperative liver biopsy without other clinical findings for systemic sarcoidosis was not sufficient to exclude the possibility of pancreatic carcinoma and to avoid pancreatic surgery. Chronic pancreatitis was less probable because there was no anamnestic evidence and pancreatic endocrine and exocrine function was normal.

Treatment and prognosis

Steroids are an important base in the treatment of pulmonary sarcoidosis. Nevertheless, the exact time point of treatment, dosage, and benefits versus side effects are still controversial[5,66-72]. Because sarcoidosis has such varied manifestations, severity, and course, there have been no valid prospective placebo-controlled treatment trials. Prolonged length of observation and a large number of patients are required[33] to prove the effectiveness of treatment strategies. In asymptomatic patients or those with mild lung disease, the side effects of systemic steroids often exceed the benefits of treatment[6], and the disease may spontaneously remit. In the literature, there are only a few reports on treatment of liver sarcoidosis[5,73,74]. These cases demonstrate that liver function may improve even though liver granulomas may persist on histology. Early steroid treatment does not preclude the development of intrahepatic cholestasis or development of portal hypertension. Therefore, the role of liver biopsies seems undoubted not only in diagnosis, but also in continued disease. In patients where liver function tests were deranged for a time, corticosteroids have been used as a first line treatment[75].

A more recent publication[5] summarizes cases of hepatic dysfunction complicated with lung disease and significant liver involvement presenting independent of pulmonary sarcoid (23 of 180 patients with a follow-up of two years (13%). Sixty-three patients were treated with corticosteroids based on current protocols 30-40

mg of prednisone daily for 8-12 wk and then gradual tapering of the dose to 10-20 mg over a period of 6-12 mo, to establish the minimal effective dose[4]. In the above-mentioned study, approximately one third had a complete clinical response, one-third a partial response and one-third no response. This study, providing the first comprehensive review of liver involvement in sarcoidosis, suggests that hepatic sarcoidosis can be a serious and rapidly progressive disease[76]. The authors conclude that in case of chronic liver disease treatment should be started irrespective of the absence of pulmonary or other extrahepatic manifestations.

Alternatives to steroids have been tried, being primarily limited to steroid refractory cases. Methotrexate[5,73,76-78] has shown the greatest promise of these alternative agents, but again, data are limited. In cases of hepatic cholestasis, ursodeoxycholic acid may be beneficial[79]. Organ transplantation is often the only treatment modality in advanced sarcoidosis of the liver. Cyclosporine has been used successfully in a cohort of nine patients transplanted for sarcoidosis with no evidence of disease recurrence. Nevertheless, recurrence of disease in the allograft has been reported in a previous case report[80-82].

No general conclusion can be drawn on medical treatment of pancreatic sarcoidosis. Up to now, in the literature, 25 cases of pancreatic sarcoidosis presenting as a pancreatic mass have been reported. Among 18 patients, treatment regimens were compared, 6 patients improved spontaneously without corticosteroids and 10 with prednisone treatment[28].

In summary, we report a case of systemic sarcoidosis with extrapulmonal involvement of the liver and the pancreas. Pancreatic surgery was required to exclude pancreatic cancer. Liver biopsies are a valuable tool for diagnosis and also for disease monitoring in patients with liver sarcoidosis. The prognosis of mild disease and pancreatic involvement is good, with high spontaneous remission rates. The exact time point of treatment, dosage, and benefits versus side effects for corticoids in extrapulmonary sarcoidosis are still discussed controversially.

REFERENCES

1 **Agostini C**, Semenzato G, James DG. Immunological, clinical and molecular aspects of sarcoidosis. *Mol Aspects Med* 1997; **18**: 91-165
2 **Sharma OP**. Sarcoidosis: clinical, laboratory, and immunologic aspects. *Semin Roentgenol* 1985; **20**: 340-355
3 **James DG**. Epidemiology of sarcoidosis. *Sarcoidosis* 1992; **9**: 79-87
4 **Newman LS**, Rose CS, Maier LA. Sarcoidosis. *N Engl J Med* 1997; **336**: 1224-1234
5 **Kennedy PT**, Zakaria N, Modawi SB, Papadopoulou AM, Murray-Lyon I, du Bois RM, Jervoise N Andreyev H, Devlin J. Natural history of hepatic sarcoidosis and its response to treatment. *Eur J Gastroenterol Hepatol* 2006; **18**: 721-726
6 **Mueller S**, Boehme MW, Hofmann WJ, Stremmel W. Extrapulmonary sarcoidosis primarily diagnosed in the liver. *Scand J Gastroenterol* 2000; **35**: 1003-1008
7 **Romboli E**, Campana D, Piscitelli L, Brocchi E, Barbara G, D'Errico A, Fusaroli P, Corinaldesi R, Pezzilli R. Pancreatic involvement in systemic sarcoidosis. A case report. *Dig Liver Dis* 2004; **36**: 222-227
8 **McCormick PA**, O'Donnell M, McGeeney K, FitzGerald O, McCormick DA, FitzGerald MX. Sarcoidosis and the pancreas. *Ir J Med Sci* 1988; **157**: 181-183
9 **Longcope WT**, Freiman DG. A study of sarcoidosis; based on a combined investigation of 160 cases including 30 autopsies from The Johns Hopkins Hospital and Massachusetts General Hospital. *Medicine* (Baltimore) 1952; **31**: 1-132
10 **Mayock RL**, Bertrand P, Morrison CE, Scott JH. Manifestations of sarcoidosis. Analysis of 145 patients, with a review of nine series selected from the literature. *Am J Med* 1963; **35**: 67-89
11 **Iwai K**, Tachibana T, Hosoda Y, Matsui Y. Sarcoidosis autopsies in Japan. Frequency and trend in the last 28 years. *Sarcoidosis* 1988; **5**: 60-65
12 **Bonhomme A**, Dhadamus A, De Bie P, Van Hoe L, Baert AL. Pancreatic involvement in systemic sarcoidosis: CT findings. *J Belge Radiol* 1997; **80**: 116-117
13 **Frank JL**, Goldman M, Nathanson I, Pierangelo D, Kaufman JL, Freeman JK, Reed WP. Surgical management of pancreatic sarcoid. *Eur J Surg* 2001; **167**: 68-72
14 **CURRAN JF**, CURRAN JF. Boeck's sarcoid of the pancreas. *Surgery* 1950; **28**: 574-578
15 **Stürmer J**, Becker V. Granulomatous pancreatitis--granulomas in chronic pancreatitis. *Virchows Arch A Pathol Anat Histopathol* 1987; **410**: 327-338
16 **Snook B**, Duggan M, Birkett D. Sarcoidosis of the pancreas: a case report. *Am Surg* 1996; **62**: 947-948
17 **Brady MS**, Garfein CF, Klimstra D, Brenann MF. Sarcoidosis of the pancreas. *J Surg Oncol* 1993; **54**: 132-137
18 **Rodriguez J**, Dyck WP. An unusual case of sarcoidosis presenting as a pancreatic mass. *Am J Gastroenterol* 1996; **91**: 2253-2254
19 **Soyer P**, Gottlieb L, Bluemke DA, Fishman E. Sarcoidosis of the pancreas mimicking pancreatic cancer: CT features. *Eur J Radiol* 1994; **19**: 32-33
20 **Cronin CC**, Dinneen SF, Mitchell TH, Shanahan FL. Sarcoidosis, the pancreas, and diabetes mellitus. *Am J Gastroenterol* 1995; **90**: 2068
21 **Friedman HZ**, Weinstein RA. Sarcoidosis of the pancreas. *Arch Intern Med* 1983; **143**: 2182-2183
22 **Maher L**, Choi H, Dodds WJ. Noncaseating granulomas of the pancreas. Probable sarcoidosis. *Am J Gastroenterol* 1981; **75**: 222-225
23 **Tsou E**, Romano MC, Kerwin DM, Soteropoulos GC, Katz S. Sarcoidosis of anterior mediastinal nodes, pancreas, and uterine cervix: three unusual sites in the same patient. *Am Rev Respir Dis* 1980; **122**: 333-338
24 **Caldwell JH**, Evans WE. Granuloma (sarcoid?) of the pancreas. A case report. *Am J Gastroenterol* 1978; **69**: 320-322
25 **Siavelis HA**, Herrmann ME, Aranha GV, Garcia G, Eubanks T, Reyes CV. Sarcoidosis and the pancreas. *Surgery* 1999; **125**: 456-461
26 **Bacal D**, Hoshal VL, Schaldenbrand JD, Lampman RM. Sarcoidosis of the pancreas: case report and review of the literature. *Am Surg* 2000; **66**: 675-678
27 **Chaun H**, King DM, Gofton JP, Sutherland WH, Bogoch A. Sarcoidosis of the pancreas. *Am J Dig Dis* 1972; **17**: 725-730
28 **Garcia C**, Kumar V, Sharma OP. Pancreatic sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis* 1996; **13**: 28-32
29 **Sagalow BR**, Miller CL, Wechsler RJ. Pancreatic sarcoidosis mimicking pancreatic cancer. *J Clin Ultrasound* 1988; **16**: 131-134
30 **Caceres M**, Sabbaghian MS, Braud R, Wilks S, Boyle M. Pancreatic sarcoidosis: unusual presentation resembling a periampullary malignancy. *Curr Surg* 2006; **63**: 179-185
31 **Büchler MW**, Wagner M, Schmied BM, Uhl W, Friess H, Z'graggen K. Changes in morbidity after pancreatic resection: toward the end of completion pancreatectomy. *Arch Surg* 2003; **138**: 1310-1314; discussion 1315
32 **Bassi C**, Dervenis C, Butturini G, Fingerhut A, Yeo C, Izbicki J, Neoptolemos J, Sarr M, Traverso W, Buchler M. Postoperative pancreatic fistula: an international study group (ISGPF) definition. *Surgery* 2005; **138**: 8-13
33 **Eguro H**, Yabuki M. Sarcoidosis of the skeletal muscle with

tumor formation. A case report and review of the literature. *Yokohama Med Bull* 1966; **17**: 75-90
34 **Johns CJ**, Michele TM. The clinical management of sarcoidosis. A 50-year experience at the Johns Hopkins Hospital. *Medicine* (Baltimore) 1999; **78**: 65-111
35 **Dupas B**, Gournay J, Frampas E, Leaute F, Le Borgne J. Anicteric cholestasis: imaging and diagnostic strategy. *J Radiol* 2006; **87**: 441-459
36 **Li Y**, Ayata G, Baker SP, Banner BF. Cholangitis: a histologic classification based on patterns of injury in liver biopsies. *Pathol Res Pract* 2005; **201**: 565-572
37 **Peyré CG**, Wakim M, Mateo R, Genyk Y, Singh G, Selby RR, Jabbour N. Unusual cases of jaundice secondary to non-neoplastic bile duct obstruction. *Am Surg* 2004; **70**: 620-624
38 **Rudzki C**, Ishak KG, Zimmerman HJ. Chronic intrahepatic cholestasis of sarcoidosis. *Am J Med* 1975; **59**: 373-387
39 **Mehrotra S**, Krishnan PV, Reddy BS, Arora A, Anuradha S, Kar P. Portal hypertension in sarcoid liver disease with hypomelanotic skin involvement. *Trop Gastroenterol* 2004; **25**: 168-169
40 **Sharma SK**, Mohan A. Uncommon manifestations of sarcoidosis. *J Assoc Physicians India* 2004; **52**: 210-214
41 **Nataline MR**, Goyette RE, Owensby LC, Rubin RN. The Budd-Chiari syndrome in sarcoidosis. *JAMA* 1978; **239**: 2657-2657
42 **Russi EW**, Bansky G, Pfaltz M, Spinas G, Hammer B, Senning A. Budd-Chiari syndrome in sarcoidosis. *Am J Gastroenterol* 1986; **81**: 71-75
43 **Stanca CM**, Fiel MI, Allina J, Caracta CF, Odin JA. Liver failure in an antimitochondrial antibody-positive patient with sarcoidosis: primary biliary cirrhosis or hepatic sarcoidosis? *Semin Liver Dis* 2005; **25**: 364-370
44 **Lidar M**, Langevitz P, Livneh A, Weiss P, Yosepovich A. Sclerosing cholangitis associated with systemic sarcoidosis. *J Clin Gastroenterol* 2003; **36**: 84-85
45 **Ishak KG**. Sarcoidosis of the liver and bile ducts. *Mayo Clin Proc* 1998; **73**: 467-472
46 **Takai A**, Fijikawa H, Kawashima Y, Kitazawa E, Watabe H, Sato S, Matsushita M, Miyakawa H. Hepatic sarcoidosis found as a small liver SOL in a patient with type C cirrhosis. *J Gastroenterol* 2005; **40**: 769-770
47 **Blich M**, Edoute Y. Clinical manifestations of sarcoid liver disease. *J Gastroenterol Hepatol* 2004; **19**: 732-737
48 **James DG**, Sherlock S. Sarcoidosis of the liver. *Sarcoidosis* 1994; **11**: 2-6
49 **Diab SM**, Karnik AM, Ouda BA, Denath FM, Fettich J, Francis IM. Sarcoidosis in Arabs: the clinical profile of 20 patients and review of the literature. *Sarcoidosis* 1991; **8**: 56-62
50 **Vatti R**, Sharma OP. Course of asymptomatic liver involvement in sarcoidosis: role of therapy in selected cases. *Sarcoidosis Vasc Diffuse Lung Dis* 1997; **14**: 73-76
51 **Peterli R**, Meyer-Wyss B, Herzog U, Tondelli P. CA19-9 has no value as a tumor marker in obstructive jaundice. *Schweiz Med Wochenschr* 1999; **129**: 77-79
52 **Rømer FK**. Clinical and biochemical aspects of sarcoidosis. With special reference to angiotensin-converting enzyme (ACE). *Acta Med Scand Suppl* 1984; **690**: 3-96
53 **Romer FK**. Angiotensin-converting enzyme activity in sarcoidosis and other disorders. *Sarcoidosis* 1985; **2**: 25-34
54 **McCluggage WG**, Sloan JM. Hepatic granulomas in Northern Ireland: a thirteen year review. *Histopathology* 1994; **25**: 219-228
55 **Loginov AS**, Aruin LI, Shepeleva SD, Shatalova OL, Tkachev VD, Petrova OP. Sarcoidosis and sarcoid-like granulomas in the liver. *Ter Arkh* 1990; **62**: 51-55
56 **Sartin JS**, Walker RC. Granulomatous hepatitis: a retrospective review of 88 cases at the Mayo Clinic. *Mayo Clin Proc* 1991; **66**: 914-918
57 **WAGONER GP**, ANTON AT, GALL EA, SCHIFF L. Needle biopsy of the liver. VIII. Experiences with hepatic granulomas. *Gastroenterology* 1953; **25**: 487-494
58 **Guckian JC**, Perry JE. Granulomatous hepatitis. An analysis of 63 cases and review of the literature. *Ann Intern Med* 1966; **65**: 1081-1100
59 **Maurer LH**. Granulomatous hepatitis associated with regional enteritis. *Gastroenterology* 1967; **53**: 301-305
60 **McMaster KR**, Hennigar GR. Drug-induced granulomatous hepatitis. *Lab Invest* 1981; **44**: 61-73
61 **Bouaziz H**, Kaffel N, Charfi N, Fourati M, Abid H, Abid M. Panhypopituitarism revealing metastasis of small-cell lung carcinoma associated with sarcoidosis. *Ann Endocrinol* (Paris) 2006; **67**: 259-264
62 **Chalasani P**, Vohra M, Sheagren JN. An association of sarcoidosis with hepatocellular carcinoma. *Ann Oncol* 2005; **16**: 1714-1715
63 **Macaya A**, Servitje O, Moreno A, Peyrí J. Cutaneous granulomas as the first manifestation of Hodgkin's disease. *Eur J Dermatol* 2003; **13**: 299-301
64 **Paydas S**, Disel U, Yavuz S, Ergin M. Granulomatous orchitis mimicking Hodgkin's lymphoma relapse. *Hematol J* 2003; **4**: 370-371
65 **Zardawi IM**, Barker BJ, Simons DP. Hodgkin's disease masquerading as granulomatous lymphadenitis on fine needle aspiration cytology. *Acta Cytol* 2005; **49**: 224-226
66 **Sharma OP**. Pulmonary sarcoidosis and corticosteroids. *Am Rev Respir Dis* 1993; **147**: 1598-1600
67 **Sharma OP**. Pulmonary sarcoidosis: management. *J Postgrad Med* 2002; **48**: 135-141
68 **Judson MA**. An approach to the treatment of pulmonary sarcoidosis with corticosteroids: the six phases of treatment. *Chest* 1999; **115**: 1158-1165
69 **Paramothayan NS**, Lasserson TJ, Jones PW. Corticosteroids for pulmonary sarcoidosis. *Cochrane Database Syst Rev* 2005: CD001114
70 **Doty JD**, Mazur JE, Judson MA. Treatment of corticosteroid-resistant neurosarcoidosis with a short-course cyclophosphamide regimen. *Chest* 2003; **124**: 2023-2026
71 **Fazzi P**. Pharmacotherapeutic management of pulmonary sarcoidosis. *Am J Respir Med* 2003; **2**: 311-320
72 **Baughman RP**, Lower EE. Therapy for extrapulmonary sarcoidosis. *Semin Respir Crit Care Med* 2002; **23**: 589-596
73 **Gary A**, Modeste AB, Richard C, Jubert C, Majour F, Nouvet G, Remond B, Joly P. Methotrexate for the treatment of patients with chronic cutaneous sarcoidosis: 4 cases. *Ann Dermatol Venereol* 2005; **132**: 659-662
74 **Hoefnagels MA**, van Leusen R, de Vries RA. Three patients with weight loss as a result of sarcoidosis presenting as liver disease. *Ned Tijdschr Geneeskd* 2003; **147**: 2453-2457
75 **du Bois RM**. Corticosteroids in sarcoidosis: friend or foe? *Eur Respir J* 1994; **7**: 1203-1209
76 **Lower EE**, Baughman RP. The use of low dose methotrexate in refractory sarcoidosis. *Am J Med Sci* 1990; **299**: 153-157
77 **Lower EE**, Baughman RP. Prolonged use of methotrexate for sarcoidosis. *Arch Intern Med* 1995; **155**: 846-851
78 **Wu JJ**, Schiff KR. Sarcoidosis. *Am Fam Physician* 2004; **70**: 312-322
79 **Baratta L**, Cascino A, Delfino M, Giorgino F, Vitolo D, Laganà B, Urani C, Rossi Fanelli F. Ursodeoxycholic acid treatment in abdominal sarcoidosis. *Dig Dis Sci* 2000; **45**: 1559-1562
80 **Neuberger J**, Jothimani D. Long-term immunosuppression for prevention of nonviral disease recurrence. *Transplant Proc* 2005; **37**: 1671-1674
81 **Papo T**, Piette JC, Valla D. Sarcoidosis, liver transplantation, and cyclosporine. *Ann Intern Med* 1993; **119**: 1148; author reply 1148-1149
82 **Bain VG**, Kneteman N, Brown NE. Sarcoidosis, liver transplantation, and cyclosporine. *Ann Intern Med* 1993; **119**: 1148; author reply 1148-1149

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
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CASE REPORT

Cecal lipoma with pseudomalignant features: A case report and review of the literature

Panagiotis Katsinelos, Grigoris Chatzimavroudis, Christos Zavos, Ioannis Pilpilidis, Georgia Lazaraki, Basilis Papaziogas, George Paroutoglou, Jannis Kountouras, Dimitris Paikos

Panagiotis Katsinelos, Ioannis Pilpilidis, Georgia Lazaraki, George Paroutoglou, Dimitris Paikos, Department of Endoscopy and Motility Unit, Central Hospital, Thessaloniki, Greece
Grigoris Chatzimavroudis, Basilis Papaziogas, 2nd Surgical Clinic, Medical School, Aristotle University of Thessaloniki, Greece
Christos Zavos, Jannis Kountouras, 2nd Clinic of Internal Medicine, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece
Correspondence to: Dr. Panagiotis Katsinelos, Department of Endoscopy and Motility Unit, Central Hospital, Ethnikis Aminis 41, 54635, Thessaloniki, Greece. gchatzim@med.auth.gr
Telephone: +30-2310-211221 Fax: +30-2310-210401
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Abstract

Colonic lipoma is a well-documented benign neoplasia, endoscopically appearing as a smooth round yellowish polyp with a thick stalk or broad-based attachment. We describe a 63-year old woman with persistent abdominal pain, in whom colonoscopy revealed a cecal mass with malignant features. Based on the colonoscopy findings, right hemicolectomy was laparoscopically performed for a presumptive diagnosis of a cecal adenocarcinoma, but histological examination revealed a colonic lipoma with overlying mucosal ulceration.

Key words: Colonic lipoma; Pseudomalignant features; Laparoscopic resection

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INTRODUCTION

Lipomas are rare and slow-growing benign nonepithelial tumors that can be found in the gastrointestinal tract[1,2]. Lipomas develop only infrequently in the colon, and are usually asymptomatic and detected incidentally at colonoscopy, surgery or autopsy[3]. The occurrence of symptoms appears to be related to the size of lipoma. Those larger than 2 cm may occasionally cause abdominal pain, changes of bowel habits, rectal bleeding and bowel obstruction, intussusception or prolapse[3-5]. The common endoscopic picture consists of a smooth, spherical polyp, usually sessile and rarely penduculated that is slightly yellow, while the overlying mucosa is intact[1-6]. In rare cases the mucosa presents necrotic and/or ulcerative lesions that resemble malignant tumors[7,8]. The decision for selecting the most suitable treatment of a colonic lipoma mainly depends on the tumor's size and is either endoscopic resection or surgical removal. Lipomas with a diameter less than 2 cm can be safely removed endoscopically, whereas larger lesions should be removed by segment resection[9-11].

We describe a patient with persistent abdominal pain who underwent laparoscopic right hemicolectomy for the presumptive endoscopic diagnosis of cecal adenocarcinoma that turned out to be a lipoma on histological examination.

CASE REPORT

A 63-year old woman with abdominal pain for several weeks was referred to our department by a private gastroenterologist for further investigation of a 2 cm broad-based cecal polypoid mass, revealed during colono-scopy.

The patient did not report any episodes of constipation, diarrhea, hematochezia or melena, and her medical history did not include consumption of alcohol or non-steroid anti-inflammatory drugs (NSAIDs). Examination of the abdomen revealed no mass or tenderness. Detailed laboratory studies were within normal ranges, including complete peripheral blood cell count, blood coagulation and carcinoembryonic antigen (CEA). Colonoscopy performed in our department revealed a sessile tumor of about 2 cm in diameter, proximal to the ileocecal valve. The overlying mucosa was ulcerated and nodular with red color, while on palpation with biopsy forceps, the mass was felt to be stiff, not compressible and easy bleeding (Figure 1A and B). Although histopathological examination of biopsy specimens showed only non-specific ulcerations, malignancy could not be excluded according to the endoscopic findings. Further investigation included an abdominal ultrasound, which was negative for liver metastases and a computed tomography (CT) scan which showed a sessile neoplasm protruding into the lumen of cecum with a medium tissue density.

Given the suspicion of the malignant nature of the tumor, the patient underwent a laparoscopic right

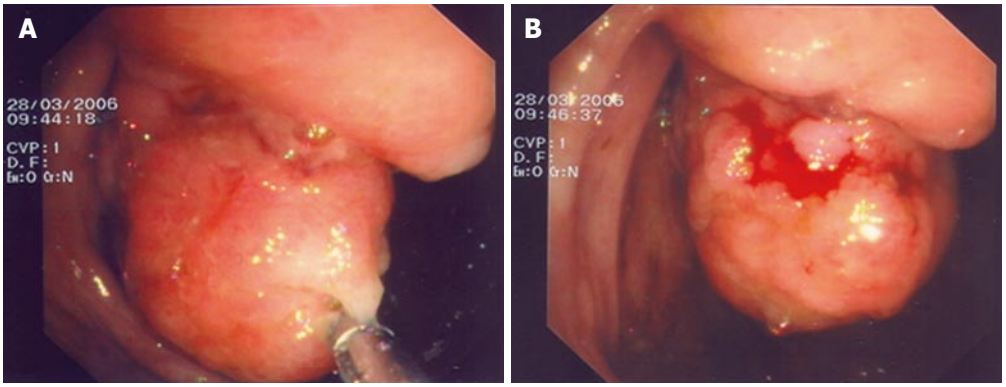

Figure 1 Endoscopic view of a broad-attachment polypoid tumor (**A**) and bleeding from the lesion on compression with biopsy forceps on compression with biopsy forceps (**B**). The overlying mucosa was nodular with ulcerations.

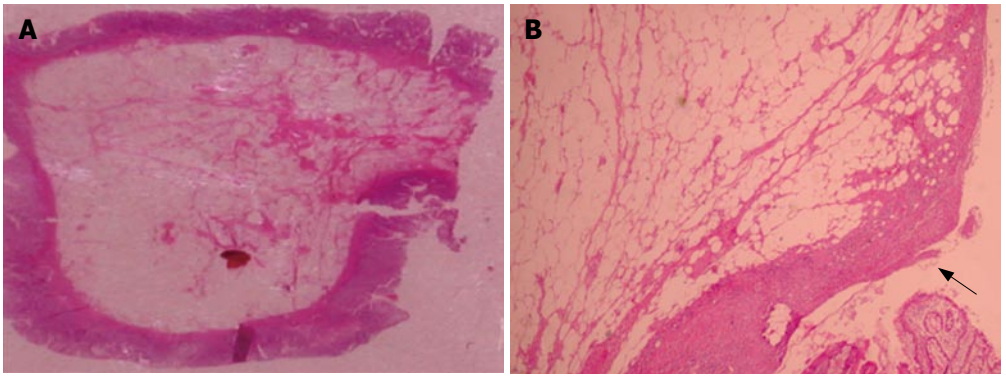

Figure 2 Endoscopic view of a broad-attachment polypoid tumor (**A**) and bleeding from the lesion on compression with biopsy forceps on compression with biopsy forceps (**B**). The overlying mucosa was nodular with ulcerations.

hemicolectomy. Macroscopic assessment of the resected specimen revealed a round polypioid tumor of 2.1 cm × 2.4 cm × 1.8 cm in size, with firm characteristics and nodular surface. Pathological examination of the resected lesion revealed a cecal lipoma with ulcerative lesions on the overlying mucosa (Figure 2A and B). The postoperative course was uneventful, and the patient was free of symptoms during a 10-month follow-up period.

DISCUSSION

Colonic lipomas are relatively uncommon tumors of mesenchymal origin, composed of well-differentiated adipose tissue supported by fibrous tissue, that rarely cause symptoms and are usually detected incidentally[1-3]. They arise from the submucosa, but occasionally extend into the muscularis propria; up to 10% are subserosal[12]. The incidence of lipomas relative to all polypioid lesions of the large intestine is reported to range from 0.035% to 4.4%[1,2]. However, they represent the third most common benign tumors after hyperplastic and adenomatous polyps[13]. They are usually solitary, but multiple lesions are reported in 10% to 25% of cases[1,3]. A rare polyposis syndrome has been described, with numerous lipomas throughout the bowel (colonic lipomatosis)[14]. Colonic lipomas are mainly found on the right-side of the colon and cecum[15]. Most of these tumors are asymptomatic and usually detected incidentally during colonoscopy and laparotomy. Only 25% of patients with colonic lipoma develop symptoms. When lipomas are larger than 2 cm in diameter, they may cause symptoms including bleeding with anemia, diarrhea or constipation, abdominal intestinal obstruction, and rarely, intussusception[16].

Various imaging modalities can imply the diagnosis of colonic lipomas. Barium enema may reveal an ovoid filling defect with well-defined borders. A so-called squeeze sign, indicating a change in size and shape of a radiolucent lesion in response to peristalsis, is frequently noted[17]. CT scans of colonic lipomas can provide a definite diagnosis because the mass typically has characteristic fatty densitometric values[18,19]. On CT, lipomas appear ovoid or pear shaped, with sharp margins and absorption densities of -40 to -120 Hounsfield units, typical of fatty composition[20,21]. However, these features are evident only in large lesions, as smaller tumors are not detectable due to artifacts and partial volume averaging[22]. Correspondingly, in our case, abdominal CT was not diagnostic for lipoma possibly due to the tumor's relatively small size. Magnetic resonance imaging (MRI) may be particularly useful in the detection of lipomas as the signal intensity is characteristic of adipose tissue on T1-weighted and fat-suppressing images[23,24]. Endoscopic ultasonography (EUS) is a potent adjunct modality for characterization of submucosal tumors. Colonic lipomas appear as hyperechoic lesions with regular borders in the three layers and can be distinguished from smooth muscle tumors, lymphangiomata, and invasive or metastatic malignancies[25]. EUS can be used to determine any extension into the muscularis propria before injection-assisted polypectomy of symptomatic lipomas[26]. Recently, CT colonographic examination (virtual colonoscopy) has been performed to detect colonic lipomas[27].

Endoscopy can usually distinguish lipomas from gastrointestinal cancer or other tumors. Lipomas are seen as smooth, rounded yellowish polyps with a thick stalk or

Table 1 Reported cases of colonic lipomas with pseudomalignant features

Author	Year	Age	Sex	Clinical presentation	Site of lipoma	Size (cm)	Treatment	Histological findings of endoscopic biopsies
Loludice[7]	1980	43	M	Hematochezia	Descending	3.9	Left hemicolectomy	Ulcerated mucosal
Lera[30]	1982	70	F	Abdominal pain	Hepatic flexure	4	Right hemicolectomy	-
Snover[8]	1984	57	M	Occult blood	Sigmoid	3	Sigmoid colectomy	Ulcerated mucosa
McGrew[31]	1985	75	M	Hematochezia	Rectosigmoid	5	Endoscopic resection	-
Taylor[32]	1987	62	M	Hematochezia	Sigmoid	10	Sigmoid colectomy	Ulcerated mucosa
Ibrazullah[33]	1992	50	M	Hematochezia	Descending	3	Left hemicolectomy	Inflammatory tissue
El-Khalil[34]	2000	64	M	Hematochezia	Sigmoid	7.8	Left hemicolectomy	Focal hemorrhagic necrosis
Caterino[35]	2002	60	M	Abdominal pain	Hepatic flexure	5	Right hemicolectomy	-
Meghoo[36]	2003	60	F	Hematochezia	Cecum	6	Right hemicolectomy	Ulcerated mucosa
Huh[16]	2006	62	M	Intussusception	Sigmoid	3.5	Anterior resection	Focal hemorrhagic necrosis and inflammation
Katsinelos	2006	62	F	Abdominal pain	Cecum	2.4	Laparoscopic right hemicolectomy	Non-specific ulceration

broad-based attachment[1-6]. Typical colonoscopic features are the "cushion sign" or "pillow sign" (pressing forceps against the lesion results in depression or pillowing of the mass) and the naked fat sign (extrusion of yellowish fat at biopsy site)[4,12,28,29]. Although the mucosa overlying a colonic lipoma is usually not involved, in rare cases, as in our patient, colonoscopy may reveal ulcerations and stiffness on palpation that may lead to a mistaken impression of carcinoma. Eleven such cases[7,8,30-36] reported over 3 decades are summarized in Table 1. Among these patients the most common indications for endoscopy were hematochezia and persistent abdominal pain. The location of lesion was in the cecum in three cases, ascending and transverse colon in one respectively, descending colon in two and rectosigmoid in four cases. In cases where biopsy specimens were taken, only hemorrhagic necrosis or ulceration of the mucosa was identified. In all but one case treatment consisted of operative resection.

The indications for performing colonoscopic snare polypectomy to remove colonic lipomas remain controversial[9,28,37]. Most authors agree that colonic lipomas with a diameter larger than 2 cm should be removed surgically, because in these cases endoscopic resection is associated with a high rate of complications[9,10,38,39]. However, especially for large penduculated lipomas, the size of the stalk seems to be a more important factor than the diameter of the lipoma when colonoscopic removal is considered[10,38]. In our opinion, if a lipoma is sessile or broadly-based, endoscopic removal is risky because the fatty tissue is an inefficient conductor for electric current and may result in a significantly high rate of complications like perforation or hemorrhage.

A wide range of operative techniques using conventional laparotomy[9] and mini-laparotomy[40] have been described including enucleation, colostomy, excision and segmental colonic resection. However, only a few cases of laparoscopic resection of colonic lipomas have been reported so far[13,15,39,41-47]. Advantages of laparoscopic surgery include less postoperative pain, shorter hospitalization and faster recovery than conventional laparotomy. The latest published data referring to the comparison of laparoscopic versus open colorectal resection for cancer[48.49] indicate that laparoscopic resection of colonic lipomas should become the gold standard method for removal of lipomas greater than 2-3 cm in diameter, even in cases where the malignancy of the tumor could not be excluded preoperatively.

In conclusion, our case emphasizes that a colonic lipoma can simulate a malignant neoplasm and underscores the laparoscopic resection as the recommended operation for symptomatic colonic lipomas.

REFERENCES

1 **Corman ML**. Colon and rectal surgery. 4th ed. Philadelphia: Lippincott-Raven, 1998: 913-915
2 **Castro EB**, Stearns MW. Lipoma of the large intestine: a review of 45 cases. *Dis Colon Rectum* 1972; **15**: 441-444
3 **Vecchio R**, Ferrara M, Mosca F, Ignoto A, Latteri F. Lipomas of the large bowel. *Eur J Surg* 1996; **162**: 915-919
4 **Pfeil SA**, Weaver MG, Abdul-Karim FW, Yang P. Colonic lipomas: outcome of endoscopic removal. *Gastrointest Endosc* 1990; **36**: 435-438
5 **Rogy MA**, Mirza D, Berlakovich G, Winkelbauer F, Rauhs R. Submucous large-bowel lipomas-presentation and management. An 18-year study. *Eur J Surg* 1991; **157**: 51-55
6 **Bahadursingh AM**, Robbins PL, Longo WE. Giant submucosal sigmoid colon lipoma. *Am J Surg* 2003; **186**: 81-82
7 **LoIudice T**A, Lang JA. Submucous lipoma simulating carcinoma of the colon. *South Med J* 1980; **73**: 521-523
8 **Snover DC**. Atypical lipomas of the colon. Report of two cases with pseudomalignant features. *Dis Colon Rectum* 1984; **27**: 485-488
9 **Chung YF**, Ho YH, Nyam DC, Leong AF, Seow-Choen F. Management of colonic lipomas. *Aust N Z J Surg* 1998; **68**: 133-135
10 **Stone C**, Weber HC. Endoscopic removal of colonic lipomas. *Am J Gastroenterol* 2001; **96**: 1295-1297
11 **de Ruijter SH**, van Marle AG, Doornewaard H, Melse JC. Submucosal lipoma of the colon: abdominal cramps with rectal bleeding and weight loss. *Ned Tijdschr Geneeskd* 2006; **150**: 1990-1993
12 **De Beer RA**, Shinya H. Colonic lipomas. An endoscopic analysis. *Gastrointest Endosc* 1975; **22**: 90-91
13 **Chiba T**, Suzuki S, Sato M, Tsukahara M, Saito S, Inomata M, Orii S, Suzuki K. A case of a lipoma in the colon complicated by intussusception. *Eur J Gastroenterol Hepatol* 2002; **14**: 701-702
14 **Tatsuguchi A**, Fukuda Y, Moriyama T, Yamanaka N. Lipomatosis of the small intestine and colon associated with intussusception in the ileocecal region. *Gastrointest Endosc* 1999; **49**: 118-121
15 **Cossavella D**, Clerico G, Rosato L, Galetto PV, Paino O, Trompetto M, Luc AR, Pozzo M. Lipoma of the colon as an unusual cause of recurring partial intestinal occlusion. Clinical case and review of the literature. *Minerva Chir* 1998; **53**: 277-280
16 **Huh KC**, Lee TH, Kim SM, Im EH, Choi YW, Kim BK, Jung DJ,

Choi WJ, Kang YW. Intussuscepted sigmoid colonic lipoma mimicking carcinoma. *Dig Dis Sci* 2006; **51**: 791-795
17 **Kaplan P**. Submucous lipoma of the colon. Report of a case. *Int Surg* 1971; **56**: 113-117
18 **Buetow PC**, Buck JL, Carr NJ, Pantongrag-Brown L, Ros PR, Cruess DF. Intussuscepted colonic lipomas: loss of fat attenuation on CT with pathologic correlation in 10 cases. *Abdom Imaging* 1996; **21**: 153-156
19 **Kakitsubata Y**, Kakitsubata S, Nagatomo H, Mitsuo H, Yamada H, Watanabe K. CT manifestations of lipomas of the small intestine and colon. *Clin Imaging* 1993; **17**: 179-182
20 **Wulff C**, Jespersen N. Colo-colonic intussusception caused by lipoma. Case reports. *Acta Radiol* 1995; **36**: 478-480
21 **Heiken JP**, Forde KA, Gold RP. Computed tomography as a definitive method for diagnosing gastrointestinal lipomas. *Radiology* 1982; **142**: 409-414
22 **Megibow AJ**, Redmond PE, Bosniak MA, Horowitz L. Diagnosis of gastrointestinal lipomas by CT. *AJR Am J Roentgenol* 1979; **133**: 743-745
23 **Younathan CM**, Ros PR, Burton SS. MR imaging of colonic lipoma. *J Comput Assist Tomogr* 1991; **15**: 492-494
24 **Liessi G**, Pavanello M, Cesari S, Dell'Antonio C, Avventi P. Large lipomas of the colon: CT and MR findings in three symptomatic cases. *Abdom Imaging* 1996; **21**: 150-152
25 **Kameyama H**, Niwa Y, Arisawa T, Goto H, Hayakawa T. Endoscopic ultrasonography in the diagnosis of submucosal lesions of the large intestine. *Gastrointest Endosc* 1997; **46**: 406-411
26 **Ochiai T**, Akahoshi K, Hamada S, Chijiiwa Y, Nawata H, Namoto M. Endoscopic resection of a colonic lipoma under endoscopic ultrasound guidance. *Endoscopy* 1998; **30**: S65-S66
27 **Johnson CD**, Hara AK, Reed JE. Computed tomographic colonography (Virtual colonoscopy): a new method for detecting colorectal neoplasms. *Endoscopy* 1997; **29**: 454-461
28 **Christie JP**. The removal of lipomas. *Gastrointest Endosc* 1990; **36**: 532-533
29 **Zhang H**, Cong JC, Chen CS, Qiao L, Liu EQ. Submucous colon lipoma: a case report and review of the literature. *World J Gastroenterol* 2005; **11**: 3167-3169
30 **Lera JM**, Gómez-Portilla A, Fakih A, Vicente F, Zornoza G. Submucosal lipomas of the colon. *Rev Med Univ Navarra* 1982; **26**: 185-187
31 **McGrew W**, Dunn GD. Colonic lipomas: clinical significance and management. *South Med J* 1985; **78**: 877-879
32 **Taylor BA**, Wolff BG. Colonic lipomas. Report of two unusual cases and review of the Mayo Clinic experience, 1976-1985. *Dis Colon Rectum* 1987; **30**: 888-893
33 **Ibrarullah M**, Aggarwal R, Choudhuri G, Tandon P, Kapoor VK. Colonic lipoma, masquerading as malignant tumour. *Indian J Cancer* 1992; **29**: 4-6
34 **El-Khalil T**, Mourad FH, Uthman S. Sigmoid lipoma mimicking carcinoma: case report with review of diagnosis and management. *Gastrointest Endosc* 2000; **51**: 495-496
35 **Caterino S**, Tiziano G, Mercantini P, Ziparo V. Rectorrhagia caused by lipoma of the hepatic flexure of the colon. A case report and review of the literature. *G Chir* 2002; **23**: 205-208
36 **Meghoo CA**, Cook PR, McDonough CA, Bowser LK, Waddell BE. Large colonic lipoma with mucosal ulceration mimicking carcinoma. *Gastrointest Endosc* 2003; **58**: 468-470
37 **Yu JP**, Luo HS, Wang XZ. Endoscopic treatment of submucosal lesions of the gastrointestinal tract. *Endoscopy* 1992; **24**: 190-193
38 **Tamura S**, Yokoyama Y, Morita T, Tadokoro T, Higashidani Y, Onishi S. "Giant" colon lipoma: what kind of findings are necessary for the indication of endoscopic resection? *Am J Gastroenterol* 2001; **96**: 1944-1946
39 **Abou-Nukta F**, Gutweiler J, Khaw J, Yavorek G. Giant lipoma causing a colo-colonic intussusception. *Am Surg* 2006; **72**: 83-84
40 **Nakagoe T**, Sawai T, Tsuji T, Tanaka K, Nanashima A, Shibasaki S, Yamaguchi H, Yasutake T. Minilaparotomy approach for removal of a large colonic lipoma: report of two cases. *Surg Today* 2004; **34**: 72-75
41 **Peters MB**, Obermeyer RJ, Ojeda HF, Knauer EM, Millie MP, Ertan A, Cooper S, Sweeney JF. Laparoscopic management of colonic lipomas: a case report and review of the literature. *JSLS* 2005; **9**: 342-344
42 **Hackam DJ**, Saibil F, Wilson S, Litwin D. Laparoscopic management of intussusception caused by colonic lipomata: a case report and review of the literature. *Surg Laparosc Endosc* 1996; **6**: 155-159
43 **Ladurner R**, Mussack T, Hohenbleicher F, Folwaczny C, Siebeck M, Hallfeld K. Laparoscopic-assisted resection of giant sigmoid lipoma under colonoscopic guidance. *Surg Endosc* 2003; **17**: 160
44 **Saclarides TJ**, Ko ST, Airan M, Dillon C, Franklin J. Laparoscopic removal of a large colonic lipoma. Report of a case. *Dis Colon Rectum* 1991; **34**: 1027-1029
45 **Jelenc F**, Brencic E. Laparoscopically assisted resection of an ascending colon lipoma causing intermittent intussusception. *J Laparoendosc Adv Surg Tech A* 2005; **15**: 173-175
46 **Chiu CC**, Wei PL, Huang MT, Wang W, Chen TC, Lee WJ. Colonoscopic-assisted laparoscopic resection of a colon lipoma. *J Laparoendosc Adv Surg Tech A* 2006; **16**: 37-40
47 **Scoggin SD**, Frazee RC. Laparoscopically assisted resection of a colonic lipoma. *J Laparoendosc Surg* 1992; **2**: 185-189
48 **Zerey M**, Burns JM, Kercher KW, Kuwada TS, Heniford BT. Minimally invasive management of colon cancer. *Surg Innov* 2006; **13**: 5-15
49 **Lezoche E**, Guerrieri M, De Sanctis A, Campagnacci R, Baldarelli M, Lezoche G, Paganini AM. Long-term results of laparoscopic versus open colorectal resections for cancer in 235 patients with a minimum follow-up of 5 years. *Surg Endosc* 2006; **20**: 546-553

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PO Box 2345, Beijing 100023, China
www.wjgnet.com
wjg@wjgnet.com

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CASE REPORT

Telerobotic-assisted laparoscopic abdominoperineal resection for low rectal cancer: Report of the first case in Hong Kong and China with an updated literature review

Simon Siu-Man Ng, Janet Fung-Yee Lee, Raymond Ying-Chang Yiu, Jimmy Chak-Man Li, Sophie Sok-Fei Hon

Simon Siu-Man Ng, Janet Fung-Yee Lee, Raymond Ying-Chang Yiu, Jimmy Chak-Man Li, Sophie Sok-Fei Hon, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong SAR, China
Correspondence to: Dr. Simon Siu-Man Ng, Department of Surgery, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, Hong Kong SAR, China. simonng@surgery.cuhk.edu.hk
Telephone: +852-26321495 Fax: +852-26377974
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Abstract

Telerobotic surgery is the most advanced development in the field of minimally invasive surgery. The da Vinci surgical system, which is currently the most widely used telerobotic device, was approved by the Food and Drug Administration of the United States of America for clinical use in all abdominal operations in July 2000. The first da Vinci surgical system in China was installed in November 2005 at our institution. We herein report the first telerobotic-assisted laparoscopic abdominoperineal resection using the 3-arm da Vinci surgical system for low rectal cancer in Hong Kong and China, which was performed in August 2006. The operative time and blood loss were 240 min and 200 mL, respectively. There was no complication, and the patient was discharged on postoperative day five. An updated review of published literature on telerobotic-assisted colorectal surgery is included in this report, with special emphasis on its advantages and limitations.

Key words: Telerobotic-assisted surgery; da Vinci; Colorectal surgery; Abdominoperineal resection; China

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INTRODUCTION

The minimally invasive or laparoscopic approach has revolutionised surgical care over the past two decades. Since the introduction of laparoscopic cholecystectomy in 1987, laparoscopic surgery has been attempted and applied to many surgical operations, including resection of benign and malignant colorectal diseases[1]. Although the laparoscopic approach in colorectal surgery has been shown to be beneficial and oncologically safe[2-4], its use is still not yet widespread[5,6]. This restriction may be due to the technical difficulties and the steep learning curve associated with these complex procedures[7]. Conventional laparoscopic surgery has many inherent technical drawbacks, including unstable video camera platform, lack of stereoscopic or three-dimensional (3D) vision, limited motion of straight instruments, long instruments that can increase physiological hand tremor, and poor ergonomics for the surgeon[8]. The introduction of the telerobotic surgical systems may offer potential solutions to the above-mentioned problems.

The da Vinci surgical system (Intuitive Surgical, Inc., Sunnyvale, CA) was developed to facilitate laparoscopic surgery and overcome its disadvantages[9]. This telerobotic system comprises three main components: the robotic cart with three or four mechanical arms, the console, and the endoscopic stack. The operating surgeon sits comfortably at the console, with his hands placed on master handles. His movements are then translated via computer software to the robotic arms at the site of the operation. The system provides a magnified 3D view and intuitively transfers movements from the handle to the tip of the instrument with tremor filtering. Dexterity is enhanced *via* EndoWrist (Intuitive Surgical, Inc., Sunnyvale, CA) technology, returning seven degrees of freedom to the surgeon, so that precise manoeuvres like anastomosis can be accomplished easily. The system can actually facilitate less experienced surgeons to perform minimally invasive surgery in a smooth and more ergonomically manner.

The Food and Drug Administration of the United States of America approved the da Vinci surgical system for clinical use in all abdominal operations in July 2000. The first reported telerobotic-assisted laparoscopic colorectal procedures were performed in late 2000 in Japan[10]. Since then, there have been a small number of series in the literature describing the use of telerobotic systems in the field of colorectal surgery[11-17]. The first da Vinci surgical system in China was installed in November 2005 at our institution in Hong Kong. We herein report the first telerobotic-assisted laparoscopic abdominoperineal resection using the 3-arm da Vinci surgical system for

low rectal cancer in Hong Kong and China, which was performed in August 2006. An updated review of published literature on telerobotic-assisted colorectal surgery is included in this report, with special emphasis on its advantages and limitations.

CASE REPORT

A 49-year-old woman with a body mass index of 25.2 kg/m^2 presented with rectal bleeding associated with constipation and tenesmus for one year. Her past medical history included hypertension and diabetes mellitus. Colonoscopy revealed a tumour at the lower rectum 3 cm above the anal verge, and biopsy confirmed the diagnosis of adenocarcinoma. Computed tomography showed no evidence of loco-regional invasion or distant metastasis. The patient underwent telerobotic-assisted laparoscopic abdominoperineal resection using the 3-arm da Vinci surgical system on August 25, 2006. The patient understood that this was the first case of its kind in Hong Kong and China, and informed consent was obtained prior to surgery. Mechanical bowel preparation was carried out one day before surgery with sodium phosphates oral solution. Systemic prophylactic antibiotics (1.5 g cefuroxime and 500 mg metronidazole) were administered intravenously at induction of general anaesthesia.

Operative strategy and techniques

The operating surgeon and the assistants were skilled in both laparoscopic and colorectal surgery. All members of the surgical team had also undergone a specific two-day robotic-assisted animal laparoscopic surgery training course before the surgery, which consisted of didactic and practical application sessions covering the components and use of the da Vinci surgical system and EndoWrist instruments on live animals. Live telerobotic surgery on patients was also demonstrated.

The patient was placed in the Lloyd-Davies position. Pneumoperitoneum was created with the open technique just below the umbilicus. A zero-degree 10-mm laparoscope was used. Additional trocars (T1-T5) were inserted as shown in Figure 1. T1 (13-mm trocar used for stapling) was inserted 5 cm lateral to the right midclavicular line above the level of the umbilicus. T2 (5-mm trocar) was inserted 5 cm lateral to the right midclavicular line above the level of the right anterior superior iliac spine (ASIS). T3 (5-mm trocar) was inserted 5 cm lateral to the left midclavicular line at the level of the umbilicus. T4 (8-mm robotic trocar) was inserted just medial to the right midclavicular line between the camera trocar and the right ASIS. Finally, T5 (8-mm robotic trocar) was inserted in the left lower quadrant of the abdomen, opposite and symmetrical to T4.

The first part of the surgery was carried out laparoscopically. The operating surgeon used T1 and T2 for dissection, while the assistants held the camera and T3. With the patient tilted head down, the sigmoid-descending colon was mobilised along the white line of Toldt. The left ureter was identified and protected. The inferior mesenteric vessels were transected distal to the left colic vessels with

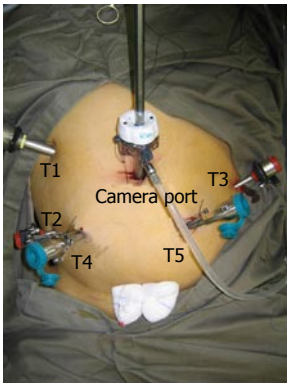

Figure 1 Trocar positions for telerobotic-assisted laparoscopic abdominoperineal resection using the 3-arm da Vinci surgical system.

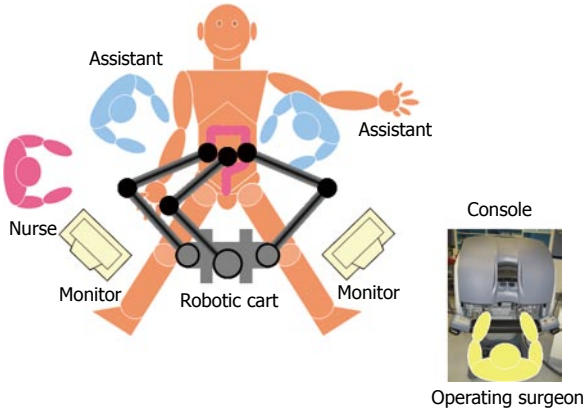

Figure 2 Operating theatre setup for telerobotic-assisted laparoscopic abdominoperineal resection using the 3-arm da Vinci surgical system.

EndoGIA staplers (Autosuture, Tyco Healthcare, Norwalk, CT).

After complete laparoscopic mobilisation of the sigmoid-descending colon and transection of the lymphovascular pedicle, the 3-arm da Vinci surgical system was brought onto the field. With the patient still in the head-down position, the three robotic arms were docked to the camera port and ports T4 and T5 as shown in Figure 2. The operating surgeon sat at the console in the operating theatre and controlled the robotic camera and the two robotic arms. The left robotic arm (T5) carried a Cadière forceps while the right robotic arm (T4) carried a monopolar cautery hook (Figure 3). The assistants stood on both sides of the patients using T1, T2, and T3 for suction and retraction. The rectosigmoid mesentery was retracted superiorly and anteriorly, and the Holy plane was entered. Total mesorectal excison was performed with the da Vinci surgical system down to the pelvic floor. The ureters, the hypogastric nerves, and the pelvic parasympathetic plexus were carefully identified and safeguarded.

The da Vinci surgical system was disengaged after rectal mobilisation was completed. The sigmoid colon was transected with EndoGIA staplers; an abdominal wall opening was created by excision of port T5, and the proximal sigmoid colon was drawn through this opening to form an end colostomy. Perineal dissection was then performed, and the resected specimen was retrieved through the perineal wound. The perineal wound was

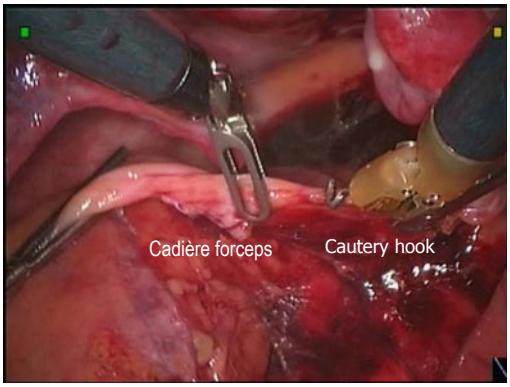

Figure 3 Intraoperative photograph showing the Cadière forceps in the left robotic arm and the monopolar cautery hook in the right robotic arm.

closed primarily with a drain put in the pelvic cavity via a separate stab wound.

The total operative time was about 240 min, with 45 min spent on insertion of trocars and laparoscopic mobilisation of the sigmoid colon, 15 min spent on docking of the da Vinci surgical system, 120 min spent on telerobotic-assisted rectal mobilisation, and 60 min spent on perineal dissection and creation of colostomy. The intraoperative blood loss was 200 mL and no blood transfusion was required.

The patient was able to resume oral diet on the next day after surgery. She opened her bowel on postoperative day four, and she was discharged on postoperative day five. There was no complication.

Pathological examination of the resected specimen revealed moderately differentiated adenocarcinoma with invasion into the muscularis propria but not through it yet. One out of sixteen removed lymph nodes showed metastasis. At the time of writing of this report (five months after the surgery), the patient is still undergoing adjuvant therapy. She remains well and asymptomatic.

DISCUSSION

Hashizume *et al*[10] from Japan were the first to publish their experiences with telerobotic-assisted colorectal resections, performing one ileocaecal resection, one left hemicolectomy, and one sigmoid colectomy for cancer in late 2000. The first telerobotic-assisted laparoscopic colectomies for benign diseases were performed by Weber *et al*[11] in March 2001. Since then, there have been a small number of published case series in the literature (Table 1) demonstrating the feasibility and safety of telerobotic-assisted colorectal procedures using the da Vinci surgical system, ranging from partial colectomies to rectal resections[12-17]. The advantages of the telerobotic surgical system including enhanced vision, facilitated dexterity, and better ergonomics were acknowledged by all the authors. The operative time, however, was long in most of the early series. Extra time was needed for setting up of the operating theatre and docking/repositioning of the telerobotic surgical system, which might decrease with experience. In a recent study by Rawlings *et al*[17], the total operative time for telerobotic-assisted right hemicolectomy had become faster after 17 cases (from > 300 min in the first case to about 200 min in the last few cases), while no definite change in operative time trend was observed after 13 cases of telerobotic-assisted sigmoid colectomies. For experienced minimally invasive surgeons, the learning curve for telerobotic-assisted colorectal surgery is believed to be about 20 cases[18].

We have chosen to start our telerobotic-assisted colorectal surgery programme with a case of rectal resection. One of the reasons is that the telerobotic surgical systems have been considered to be particularly useful when the operative field is small and precise dissection is required. In colonic surgery, most of the dissection entails up-and-down and left-to-right movement in different quadrants of the peritoneal cavity; the small excursion arcs of the robotic arms may not adequately encompass this wide field of dissection, and thus repositioning of the patient and the robotic cart is often necessary. We feel that the telerobotic surgical system may be more useful in rectal surgery because the robotic arms can allow for very accurate dissection in the confined pelvic space, and the magnified 3D vision can give an excellent view of the pelvic anatomy. In order to avoid repositioning of the robotic cart during surgery, we mobilised the sigmoid-descending colon with conventional laparoscopic techniques first, before we brought in the robotic cart for telerobotic-assisted rectal mobilisation. With this hybrid technique, our case can be finished within 240 min, which compares favourably with the mean operative time of 215 min in our previous report on laparoscopic-assisted abdominoperineal resection for low rectal cancer[19].

Table 2 summarises the published studies comparing telerobotic-assisted colorectal surgery using the da Vinci surgical system versus conventional laparoscopic colorectal surgery[20-23]. In the largest comparative study to date, D'Annibale *et al*[21] compared 53 telerobotic-assisted with 53 conventional laparoscopic colorectal procedures, ranging from partial/total colectomies to abdominoperineal resections. No differences were observed in the total time of surgery (telerobotic group, 240 min *vs* laparoscopic group, 222 min), time to recovery of bowel function, and duration of hospital stay. The only significant difference was a longer setup time to prepare for the operating theatre and the patient in the telerobotic group (24 min *vs* 18 min; $P = 0.002$). In a more recent study by Pigazzi *et al*[23], 6 telerobotic-assisted total mesorectal excisions (TME) were compared with 6 conventional laparoscopic TME. The two groups were not different in total operative time (telerobotic group, 4.4 h *vs* laparoscopic group, 4.3 h), complication rates, and duration of hospital stay. However, surgeons in the telerobotic group reported less fatigue and less strain after surgery. Interestingly, the issue of cost was only addressed in the first comparative study[20], which reported additional direct equipment costs of approximately US$ 350 per case for the robotic group, without including acquisition and maintenance costs for the telerobotic surgical system.

Although most of the published studies on telerobotic-

Table 1 Published case series and reports on telerobotic-assisted colorectal surgery using the da Vinci surgical system

First author (yr)	Country	Types and number of surgery					Operative time (min)	Conversion to open (number)	Complications
		RH	SC	AR/LAR	APR	Others			
Hashizume *et al*[10] (2002)	Japan		1			1 ileocaecal resection (IC) 1 left hemicolectomy (LH)	IC: 335 SC: 180 LH: 265	0	None
Weber *et al*[11] (2002)	USA	1	1				RH: 228 SC: 340	0	None
Giulianotti *et al*[12] (2003)	Italy	5	1	6	2	2 ileocaecal resections	Mean 211	0	1 leak after AR
Vibert *et al*[13] (2003)	France		1	1		1 Hartmann reversal (HR)	SC: 330 LAR: 450 HR: 360	0	1 prolonged ileus after LAR
Talamini *et al*[14] (2003)	USA					17 'bowel resections'	Not clear	Not clear	Not clear
Braumann *et al*[15] (2005)	Germany	1	2	2			Mean 201	2	None
Ruurda *et al*[16] (2005)	Netherlands					5 ileocaecal resections 2 sigmoid colostomies 16 rectopexies	Median 95 Median 75 Median 150	0	1 bowel injury during rectopexy 1 wound infection after IC
Rawlings *et al*[17] (2006)	USA	17	13				RH: 219 SC: 225	2	6 complications, including 2 bowel injuries and 1 leak
Present case	Hong Kong, China				1		240	0	None

USA: United States of America; RH: Right hemicolectomy; SC: Sigmoid colectomy; AR: Anterior resection; LAR: Low anterior resection; APR: Abdominoperineal resection.

Table 2 Published studies comparing telerobotic-assisted colorectal surgery using the da Vinci surgical system versus conventional laparoscopic colorectal surgery

First author (yr)	Procedures	Number of patients	Total operative time	Blood loss (mL)	Complications	Hospital stay (d)	Remarks
Delaney *et al*[20] (2003)	RH, SC, rectopexies	Telerobotic 6	216.5 min[a]	100	1 atelectasis	3	Additional direct equipment costs of US$ 350 per case for telerobotic surgery
		Laparoscopic 6	150 min[a]	87.5	1 incisional hernia	2.5	
D'Annibale *et al*[21] (2004)	Partial/total colectomies and rectal resections	Telerobotic 53	240 min	21	1 reoperation for bowel injury	10	Significantly longer setup time in the telerobotic group
		Laparoscopic 53	222 min	37	1 reoperation for bleeding	10	
Woeste *et al*[22] (2005)	SC for sigmoid diverticulitis	Telerobotic 4	236.7[c]	60	1 (25%)	/	/
		Laparoscopic 23	172.4[c]	58.9	5 (21.7%)	/	
Pigazzi *et al*[23] (2006)	TME for rectal cancer	Telerobotic 6	4.4 h	104	1 prolonged ileus	4.5	Telerobotic surgery caused less strain for surgeons
		Laparoscopic 6	4.3 h	150	1 pelvic abscess	3.6	

RH: Right hemicolectomy; SC: Sigmoid colectomy; TME: Total mesorectal excision. [a]$P < 0.05$ by Mann-Whitney *U* test; [c]$P < 0.05$ by Student's *t*-test. No significant differences were noted in all the other parameters.

assisted colorectal surgery have shown comparable results when compared to conventional laparoscopic surgery, none have yet demonstrated a clear advantage to using the telerobotic system in colorectal surgery. Other limitations of applying this technology to the field of colorectal surgery include lack of tactile feedback, limited instrumentation, inconvenience of rotating or tilting the patient during surgery, and need for repositioning to facilitate dissection in different quadrants of the abdomen during surgery. Besides, precise manoeuvres like anastomosis are seldom required in colorectal surgery. Nevertheless, with continued refinement in technologies and techniques, many of the above-mentioned limitations can be overcome. Undoubtedly, the telerobotic system can benefit the surgeons by providing excellent dexterity, vision, and ergonomics, but whether this can be translated into better patient outcomes still needs further evaluation.

REFERENCES

1 **Kwok SP**, Lau WY, Carey PD, Kelly SB, Leung KL, Li AK. Prospective evaluation of laparoscopic-assisted large bowel excision for cancer. *Ann Surg* 1996; **223**: 170-176

2 **Leung KL**, Kwok SP, Lam SC, Lee JF, Yiu RY, Ng SS, Lai PB, Lau WY. Laparoscopic resection of rectosigmoid carcinoma: prospective randomised trial. *Lancet* 2004; **363**: 1187-1192

3 **Ng SS**, Leung KL, Lee JF, Yiu RY, Li JC. MRC CLASICC trial. *Lancet* 2005; **366**: 713; author reply 713-714

4 **Ng SS**, Li JC, Lee JF, Yiu RY, Leung KL. Laparoscopic total colectomy for colorectal cancers: a comparative study. *Surg Endosc* 2006; **20**: 1193-1196

5 **Lauter DM**, Serna S. Surgeon experience with laparoscopic-assisted colorectal surgery in Washington State. *Am J Surg* 2003; **186**: 13-16

6 **Harinath G**, Shah PR, Haray PN, Foster ME. Laparoscopic colorectal surgery in Great Britain and Ireland--where are we now? *Colorectal Dis* 2005; **7**: 86-89

7 **Tekkis PP**, Senagore AJ, Delaney CP, Fazio VW. Evaluation of the learning curve in laparoscopic colorectal surgery:

comparison of right-sided and left-sided resections. *Ann Surg* 2005; **242**: 83-91
8 **Hance J**, Rockall T, Darzi A. Robotics in colorectal surgery. *Dig Surg* 2004; **21**: 339-343
9 **Rockall TA**. The da Vinci telerobotic surgical system. In: Ballantyne GH, Marescaux J, Giulianotti PC. Primer of robotic & telerobotic surgery. Philadelphia: Lippincott Williams & Wilkins, 2004: 57-60
10 **Hashizume M**, Shimada M, Tomikawa M, Ikeda Y, Takahashi I, Abe R, Koga F, Gotoh N, Konishi K, Maehara S, Sugimachi K. Early experiences of endoscopic procedures in general surgery assisted by a computer-enhanced surgical system. *Surg Endosc* 2002; **16**: 1187-1191
11 **Weber PA**, Merola S, Wasielewski A, Ballantyne GH. Telerobotic-assisted laparoscopic right and sigmoid colectomies for benign disease. *Dis Colon Rectum* 2002; **45**: 1689-1694; discussion 1695-1696
12 **Giulianotti PC**, Coratti A, Angelini M, Sbrana F, Cecconi S, Balestracci T, Caravaglios G. Robotics in general surgery: personal experience in a large community hospital. *Arch Surg* 2003; **138**: 777-784
13 **Vibert E**, Denet C, Gayet B. Major digestive surgery using a remote-controlled robot: the next revolution. *Arch Surg* 2003; **138**: 1002-1006
14 **Talamini MA**, Chapman S, Horgan S, Melvin WS. A prospective analysis of 211 robotic-assisted surgical procedures. *Surg Endosc* 2003; **17**: 1521-1524
15 **Braumann C**, Jacobi CA, Menenakos C, Borchert U, Rueckert JC, Mueller JM. Computer-assisted laparoscopic colon resection with the Da Vinci system: our first experiences. *Dis Colon Rectum* 2005; **48**: 1820-1827
16 **Ruurda JP**, Draaisma WA, van Hillegersberg R, Borel Rinkes IH, Gooszen HG, Janssen LW, Simmermacher RK, Broeders IA. Robot-assisted endoscopic surgery: a four-year single-center experience. *Dig Surg* 2005; **22**: 313-320
17 **Rawlings AL**, Woodland JH, Crawford DL. Telerobotic surgery for right and sigmoid colectomies: 30 consecutive cases. *Surg Endosc* 2006; **20**: 1713-1718
18 **Ewing DR**, Pigazzi A, D'Annibale A, Morpurgo E, Fiscon V, Haag BL, Ballantyne GH. Telerobotic laparoscopic colorectal surgery. In: Ballantyne GH, Marescaux J, Giulianotti PC. Primer of robotic & telerobotic surgery. Philadelphia: Lippincott Williams & Wilkins, 2004: 171-180
19 **Leung KL**, Kwok SP, Lau WY, Meng WC, Chung CC, Lai PB, Kwong KH. Laparoscopic-assisted abdominoperineal resection for low rectal adenocarcinoma. *Surg Endosc* 2000; **14**: 67-70
20 **Delaney CP**, Lynch AC, Senagore AJ, Fazio VW. Comparison of robotically performed and traditional laparoscopic colorectal surgery. *Dis Colon Rectum* 2003; **46**: 1633-1639
21 **D'Annibale A**, Morpurgo E, Fiscon V, Trevisan P, Sovernigo G, Orsini C, Guidolin D. Robotic and laparoscopic surgery for treatment of colorectal diseases. *Dis Colon Rectum* 2004; **47**: 2162-2168
22 **Woeste G**, Bechstein WO, Wullstein C. Does telerobotic assistance improve laparoscopic colorectal surgery? *Int J Colorectal Dis* 2005; **20**: 253-257
23 **Pigazzi A**, Ellenhorn JD, Ballantyne GH, Paz IB. Robotic-assisted laparoscopic low anterior resection with total mesorectal excision for rectal cancer. *Surg Endosc* 2006; **20**: 1521-1525

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Luc JW van der Laan, PhD, Associate Professor
Departements of Surgery and Gastroenterology&Hepatology, Erasmus MC-University Medical Centre, 's Gravendijkwal 230, Room L458, Rotterdam, 3015 CE, The Netherlands

Robert Thimme, MD, Professor
Department of Medicine II, University Hospital Freiburg, Hugstetter Strasse 55, D-79106 Freiburg, Germany

Hisato Nakajima, MD
Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

Yogesh K Chawla, Dr, Professor
Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

Edoardo G Giannini, Assistant Professor
Department of Internal Medicine, Gastroenterology Unit, Viale Benedetto XV, no. 6, Genoa, 16132, Italy

Paolo Del Poggio, Dr
Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

Manuel Romero-Gómez, MD, Professor
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Markus Peck-Radosavljevic, Professor
Department of Internal Medicine IV, Division of Gastroenterology and Hepatology, University of Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Luc JW van der Laan, PhD, Associate Professor
Departements of Surgery and Gastroenterology&Hepatology, Erasmus MC-University Medical Centre, 's Gravendijkwal 230, Room L458, Rotterdam, 3015 CE, The Netherlands

Yukihiro Shimizu, Dr
Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan

Fernando Alvarez, Professor
Service de gastroentérologie, hépatologie et nutrition, Hôpital Sainte-Justine, 3175 Côte Ste-Catherine, Montréal, Québec, Canada H3T 1C5, Canada

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Masato Kusunoki, Professor and Chairman
Second Department of Surgery, Mie University School of Medicine, Mie, 2-174 Edobashi, Tsu MIE 514-8507, Japan

Ignacio Gil-Bazo, MD, PhD
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Akihito Tsubota, Assistant Professor
Institute of Clinical Medicine and Research , Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

Rick Greupink, Dr
University of Groningen, A. Deusinglaan 1, Groningen 9713AV, The Netherlands

Inge Irma Depoortere, PhD
Centre for Gastroenterological Research, Gasthuisberg OandN, bus 701, Leuven 3000, Belgium

Valentin Fuhrmann, MD
Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Waehringer Guertel 18-20, A-1090 Vienna, Austria

Massimo Raimondo, Dr
Division of Gastroenterology and Hepatology, Mayo Clinic, 4500 San Pablo Road, Jacksonville, FL 32224, United States

John K Marshall, MD, Associate Professor of Medicine
Division of Gastroenterology (4W8), McMaster University Medical Centre, 1200 Main Street West, Hamilton, Ontario L8N 3Z5, Canada

Thierry Piche , MD, PhD
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Marc Basson, MD, PhD, MBA
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Indra Neil Guha, MD
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Centre for Integrated Health Care Research, Wolfson Research Institute, University of Durham, Queen's Campus, Stockton on Tees TS17 6BH, United Kingdom

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