Virchows Arch (1996) 429:353–358

ORIGINAL ARTICLE

Gieri Cathomas · Christine E. McGandy
Luigi M. Terracciano · Fred Gudat
Leonardo Bianchi

Detection and typing of hepatitis C RNA in liver biopsies and its relation to histopathology

Received: 30 April 1996 / Accepted: 10 September 1996

Abstract This paper describes the correlation of hepatitis C genotypes detected in liver tissue with histological grading (inflammatory activity) and staging (degree of fibrosis/cirrhosis). The viral genotype was analysed by type-specific polymerase chain reaction (PCR) and correlated with histology and age of patients. In 69 patients with chronic hepatitis C (HCV) infection, genotypes 1a and 1b were detected in 13 (18.8%) and 31 (44.9%) liver biopsies, respectively. Genotypes 2a and 2b were each detected once (1.5%) and 12 (17.4%) tissue samples showed a mixed infection with two genotypes. In 11 (15.9%) biopsies, no genotype could be established. The liver specimens were grouped according to the presence or absence of genotype 1b: group A consisted of specimens infected with genotypes 1a, 2a, and 2b (n=16), Group B contained biopsies infected with genotype 1b (n=42), and group C were biopsies with no detectable genotype (n=11). Activity (grade) of chronic hepatitis was not different in these three groups. However, advanced fibrosis/cirrhosis was observed in 16 (38.1%) biopsies in group B (containing genotype 1b), compared with none in group A (P=0.01). The mean age of patients in group B was significantly higher than that in group A (P=0.038), and the mean age of patients with advanced fibrosis was higher than that of patients with low fibrosis scores within these two groups (P=0.004). Stepwise logistic regression revealed an independent association of age and genotype 1b (group B) with advanced fibrosis/cirrhosis. These data indicate that patients infected with genotype 1b have an higher risk of developing cirrhosis than do patients with other genotypes.

Key words HCV · Genotypes · Histopathology · Grading · Staging

G. Cathomas (✉) · C.E. McGandy · L.M. Terracciano
F. Gudat · L. Bianchi
Institute for Pathology, University of Basel, Basel, Switzerland
Kantonsspital, Schönbeinstrasse 40,
CH-4003 Basel, Switzerland
Tel.: (41) 61-265 25 25, Fax: (41) 61-265 31 94

Introduction

Hepatitis C virus (HCV) is the major cause of post-transfusion, intravenous drug use, and of sporadic, community-acquired non-A, non-B hepatitis. Chronic infection with this virus may lead to cirrhosis and hepatocellular carcinoma [1, 9, 20]. The diagnosis of HCV is based on the detection of specific anti-HCV antibodies against recombinant proteins and the detection of viral RNA by the polymerase chain reaction (PCR) in the serum [13, 22]. Although earlier reports have described the histological features of non-A, non-B hepatitis [5, 11, 34], it was not until the introduction of appropriate diagnostic tests that specific findings of lymphoid follicles, bile duct lesions and steatosis were shown to be preferentially associated with chronic hepatitis C [3, 16, 19, 24, 37].

HCV is a positive strand RNA virus of about 9500 bases and is related to pestivirus and flavivirus [9, 26]. Nucleotide sequences of clinical HCV isolates have revealed considerable nucleotide differences. Based on this nucleotide variability, it has been proposed that HCV be classified into different genotypes [6, 31]. Recently, a consensus system for nomenclature has been proposed by an international group, and this nomenclature has been used in this paper [39]. Several studies have focused on the relationship of specific HCV genotypes detected in the serum and the clinical course of chronic hepatitis, and also on the sensitivity to antiviral therapy by interferon alpha (IFN-α). A higher incidence of cirrhosis and a lower rate of response to IFN-α were reported in patients infected by genotype 1b [7, 27, 30, 35, 41, 44]. Other studies, however, have claimed the virus load to be predictive of sustained response to IFN-α therapy independent of the viral genotype [29, 43].

In this study we analysed the genotype of HCV in liver biopsies from patients with chronic hepatitis C infection. We have correlated the genotype with patient age and the histological activity, stage and the presence of individual features considered characteristic for hepatitis C.

Materials and methods

Sixty-nine consecutive liver biopsies of patients with chronic hepatitis C in which frozen liver tissue was available were studied. All were positive for anti HCV antibodies (second-generation enzyme immunoassay) and negative for HBsAg or anti-HBc IgM antibodies. None of the patients had been treated with IFN-α within the past 12 months. All patients underwent liver biopsy for diagnostic purposes because of elevated liver function tests. One part of the biopsy (0.5–1 cm) was frozen at –70°C. For conventional histology, the liver tissue was fixed in 4% neutral buffered formalin and stained with haematoxylin and eosin, chromotrope aniline blue, orcein, periodic acid–Schiff following diastase digestion, sirius red and Prussian blue.

The grade of activity of chronic hepatitis was categorized using the following four groups as recently described [4]: minimal chronic active hepatitis (CAH); mild CAH; moderate CAH; and severe CAH. The stage of fibrosis/cirrhosis was divided into two groups [4]: minimal to mild fibrosis (including none or mild portal fibrosis, portal fibrosis with incomplete septa and portal fibrosis with septa bridging portal-portal) and advanced fibrosis/cirrhosis (including septa bridging portal-central or focal incomplete cirrhosis, diffuse incomplete and/or focal complete cirrhosis).

In addition, three features typically associated with hepatitis C have been analysed: the presence of lymphoid follicles, the presence and extent of steatosis (as percentage of parenchymal area involved) and the presence of bile duct lesions [3, 16, 19, 24, 34, 37].

RNA preparation: Total RNA was isolated from liver tissue as described elsewhere [8]. The frozen liver tissue was homogenized with 500 ml denaturing solution containing 4 M guanidinium thiocyanate. The RNA fraction was purified by chloroform–isoamyl alcohol (49:1), precipitated by isopropyl alcohol and resuspended in 10–15 ml RNase-free H_2O.

The quality of the extracted RNA was tested by PCR using oligonucleotide primers for albumin mRNA [38]. All samples were tested by nested PCR using primers for the 5′ non-coding region (NCR) of the HCV genome. Complementary DNA was reverse transcribed by random hexamer priming and 10–15% of the c-DNA mix was applied to the PCR as previously described. The PCR product was analysed on a 2% agarose gel, revealing a 146-bp DNA fragment [15].

The HCV genotype was determined essentially by the technique described by Okamoto et al. [32]. To overcome nonspecific PCR products [21], 2% formamide was added to the second round of PCR. All PCR products were analysed on a 4% NuSieve 3:1 agarose gel (FMC BioProducts, Europe) and visualized by ethidium bromide staining. Four type-specific DNA fragments of 57 bp, 144 bp, 174 bp and 123 bp for genotypes 1a, 1b, 2a and 2b, respectively could be detected (Fig. 1).

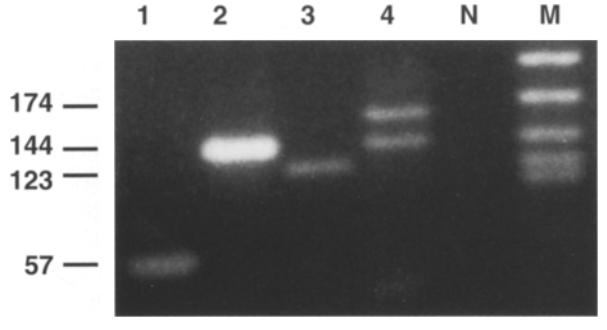

Fig. 1 Genotyping of HCV-RNA in liver biopsies: ethidium bromide-stained agarose gel showing the PCR products of different genotypes (*lane 1* genotype 1a (57 base pairs (bp), *lane 2* genotype 1b (144 bp), *lane 3* genotype 2b (123 bp), *lane 4* mixed infection with genotype 1b and 2a (144 and 174 bp, respectively, *N* negative control, *M* molecular weight marker)

The fibrosis data were analysed using the chi-square test. The ages within the groups were analysed by the Mann–Whitney U-test. For the independent analysis of fibrosis and age a stepwise logistic regression was performed.

Results

HCV RNA was detected in 65 (94.2%) of 69 biopsies from patients with chronic HCV infection by either the NCR or the genotype oligonucletide primer sets. Albumin was detected by PCR in all biopsies analysed. Genotyping was succesful in 58 (84.1%) of the samples; 43 (74.1%) of 58 were also positive by the NCR primers. Genotypes 1a and 1b were detected in 13 (18.8%) and 31 (44.9%) of 69 liver biopsies respectively (Table 1). Genotypes 2a and 2b were each found once (1.5%). Twelve (17.4%) of 69 biopsies revealed a mixture of two genotypes: genotype 1a combined with 1b or 2a in 4 (5.8%) and 1 (1.5%) of samples, respectively, and genotype 1b combined with 2a or 2b in 2 (2.9%) and 5 (7.2%) of liver specimens, respectively.

In 57 (82.6%) of 69 liver biopsies analysed, a mild to moderate CAH was seen (Table 2). Eighteen cases (26.1%) showed an advanced fibrosis and/or cirrhosis. Lymphoid follicles were seen in 33 (47.8%), steatosis in 36 (52.2%) and bile duct lesions in 20 (29.0%) of 69 biopsies.

For further analyses the samples were divided into three groups according to the presence or absence of genotype 1b: group A, comprised of 16 liver biopsies infected by genotype 1a, 2a or 2b (including one mixed infection) and group B, consisting of 42 liver biopsies infected with genotype 1b (including 11 mixed infections). Group C was made up of 11 samples in which no genotype could be established (Table 1).

A mild to moderate form of CAH was present in 16 (100%) and 34 (80.9%) of 42 liver biopsies in group A and B, respectively (Table 2). In addition, group B revealed 2 (4.8%) biopsies with minimal CAH and 6 (14.3%) with severe CAH. None of these differences was statistically significant. In group C, minimal CAH was found in 1 (9.1%), mild/moderate CAH in 7 (63.6%) and severe CAH in 3 (27.3%) samples.

All liver biopsies in group A revealed minimal to mild fibrosis without portal-central bridging septa (score 1 to 3), and none of the specimens in this group showed evidence of advanced fibrosis (score 4 or 5; Table 2). In group B, minimal/mild fibrosis was observed in 26 (61.9%) of 42 biopsies, and 16 (38.1%) tissue samples showed advanced fibrosis with porto-central bridging septa and/or cirrhosis. The presence of advanced fibrosis was significantly different between group A and B (P=0.01). In group C, advanced fibrosis/cirrhosis was detected in 2 (18.2%) of 11 biopsies; this finding was not significantly different from either group A or B. Lymphoid follicles were found in 5 of the 16 (31.3%) biopsies in group A, in 24 of the 42 (57.1%) biopsies in group B and in 4 of the 11 (36.4%) biopsies in group C.

Table 1 Detection of HCV genotypes in 69 liver biopsies. Single (*bold numbers*) and mixed infections are listed and the groups are indicated: group A (genotype 1a, 2a or 2b, including mixed infections), group B, *hatched area* (genotype, 1b, including mixed infections) and group C (nontypable)

Genotype	1a	1b	2a	2b	Total	Group
1a	**13 (18.8%)**	4 (5.8%)	1 (1.5%)	0	18 (26.1%)	A
1b		**31 (44.9%)**	2 (2.9%)	5 (7.2%)	38 (55.0%)	B
2a			**1 (1.5%)**	0	1 (1.5%)	A
2b				**1 (1.5%)**	1 (1.5%)	A
Nontypable					**11 (15.9%)**	C

Table 2 Patient data and histological characteristics of liver biopsies infected with different genotypes

	Total (n=69)	Group A (genotype 1a, 2a or 2b, n=16)	Group B (genotype 1b, n=42)	Group C (nontypable, n=11)
Age (years) of patients (range)	39.5 (18–71)	34.5[a] (26–51)	42.7[a] (18–71)	34.2 (21–58)
Female/male	33/36	4/12	22/20	4/7
Acitivity of hepatitis				
CAH minimal	3 (4.4%)	0	2 (4.8%)	1 (9.1%)
CAH mild/moderate	57 (82.6%)	16 (100%)	34 (80.9%)	7 (63.6%)
CAH severe	9 (13.0%)	0	6 (14.3%)	3 (27.3%)
Fibrosis				
Minimal/mild	51 (73.9%)	16 (100%)	26 (61.9%)	9 (81.8%)
Advanced	18 /26.1%)	0[b]	16[b] (38.1%)	2 (18.2%)
Lymphoid follicles	33 (47.8%)	5 (31.3%)	24 (57.1%)	4 (36.4%)
Steatosis	36 (52.2%)	6 (37.5%)	22 (52.4%)	8 (72.7%)
Bile duct lesion	20 (29.0%)	3 (18.8%)	13 (30.9%)	4 (36.4%)

[a] The mean age of patients in group A was significantly lower than the age in group B (P=0.038)
[b] Advanced fibrosis was seen significantly more often in patients of group B than in group A patients (P=0.01)

Steatosis was observed in 6 of 16 (37.5%) in group A, 22 of 42 (52.4%) in group B and 8 of 11 (72.7%) biopsies in group C. Bile duct lesions appeared in 3 (18.8%), 13 (30.9%) and 4 (36.4%) liver biopsies of groups A, B and C, respectively (Table 2). None of these differences between the study groups was statistically significant.

The mean age of patients was significantly higher in group B than in group A (42.7 vs 34.5 years; P=0.038; (Fig. 2). No significant difference was seen with respect to the age of the patients in these two groups compared with group C. The mean age of patients with advanced fibrosis/cirrhosis was much higher than that of those with a low fibrosis score within group B (51.1 vs 37.6 years; P=0.0025) and of all patients with low fibrosis scores in groups A and B (51.1 vs 36.4 years; P=0.004;

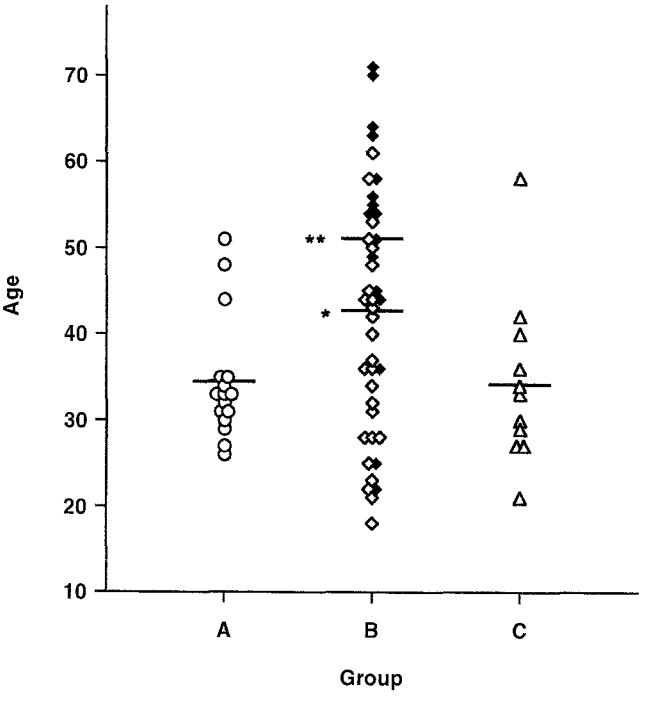

Fig. 2 Age of patients in different groups of genotypes: In group B, *open diamonds* indicate patients with low fibrosis score, and *closed diamonds* indicate patients with advanced fibrosis/cirrhosis. *The mean age of patients was significantly higher in group B than in group A (42.7 vs 34.5; P=0.038). **The mean age of patients with advanced fibrosis/cirrhosis was significantly higher than the mean age of all patients in which a genotype has been established (51.1 vs 36.4; P=0.004)

Fig. 2). No difference was seen in the mean age of patients with low fibrosis scores between groups A and B (34.5 vs 37.6 years; $P>0.05$). In a stepwise logistic regression analysis, both the age of patients and infection by genotype 1b (group B) were independently associated with advanced fibrosis ($P=0.006$ and 0.0027, respectively). No differences were detected with respect to the sex of the patients within the three groups analysed (Table 2).

Discussion

In this study we have shown that HCV genotyping by PCR, using type-specific oligonucleotide primers, can be applied directly to needle liver biopsies. Using the oligonucleotide primers of the highly conserved 5′-untranslated region and the less highly conserved core region, the sensitivity for detecting HCV RNA in frozen liver tissue was 94.2%, similar to the sensitivity reported previously [18, 38]. In our population, genotypes 1a and 1b were the predominant genotypes, comprising 26.1% and 60.8%, respectively, of all genotypes analysed, including mixed infections (Table 1). Similar results have been obtained by two Italian groups who examined sera of patients with chronic hepatitis C [33, 36]. In contrast, genotype 1a is very rare in Japan, while in North America genotype 1a has been reported to be predominant or of similar prevalence to genotype 1b [2, 12, 23, 40].

Although widely used, the specificity in detecting genotype 1b by the typing assay using type-specific primers of the core region has recently been challenged [23]. However, only one of the additional tests used in this paper was able to discriminate genotype 1a and 1b and mixed infections at the same time. Furthermore, Feray et al. found a high concordance of the specific nested PCR and the line probe assay for the detection of genotype 1b, and only some discordance in non-1b genotypes, which would not alter the results of our study [14]. In a very recent study the high concordance between these two tests was confirmed [17]. We were able to reanalyse 9 of the 11 samples of group C by the line probe assay and found HCV genotype 3a in 7 (77.8%) and 4a in 1 (11.1%) samples (data not shown), indicating that group C indeed includes other genotypes not detected by the type-specific PCR assay.

Several authors have claimed that the viral genotype is predicitive of the efficacy of IFN-α therapy. Genotype 1b has been associated with a lower therapeutic response rate [7, 27, 41, 42, 44]; an association between serum concentration of viral RNA and the histological activity index has been reported for genotype 1b but not for genotypes 2a and 2b [25]. Moreover, genotype 1b has been associated with a higher level of viral RNA in the serum, indicating that genotype 1b may be associated with an increased virus load and therefore show a more aggressive course as well as a decreased susceptibility to antiviral treatment [27, 30, 42]. We therefore divided the samples into three groups according to the presence of genotype 1b. On analysing the grade (activity) and stage (fibrosis/cirrhosis) of chronic hepatitis separately, as recently suggested [10], we found a higher degree of fibrosis/cirrhosis in group B (biopsies infected with genotype 1b) than in group A. Activity of chronic hepatitis, however, was not different between these two groups.

Recently, Nousbaum et al. showed an increased incidence of genotype 1b in patients with cirrhosis, whereas other investigators have not reported an association between fibrosis/cirrhosis and the genotype [28, 30, 41]. One report records increased necroinflammatory activity in patients infected by genotype 2 without advanced fibrosis; however this work did not distinguish between genotypes 1a and 1b [23]. Furthermore, it is important to note that many of these studies have been performed in Japan, where genotype 1a is very rare [21, 27, 42]. In our material, however, genotype 1a was the prevailing viral strain in the group of non-type-1b genotypes.

Other studies have not reported an association between genotypes and progression of disease, but have noted an association of viral load in the serum with advanced liver disease [29, 42, 43]. Although the reason for this difference is not fully understood, the fact that genotype 1a is almost absent in Japan may explain at least in part some of these differences, thus indicating that genotype 1a may be associated with a low risk of developing advanced liver fibrosis.

In our study, the mean age of patients in group B is significantly higher than that in group A (Fig. 2), suggesting that the more advanced stage of fibrosis in group B might merely reflect the longer duration of the disease. However, the lack of patients with higher scores for fibrosis within group A cannot be completely explained. One might therefore speculate that the genotype 1b promotes progression of liver disease, whereas in patients infected with genotype 1a, 2a and 2b the course of disease would be less dramatic, so that these patients would present less frequently with symptomatic liver disease at a higher age. In this context it is noteworthy that cases of severe inflammatory activity are also lacking in group A, although this difference does not reach statistical significance. In addition, there was no difference in the mean age of patients with a low fibrosis score within groups A and B, indicating no difference with respect to the age at infection. Finally, statistical analysis reveals that both the age of patients and infection by genotype 1b (group B) were each independently associated with advanced fibrosis.

With respect to the histological features associated with HCV infection, no differences were seen among the three groups, although lymphoid follicles were slightly more frequent in liver specimens infected with genotype 1b. However, this finding was not statistically significant. Thus, we could not find any evidence for association of one or several characteristic histological features described for hepatitis C with a specific genotype.

In summary, patients infected by HCV genotype 1b have an increased risk of developing fibrosis and cirrhosis. Conversely, patients infected with other genotypes,

especially genotype 1a, may be at lower risk of developing end-stage liver disease.

References

1. Alter HJ, Purcell RH, Shih JW, Melpolder JC, Houghton M, Choo QL, Kuo G (1989) Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N Engl J Med 321:1494–1500
2. Andonov A, Chaudhary RK (1994) Genotyping of Canadian hepatitis C virus isolates by PCR. J Clin Microbiol 32:2031–2034
3. Bach N, Thung SN, Schaffner F (1992) The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. Hepatology 15:572–577
4. Bianchi L, Gudat F (1994) Chronic hepatitis. In: Mc Sween RNM, Anthony PP, Scheuer PJ, Portmann B, Burt AD (eds) Pathology of the liver. Churchill Livingstone, Edinburgh, pp 349–395
5. Bianchi L, Desmet VJ, Popper H, Scheuer PJ, Aledort LM, Berk P (1987) Histological patterns of liver disease in hemophiliacs, with special reference to morphological characteristics of non-A, non-B hepatitis. Semin Liver Dis 7:203–209
6. Chan SW, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL, Simmonds P (1992) Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. J Gen Virol 73:1131–1141
7. Chemello L, Alberti A, Rose K, Simmonds P (1994) Hepatitis C serotype and response to interferon therapy (letter). N Engl J Med 330:143
8. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159
9. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244:359–362
10. Desmet JV, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ (1994) Classification of chronic hepaitits: diagnosis, grading and staging. Hepatology 19:1513–1520
11. Dienes HP, Popper H, Arnold W, Lobeck H (1982) Histologic observations in human hepatitis non-A, non-B. Hepatology 2:562–571
12. Dusheiko GM, Schmilovitz Weiss H, Brown D, McOmish F, Yap PL, Sherlock S, McIntyre N, Simmonds P (1994) Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology 13–18
13. Esteban JI, Esteban R, Viladomiu L, Lopez TJC, Gonzalez A, Hernandez JM, Roget M, Sanchez C, Vargas V, Genesca J, Buti M (1989) Hepatitis C virus antibodies among risk groups in Spain. Lancet II:294–296
14. Feray C, Gigou M, Samuel D, Paradis V, Mishiro S, Maertens G, Reynes M, Okamoto H, Bismuth H, Brechot C (1995) Influence of the genotypes of hepatitis C virus on the severity of recurrent liver disease after liver transplantation (see comments). Gastroenterology 108:1088–1096
15. Garson JA, Ring C, Tuke P, Tedder RS (1990) Enhanced detection by PCR of hepatitis C virus RNA. Lancet 337:878–879
16. Gerber MA, Krawczynski K, Alter MJ, Sampliner RE, Margolis HS (1992) Histopathology of community acquired chronic hepatitis C. The Sentinel Counties Chronic Non-A, Non-B Hepatitis Study Team. Mod Pathol 5:483–486
17. Giannini C, Thiers V, Nousbaum J-B, Stuyver L, Maertens G, Brechot C (1995) Comparative analysis of two assays for genotyping hepatitis C virus based on genotype-specific primers or probes. J Hepatol 23:246–253
18. Hosoda K, Omata M, Yokosuka O, Kato N, Ohto M (1992) Non-A, non-B chronic hepatitis is chronic hepatitis C: a sensitive assay for the detection of hepatitis C virus RNA in the liver. Hepatology 15:777–781
19. Ishak KG (1994) Chronic hepatitis: morphology and nomenclature. Mod Pathol 7:690–713
20. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH, Alter HJ (1990) Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 12:671–675
21. Kobayashi Y, Watanabe S, Konishi M, Yokoi M, Kakehashi R, Kaito M, Kondo M, Hayashi Y, Jomori T, Suzuki S (1993) Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with interferon-beta. Hepatology 18:1319–1325
22. Kuo G, Choo QL, Alter HJ, Gitnick GL, Redeker AG, Purcell RH, Miyamura T, Dienstag JL, Alter MJ, Stevens CE, Tegtmeier GE, Bonino F, Colombo M, Lee WS, Kuo C, Berger K, Shuster JR, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989) An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. Science 244:362–364
23. Lau JY, Mizokami M, Kolberg JA, Davis GL, Prescott LE, Ohno T, Perrillo RP, Lindsay KL, Gish RG, Qian KP, et al (1995) Application of six hepatitis C virus genotyping systems to sera from chronic hepatitis C patients in the United States. J Infect Dis 171:281–289
24. Lefkowitch JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HCJ, Balart LA, Ortego TJ, Payne J, Dienstag JL, Gibas A, Jacobson IM, Tamburro CH, Carey W, O'Brien C, Sampliner R, vanThiel D, Feit D, Albrecht J, Meschievitz C, Sanghvi B, Vaughan RD, group Hit (1993) Pathological diagnosis of chronic hepatitis C: a multicenter comparative study with chronic hepatitis B. The Hepatitis Interventional Therapy Group. Gastroenterology 104:595–603
25. Matsumoto A, Tanaka E, Suszuki T, Ogata H, Kiyosawa K (1994) Viral and host factors that contribute to efficacy of Interferon-alpha 2a therapy in patients with chronic hepatitis C. Dig Dis Sci 39:1273–1280
26. Miller RH, Purcell RH (1990) Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc Natl Acad Sci 87:2057–2061
27. Mita E, Hayashi N, Hagiwara H, Ueda K, Kanazawa Y, Kasahara A, Fusamoto H, Kamada T (1994) Predicting interferon therapy efficacy from hepatitis C virus genotype and RNA titer. Dig Dis Sci 39:977–982
28. Mita E, Hayashi N, Kanazawa Y, Hagiwara H, Ueda K, Kasahara A, Fusamoto H, Kamada T (1994) Hepatitis C virus genotype and RNA titer in the progression of type C chronic liver disease. J Hepatol 21:468–473
29. Nakagawa H, Shimomura H, Hasui T, Tsuji H, Tsuji T (1994) Quantitative detection of hepatitis C virus genome in liver tissue and circulation by competitive reverse transcription-polymerase chain reaction. Dig Dis Sci 39:225–233
30. Nousbaum J-B, Pol S, Nalpas B, Landais P, Berthelot P, Bréchot C, group atcs (1995) Hepatitis C virus type 1b (II) infection in France and Italy. Ann Intern Med 122:161–168
31. Okamoto H, Kurai K, Okada S, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F, Mishiro S (1992) Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virology 188:331–341
32. Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M (1992) Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 73:673–679
33. Pistello M, Maggi F, Vatteroni L, Cecconi N, Panicucci F, Bresci GP, Gambardella L, Taddei M, Bionda A, Tuoni M, Bendinelli M (1994) Prevalence of hepatitis C virus genotypes in Italy. J Clin Microbiol 32:232–234
34. Poulsen H, Christoffersen P (1972) Abnormal bile duct epithelium in chronic aggressive hepatitis and cirrhosis. A review of

morphology and clinical biochemical and immunological features. Hum Pathol 3:227–235
35. Pozzato G, Moretti M, Franzin F, Crocè LS, Titribelli C, Masayu T, Kaneko S, Unoura M, Kobayashi K (1991) Severity of liver disease with different hepatitis C viral clones. Lancet 338:509
36. Ravaggi A, Zonaro A, Marin MG, Puoti M, Albertini A, Cariani E (1994) Distribution of viral genotypes in Italy determined by hepatitis C virus typing by DNA immunoassay. J Clin Microbiol 2280–2284
37. Scheuer PJ, Ashrafzadeh P, Sherlock S, Brown D, Dusheiko GM (1992) The pathology of hepatitis C. Hepatology 15:567–571
38. Shieh YSC, Shim K-S, Lampertico P, Balart LA, Jeffers LJ, Thung SN, Regenstein F, Reddy KR, Farr G, Schiff ER, Gerber MA (1991) Detection of hepatitis C virus sequences in liver tissue by the polymerase chain reaction. Lab Invest 65: 408–411
39. Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan S-W, Chayama K, Chen D-S, Choo Q-L, Colombo M, Cuypers HTM, Date T, Dusheiko GM, Esteban JI, Fay O, Hadziyannis SJ, Han J, Hatzakis A, Holmes EC, Hotta H, Houghton M, Irvine B, Kohara M, Kolberg JA, Kuo G, Lau JYN, Lelie PN, Maertens G, McOmish F, Miyamura T, Mizokami M, Nomoto A, Prince AM, Reesink HW, Rice C, Roggendorf M, Schalm SW, Shikata T, Shimotohno K, Stuyver L, Trepo C, Weiner A, Yap PL, Urdea MS (1994) A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatology 19:1321–1324
40. Takada N, Takase S, Takada A, Date T (1993) Differences in the hepatitis C virus genotypes in different countries. J Hepatol 17:277–283
41. Tsubota A, Chayama K, Ikeda K, Yasuji A, Koida I, Saitoh S, Hashimoto M, Iwasaki S, Kobayashi M, Hiromitsu K (1994) Factors predictive of response to interferon-alpha therapy in hepatitis C virus infection. Hepatology 19:1088–1094
42. Yamada G, Takahashi M, Miyamoto R, Tsuji T, Yoshizawa H, Okamoto H (1994) Prediction of interferon effect in chronic hepatitis C by both quantification and genotyping of HCV-RNA (letter). Dig Dis Sci 39:441
43. Yamada M, Kakumu S, Yoshioka K, Higashi Y, Tanaka K, Ishikawa T, Takayanagi M (1994) Hepatitis C virus genotypes are not responsible for development of serious liver disease. Dig Dis Sci 39:234–239
44. Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992) Detection of hepatitis C virus by polymerase chain reaction and response to interferon-alpha therapy: relationship to genotypes of hepatitis C virus. Hepatology 16:293–299