Lack of Correlation Between Hepatitis C Virus Genotypes and Clinical Course of Hepatitis C Virus–Related Cirrhosis

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The influence of the hepatitis C virus (HCV)–genotype on liver disease severity was evaluated in 429 consecutive patients with chronic hepatitis C, including 109 with cirrhosis who were followed up prospectively, allowing for the assessment of the role of the HCV–genotype on disease outcome and on the development of hepatocellular carcinoma (HCC). HCV-1 was detected in 147 (46%) patients without cirrhosis and in 47 (43%) with cirrhosis (*P*: not significant), being mainly HCV-1b. HCV-2 was found in 103 (32%) cases without cirrhosis and in 30 (27.5) with cirrhosis (*P*: not significant), being mainly HCV-2a. HCV-3 was detected in 32 (10%) patients without cirrhosis and in 2 (2%) with cirrhosis ($P < 0.005$). Infection with more than one genotype (HCV-1/HCV-2 and HCV-1/HCV-3) was observed only in cirrhotic patients (6 of 109; 5.5%). During a mean follow-up of 67 ± 22 months, 21 (19%) patients with cirrhosis showed worsening in Child's stage, 5 (4.5%) underwent liver transplantation, 23 (21%) developed HCC, and 24 (22%) died of complication of liver disease; the overall incidence of at least one of these events was 38.5%. By the Kaplan-Meier method and log-rank test, the cumulative probability of developing each or at least one of the above events did not differ in relation to the genotype of infecting HCV, apart from patients with mixed genotype infection who showed a significantly higher incidence of death ($P < .05$). These data indicate that HCV–genotypes do not have a significant effect on the severity and outcome of liver disease in patients with chronic HCV–infection. Patients with cirrhosis who are also infected by HCV-1 and HCV-2 had a similar prognosis and progression to HCC, while patients infected by more than one genotype showed the most unfavorable course of disease. (HEPATOLOGY 1997;25:211-215.)

Hepatitis C virus (HCV)–infection is a major cause of chronic liver disease worldwide and greatly contributes to the etiology of cirrhosis[1-4] and of hepatocellular carcinoma (HCC).[5-8] However, in view of the rather high prevalence of chronic infection in the general population, it has been suggested that only a subgroup of infected individuals has progressive liver damage leading to end-stage disease. The determinants of the more severe evolution of chronic HCV–infection have not been yet identified and are likely to include both virus and host factors. The hepatitis C virus exists as a family of distinct variants that have been classified recently into at least 6 major genotypes on the basis of genomic nucleotide sequence–analysis.[9-14] Some evidence has been provided that the genotype of HCV may be one of the factors influencing the severity and outcome of liver disease.[15-17] Genotype 1b, the most prevalent variant found in several parts of the world, including the United States, Europe, and Japan, has been associated with more advanced liver damage, with cirrhosis, and with HCC,[18-20] leading to the conclusion that its pathogenicity may be greater than that of other HCV types, such as HCV-2 and HCV-3, which are also rather common. Genotype 1b has also been found in patients with the most poor response to interferon, supporting the idea that infection by this virus type may be particularly unfavorable for the host.[18,20-24] Despite these assumptions, there have been no prospective studies on the relationship between the HCV–genotype and the outcome of liver disease. This study assesses the prevalence of major HCV–genotypes in 429 consecutive patients with chronic HCV–infection seen in our unit over a period of 4 years. The relation between the genotype of HCV and liver disease severity and outcome has been evaluated in two ways: 1) by a cross-sectional analysis of all patients to assess the relation between virus type and activity and stage of liver damage; and 2) by a longitudinal study of cirrhotic patients aimed at evaluating the influence of the HCV–genotype on the natural course of the disease, on its complications, and on the development of HCC.

PATIENTS AND METHODS

Patients. Patients with chronic liver disease that were observed in our institution between 1986 and 1990 were retrospectively analyzed: those infected by HCV were identified on the basis of serum anti-HCV positivity by enzyme-linked immunosorbent assay-2, using stored samples. Patients with other potential causes of liver disease concurrent with HCV, such as hepatitis B surface antigen–positive patients, those with excess alcohol intake, or with features of autoimmune or metabolic liver disease, were excluded, even though they were anti-HCV–positive. Patients presenting with HCC were also excluded.

A total of 429 patients were eligible for this study. There were 269 men and 160 women ranged in age from 18 to 81 years. All patients had undergone diagnostic liver biopsy, apart from 10 patients with clinically evident cirrhosis. In all cases liver function tests were performed at the time of diagnosis. Stored serum samples were available in all cases and were tested for HCV-RNA by polymerase chain reactions (PCR), as described in later paragraphs. When virus sequences were amplifiable by this technique, they were further characterized for genotype and subtype, as described in later paragraphs in detail.

Follow-Up. Most patients with chronic hepatitis and without cirrhosis received treatment with α interferon during follow-up. Furthermore, although a subgroup of patients remained untreated, the available follow-up studies were judged as insufficient, unable to provide any conclusions about the evolution of disease, considering that they were the cases with less active, and therefore less progressive, disease. For these reasons, we decided to evaluate the influence of HCV genotypes on the outcome of liver disease only in the cirrhotic patients, as in this group the majority of cases did not receive antivi-

Abbreviations: HCV, hepatitis C virus; HCC, hepatocellular carcinoma; ALT, alanine aminotransferase; PCR, polymerase chain reaction.

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ral therapy and all had been followed regularly after diagnosis in a prospective study aimed to evaluate the natural course of the disease and the development of HCC.[25]

For this purpose, patients were evaluated at six-month intervals by abdominal ultrasound examinations, liver function tests, and alpha-fetoprotein levels in order to follow the course of liver disease and its complications. The events considered during the follow-up period of clinical observation were: 1) activity of liver disease, based on the behavior of serum alanine aminotransferase–(ALT) levels; 2) the worsening of liver function (change of cirrhosis's grade according to Child's classification); 3) liver transplantation; 4) development of HCC; and 5) death due to liver disease.

Serologic Testing. Anti-HCV was determined by second generation enzyme-linked immunosorbent assay (enzyme-linked immunosorbent assay, Ortho Diagnostic System, Raritan, NJ) and by second generation recombinant immunoblotting assay (recombinant immunoblotting assay, Chiron Corporation, Emeryville, CA). Hepatitis B surface antigen, antibody to hepatitis B surface antigen, and antibody to hepatitis B core antigen were analyzed by commercially available kits.

HCV-RNA in Serum. HCV RNA was determined in serum by nested PCR after reverse transcription, using primers derived from the 5′-nontranslated region.[17] The synthesis of complementary DNA was performed directly from 3 μL of serum mixed with 17 μL of buffer containing 10 mmol/L TRIS-HCV (pH 8.4), 50 mmol/L KCl, 2.5 mmol/L $MgCl_2$, 1 mmol/L of the four dinucleotide triphosphates (Pharmacia, Uppsala, Sweden), and 5 pmol/L of the reversal external primer. Reverse transcription was carried out using 10 U of murine myeloblastosis virus–reverse transcriptase (Gibco BRL, Milan, Italy), and PCR was performed in a DNA thermal cycler (Perkin Elmer/Cetus, Norwalk, CT and Emeryville, CA) using 2.5 U of Taq polymerase (Amplitaq, Perkin Elmer/Cetus) and 50 pmol/L of the sense external primer. The second round of PCR was made by amplifying a 1-μL aliquot of the first PCR reaction using 50 pmol/L of each internal primer. The amplified products were visualized by ethidium bromide staining after electrophoresis in a 1.5% agarose gel. Water and serum from normal healthy subjects were used as controls. All reagents were ultraviolet treated, and positive displacement pipettes (Pipetman, Gibco BRL) were used to minimize contamination. Sixty two of the 429 serum samples tested by PCR were HCV-RNA–negative, allowing for the determination of HCV–genotypes in 367 patients.

HCV Genotyping. HCV–genotypes were identified using a dot-blot assay, in which the products of PCR amplification, immobilized on nylon filters in triplicate, were hybridized with oligonucleotide probes specific for different HCV–genotypes.[17] To test our patients, we used probes specific for the three major genotypes that are present in Italy: HCV types 1, 2, and 3, according to the Simmonds' classification.[14] The probes were derived from the highly variable sequence of the 5′-noncoding region (nucleotides -135 to -155), were labeled with fluorescein-deoxyuridine triphosphate, and specific hybridization revealed by the enhanced chemiluminescent method (enhanced chemiluminescent method, Amersham International, Amersham, UK). Briefly, prehybridization was performed at 42°C for 1 hour, followed by 2 hours of hybridization at 42°C. Washes were carried out twice with 6 × SSC, .1% sodium dodecyl sulfate at room temperature for 5 minutes, twice with 3 × SSC and 0.1% sodium dodecyl sulfate at room temperature for 10 minutes, and once with .1 × SSC and .1% sodium dodecyl sulfate at 50°C for 10 minutes. Hybridized probes were detected by the development of the fluorescein hapten by incubation with an antifluorescein horseradish peroxidase conjugate and by the identification of the bound peroxidase using the enhanced chemiluminescent detection reagents. The enzymatic reduction of peroxide, together with the oxidation of luminol, induces light emittance which is detected by autoradiographic film (Amersham International) after 1 to 5 minutes of exposure. HCV-1a and HCV-1b subtypes were identified by the BstU I endonuclease digestion of PCR–amplified products, and the results revealed by agarose gel electrophoresis are that only 1 restriction site is recognized by HCV-1a and that 2 restriction sites are recognized by HCV-1b.[26] Ten μL of amplified products were mixed with 2.5 μL of buffer containing .4 μL of BstU I (10 U/μL) and 12.1 μL H_2O and incubated for 30 minutes at 37°C. After electrophoresis, 2 bands for HCV-1a and 3 bands for HCV-1b were detectable by ethidium bromide staining. HCV-2a–subtype identification was performed using a HCV-2a–specific probe, and the minority of samples that did not react with our probes, including HCV-2b, were characterized by direct sequencing (Sequenase United States Biochemical, Denver, CO) according to the manufacturer's instructions. Thirty eight randomly chosen samples were also tested by the commercial INNO-LiPA HCV kits (Innogenetics N.V., Zwijndrecht, Belgium), following the manufacturer's instructions, and the results were concordant with our methods in all cases.

TABLE 1. HCV–Genotypes Prevalence in 429 Patients With Chronic HCV–Infection, According to Presence or Absence of Cirrhosis

HCV–Genotype	Chronic Hepatitis (n) (%)	Cirrhosis N° (%)	P*
HCV-RNA–Negative	38 (11.9)	24 (22.0)	.017
HCV-1a	14 (4.3)	3 (2.7)	n.s.
HCV-1b	133 (41.5)	44 (40.3)	n.s.
HCV-2a	94 (29.4)	30 (27.5)	n.s.
HCV-2b	9 (2.8)	– (0)	n.s.
HCV-3	32 (10)	2 (1.8)	.005
Mixed	– (0)	6 (5.5)	.005
All Cases	320 (100)	109 (100)	

Abbreviation: n.s., not significant; n, number of cases.
* Fisher's Exact test.

Statistical Analysis. Fisher's exact test and the χ^2 test were used to compare: 1) the HCV–genotypes distribution in patients with chronic hepatitis and in patients with cirrhosis; 2) the sex and Child's stage distribution in relation to HCV–genotypes in patients with cirrhosis; 3) the frequency of worsening Child's stage, death, or liver transplantation and the development of HCC during follow-up in patients with cirrhosis in relation to HCV–genotypes; and 4) the serum ALT behavior during follow-up in cirrhotics in relation to HCV–genotypes. Student's t test was used to define the mean age and duration of cirrhosis in cirrhotic patients grouped in relation to the different HCV–genotypes. Univariate analysis by the Kaplan-Meier method and log-rank test was used to evaluate the influence of HCV–genotypes in cirrhotic patients in cases of worsening Child's stage, occurrence of death, or liver transplantation and development of HCC, as well as in the occurrence of at least one of these events during follow-up.

RESULTS

Prevalence of HCV–Genotypes in Patients With Chronic HCV Infection. Among 429 consecutive patients with chronic HCV–infection, 194 (45%) were found infected by HCV-1 (17 by HCV-1a and 177 by HCV-1b), 133 (31%) by HCV-2 (124 by HCV-2a and 9 by HCV-2b), and 34 (8%) by HCV-3. Six additional patients had a mixed infection (1 patient had HCV-1a + HCV-2a, 2 cases had HCV-1b + HCV-2a, 1 patient had HCV-1b + HCV-3, and 2 patients had HCV-1a + HCV-1b + HCV-2a), while 62 were HCV-RNA–negative at the time of testing and could not be genotyped. The distribution of the different HCV types and subtypes, and the comparison of their prevalence in patients with or without cirrhosis are described in Table 1. HCV-1b and HCV-2a were the most prevalent subtypes seen in our patients. There were no significant differences between cirrhotic and noncirrhotic patients as to HCV-1 and HCV-2 types and subtypes, while HCV-3 was more frequent in patients without cirrhosis ($P < .005$). Thus, we could not confirm in our patients the higher prevalence of infection by HCV-1b in cirrhotic patients reported by other authors[19] in similar studies. Furthermore, when patients infected with HCV-1b were compared with those infected by other genotypes, a similar prevalence of cases of cirrhosis was observed (44/177: 24.8% vs. 41/190: 21.5%; not significant). Even assuming that all HCV-RNA–negative patients had HCV-1b, the differences did not reach statistical significance.

Table 2 describes in more detail the base-line characteristics of our cirrhotic patients in relation to the infecting–HCV types and subtypes. Mean age, sex distribution, and known duration of cirrhosis did not differ significantly in relation to the HCV–genotype. Furthermore, the percentages of patients presenting with Child's A stage of cirrhosis were also

TABLE 2. Base-Line Characteristics of Cirrhotic Patients in Relation to HCV–Genotypes

HCV–Genotype	n°	Mean Age (y)	Sex M/F	Duration of Cirrhosis (y)	Child's Stage A	Child's Stage B
HCV-RNA–Negative	24	57.3	18/6	1.8	22	2
HCV-1	47	57	30/17	2.5	36	11
HCV-2	30	60.2	15/15	1.8	24	6
HCV-3	2	57	1/1	2	1	1
Mixed	6	55	3/3	2	3	3
All Cases	109	57.7	67/42	2.2	86	23

NOTE. Differences were not statistically significant.

TABLE 4. Serum ALT–Behavior During Follow-Up in Patients With Cirrhosis in Relation to HCV–Genotypes

HCV–Genotype	n°	Serum ALT–Levels During Follow-Up: Normal or Normalized	Serum ALT–Levels During Follow-Up: Always High or Fluctuating
HCV-RNA–negative	24	13 (54%)	11 (46%)
	$P < .01$	$P < .01$	
HCV-1	47	9 (19.1%)	38 (80.9%)
HCV-2	30	9 (30%)	21 (70%)
HCV-3	2	1 (50%)	1 (50%)
Mixed	6	3 (50%)	3 (50%)
All cases	109	35 (32.1%)	74 (67.9%)

* Fisher's Exact test.

similar. The long-term clinical outcome in these 109 patients with cirrhosis, classified according to the 3 major types of infecting–HCV, was then analyzed to assess whether the HCV strain could have a major influence on the natural course of the liver disease and on the development of its complications.

Follow-Up of Patients With Cirrhosis in Relation to Base-Line HCV-RNA–Profile and HCV Genotypes. As shown in Table 3, during a mean follow-up period of 66.9 ± 22.5 months (range, 12-119 months), 21 (19.2%) of the 109 patients with cirrhosis showed a worsening of their liver disease and a progression of the stage of cirrhosis according to Child's classification. Five (4.5%) cases were subjected to liver transplantation, 23 (21.1%) developed HCC, and 24 (22%) died from complications of liver disease. The overall incidence of at least one of the above-mentioned events in the whole group was 38.5% (42 of 109 cases). During follow-up studies, serum ALT–levels remained normal or below 1.5 times the upper normal limit in 35 (32.1%) patients, while in the remaining 74 cases (67.9%) ALT persisted at high levels or fluctuated during the whole period of observation (Table 4). The frequencies of worsening cirrhosis, of HCC–development, of death or liver transplantation, as well as the occurrence of at least one event during follow-up, in relation to the genotype of infecting HCV are shown in Table 3. The worsening of cirrhosis occurred more frequently in patients with mixed genotypes infection (50.0%) than in those with HCV-1 or HCV-2, but the difference was not statistically significant. Death from hepatic failure or liver transplantation was observed more frequently with mixed infections (66.7%) and was rare in patients with HCV-2–infection (16.7%) ($P < .05$). The incidence of HCC–development showed no significant differences among the subgroups of cirrhotic patients. During follow-up, HCC appeared in 29.1% of HCV-RNA–negative patients, in 19.1% of cases infected by HCV-1, in 20% of subjects infected by HCV-2, and in 16.6% of patients with mixed genotypes. The incidence of at least one of the mentioned events during follow-up studies was higher in patients infected by mixed genotypes (66.7%) and in HCV-RNA–negative patients (50%) than in HCV-1– and HCV-2–infected cases (31.9% and 33.3%, respectively); however, these differences were not statistically significant.

TABLE 3. Frequency of Worsening of Cirrhosis, HCC–Development, OLT, and/or Death and of At Least One of These Events During Follow-Up in Patients With Cirrhosis, in Relation to HCV–Genotypes

HCV–Genotype	n°	Worsening of Child's Stage N° (%)	HCC (n) (%)	Death and/or OLT (n) (%)	At Least One Adverse Event (n) (%)
HCV-RNA–Negative	24	6 (25)	7 (29.1)	7 (29.1)	12 (50)
HCV-1	47	7 (14.8)	9 (19.1)	12 (25.5)	15 (31.9)
HCV-2	30	4 (13.3)	6 (20)	5 (16.7)	10 (33.3)
HCV-3	2	1 (50)	(0)	1 (50)	1 (50)
Mixed	6	3 (50)	1 (16.6)	4 (66.7)	4 (66.7)
All Cases	109	21 (19.2)	23 (21.1)	24 (22)	42 (38.5)

Abbreviation: OLT, orthotopic liver transplantations.

As shown in Table 4, persistently high or fluctuating levels of ALT during follow-up were more frequently observed in patients infected by HCV-1 genotype (80.9%), while this profile was rarely seen in HCV-RNA–negative patients (46%) ($P < .01$).

All the above described events were analyzed by univariate analysis according to Kaplan-Meier method and log-rank test. As shown in Fig. 1, the cumulative probability of the worsening of cirrhosis and the appearance of HCC showed no significant differences in the four subgroups of patients considered, while patients infected by a mixed HCV–population had the highest probability of death and of liver transplantation compared to all other subgroups ($P < .05$). However, when all adverse events were considered together, the probability of developing at least one of them during follow-up did not differ significantly in relation to the genotype of infecting–HCV. Even assuming that all HCV-RNA–negative cases were infected by HCV-1, the outcomes in this group did not appear significantly different.

Since part of our cirrhotic patients had received interferon therapy during follow-up, we have analyzed the different outcomes in treated and untreated patients. Overall, 36 patients received a course of interferon therapy with a dose of 3 mU to 6 mU given thrice weekly for 6 to 12 months. The percentage of treated patients was not significantly different among the cases infected with the different HCV–genotypes. Six patients (16.6%) showed a persistent normalization of ALT that was maintained after therapy, and only 2 (5.5%) became and remained HCV-RNA–negative for the entire posttreatment follow-up. Despite this low rate of sustained response, patients treated with interferon showed lower rates of adverse events during follow-up (HCC development: 5.5% in treated cases vs. 30.2% in untreated cases; worsening of Child's stage: 5.5% vs. 26%; death and/or liver transplantation: 2.7% vs. 31.5%; at least one adverse event: 11.1% vs. 52%). These differences, however, may merely reflect that patients who had been treated were in fact in an earlier stage of disease compared to those who were left untreated.

DISCUSSION

The HCV exists as a family of different genotypes and subtypes, and several recent studies have suggested that the HCV strain may play a relevant role in determining the severity and outcome of liver disease in HCV–infected individ-

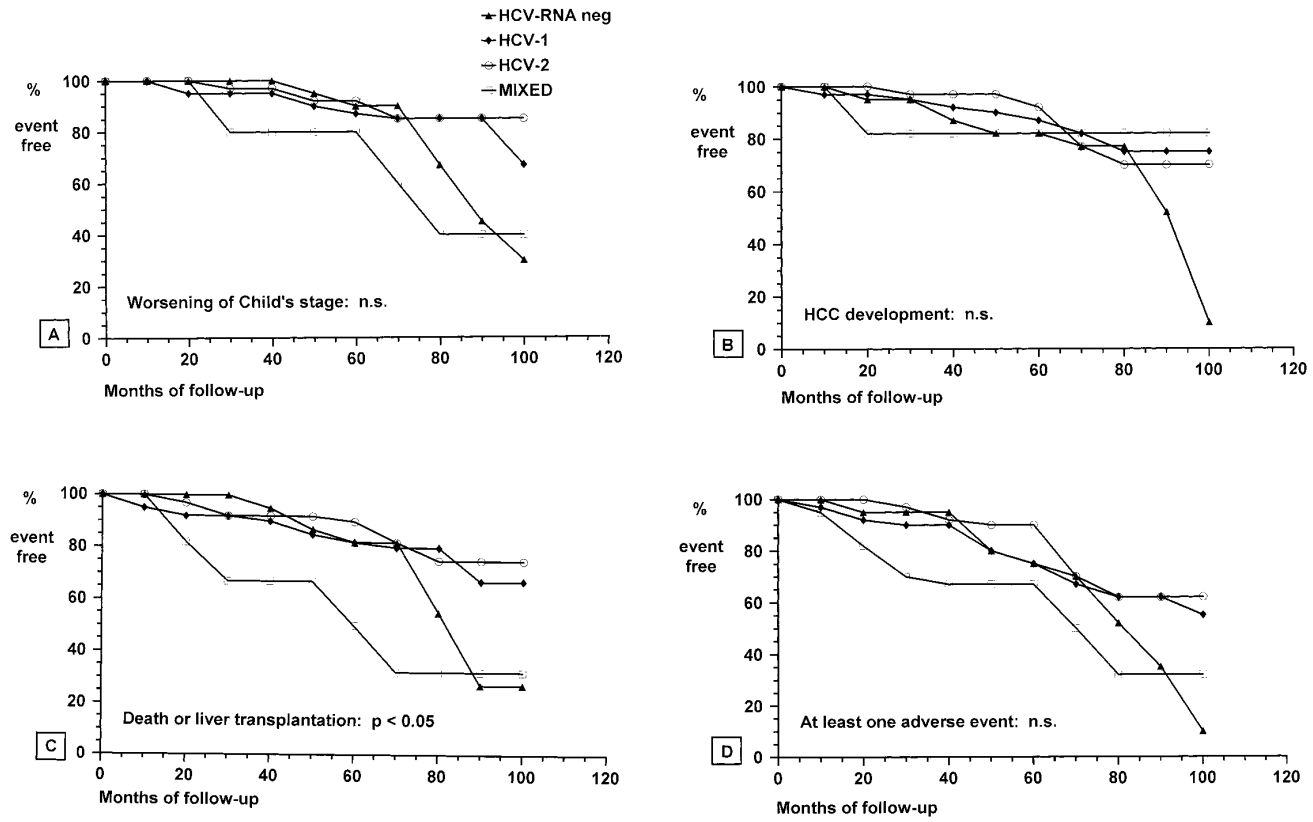

FIG. 1. Cumulative probability of: (A) worsening of stage of cirrhosis, according to Child's classification; (B) HCC; (C) death or liver transplantation; and (D) at least one adverse event in 109 patients with cirrhosis in relation to HCV–genotype (Kaplan-Meier method and log-rank test). n.s., not significant.

uals.[15-17,27,28] These assumptions have been reached mainly by following the observation that the distribution of HCV–genotypes differs in relation to the type of patients investigated. Indeed several authors have described a higher prevalence of infection by HCV-1b in patients with more advanced liver disease, such as cirrhosis and HCC, as compared to control populations of patients with chronic hepatitis type C that has not yet evolved into the cirrhotic stage.[16,18-20] Other studies have shown that patients with mild forms of chronic hepatitis C, such as those with persistent infection and normal transaminases, are often infected by genotypes other than that of HCV-1b, such as HCV-2 or HCV-3.[29] Fèray et al.[30] have reported that after liver transplantation, the recurrence of hepatitis C is more severe and devastating when HCV-1b is involved, further supporting the idea that this particular HCV–subtype may be more cytopathic than any other type of HCV.

There are, however, other possibilities that may explain the association between genotype 1b and more advanced chronic liver–disease. It has been suggested that HCV-1b may have existed for a longer period than most HCV–genotypes, and this may explain why it is frequently found in patients with cirrhosis and HCC who have had a longer duration of infection.

In this study, we have not seen a higher frequency of infection by HCV-1b in patients with cirrhosis in comparison with those who had chronic hepatitis suggesting that, at least in our geographic region, where HCV–genotypes other than HCV-1b and particularly HCV-2 and HCV-3 are also frequently found, the genotype of infecting–HCV does not have a major role in determining the progression of chronic hepatitis into the cirrhotic stage.

This is consistent with our previous data, obtained in a large cohort of Italian patients with chronic HCV–infection[31] in whom severity and stage of chronic liver disease were not found to show a definitive association with the genotype of infecting–HCV. This is true at least for patients who present with evidence of liver disease, as were those included in our study, and does not exclude the possibility that in the subgroup of HCV carriers, who have normal ALT, HCV-2 may be more frequently detected, as recently shown by Prati et al.[32] In the present study, we have also tried to address the issue of whether the course and the outcome of chronic liver disease, at least in cirrhotic patients, is significantly influenced by the type of HCV with which they are infected. For this purpose, a cohort of 109 cirrhotic patients, who were followed prospectively, was evaluated. The disease outcome was not different in patients infected by the different HCV genotypes; this is in agreement with what has been recently reported also in Japanese patients by Takano et al.[33] In our own study, patients with HCV-1, most having HCV-1b, did not show a higher risk for the worsening of cirrhosis, for HCC, or for death compared with patients who were infected by other HCV–types. On the other hand, most patients with HCV-1 maintained elevated or fluctuating ALT–levels during follow-up, while persistent enzyme normalization was observed in several patients who were HCV-RNA–negative in the serum upon entry into the follow-up study, suggesting that the remission of biochemical activity of chronic hepatitis C may occur when virus activity is minimal or nonexistent. Interestingly, the course of liver disease was somehow more severe in the small group of cirrhotics who were infected by a mixed HCV–population in comparison with those with a single HCV–type, suggesting that there could be a synergistic effect on the pathogenicity, as already seen by us in cirrhotic patients coinfected by hepatitis B virus and HCV.[25,34] Cirrhotic patients who had received interferon therapy showed a reduced incidence of HCC–development, as

well as of all other major adverse events. However, these observations might be difficult to interpret, due to the retrospective, nonrandomized nature of our analysis.

In conclusion, our study does not support the idea that HCV-1b is associated with a more severe activity and outcome of chronic liver disease, thus excluding that HCV–genotyping may have a significant impact in the prognostic evaluation of patients with chronic HCV–infection; this is in contrast with its undoubted value in predicting response to interferon therapy. Whether or not HCV–types have different pathogenicity remains to be determined by future studies.

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