Concurrent Hepatitis B and C Virus Infection and Risk of Hepatocellular Carcinoma in Cirrhosis

A Prospective Study

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Background. **Patients with cirrhosis have a high risk of hepatocellular carcinoma (HCC) but it is unclear how the etiology of liver disease influences tumor development. The authors evaluated hepatitis B and C virus (HBV, HCV) infection in cirrhosis in relation to the risk of HCC.**

Methods. **Two hundred and ninety consecutive cirrhotic patients were followed prospectively with periodic ultrasound examination. At entry, patients were tested for markers of HBV and HCV to assess relation to tumor development during follow-up.**

Results. **Twenty and five-tenths percent of patients were hepatitis B surface antigen (HBsAg) positive and 68.9% were positive for HCV antibodies. Previous alcohol abuse was present in 26.2%. During follow-up (46.3 ± 21.4 months), HCC developed in 32 patients (11.0%) (annual incidence approximately 3%) including 19.6% of HBsAg-positive patients, 12.2% of HCV antibody positive patients and 14.4% of patients with a history of alcohol abuse. The highest rate of HCC was in patients with dual HBsAg and anti-HCV positivity with or without previous alcohol abuse, whereas the lowest incidence (0%) was in cases without risk factors. By univariate analysis, age older than 59 years ($P < 0.005$), longer duration of cirrhosis ($P < 0.005$), serum alpha-fetoprotein levels higher than 20 ng/ml ($P < 0.05$), and dual HBsAg and HCV positivity ($P < 0.02$) appeared to be associated with HCC. By multivariate analysis, age ($P < 0.01$), positivity for HBsAg and HCV antibodies ($P < 0.05$), male sex ($P < 0.05$), and previous alcohol abuse ($P < 0.08$) were independently related to tumor appearance.**

Conclusions. **These results, although confirming that male sex and previous alcohol abuse are risk factors for hepatocellular carcinoma in cirrhosis, indicate that concurrent hepatitis B and C virus infection determines the highest risk of developing hepatocellular carcinoma.** ***Cancer*** **1994;74:2442–8.**

Key words: hepatocellular carcinoma, hepatitis B virus, hepatitis C virus, cirrhosis.

Patients with chronic liver disease, and particularly those with cirrhosis, have an increased risk of having hepatocellular carcinoma (HCC) develop. Several studies have shown that HCC develops mainly in cirrhotic livers, with this association being seen in more than 80% of cases in Western countries, and that the incidence of HCC in patients with cirrhosis is approximately 10 times higher than in the noncirrhotic population.[1–4]

Other factors that have been implicated as predisposing or etiologic factors for the development of HCC are hepatitis B virus (HBV) and, more recently, hepatitis C virus (HCV) infection, male sex, alcohol abuse, cigarette smoking, and aflatoxin exposure.[4–10] Chronic infection with HBV, a DNA virus able to integrate into the hepatocyte genome, is thought to have the potential of direct oncogenicity.[11–14] More recently, several studies have revealed a high prevalence of infection by HCV in patients with HCC, and this has generated the hypothesis that HCV also may be directly involved in HCC development.[15–20] However, HBV and HCV also are major causes of cirrhosis, and most infected patients have HCC develop only after their disease has progressed to cirrhosis. It is unknown whether the risk of progression to HCC is different in cirrhosis caused by HBV or HCV than in cirrhosis of other etiologic factors

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because there have been no prospective studies from Western countries in which HBV and HCV infection have been directly evaluated as risk factors for HCC. Our study prospectively assesses the risk of HCC in a cohort of consecutive adult patients with cirrhosis and attempts to define whether this risk is dependent on the etiology of cirrhosis, particularly in relation to the role of hepatitis viruses.

Patients and Methods

Design of the Study

This was a prospective study of a cohort of 290 consecutive patients with cirrhosis. At inclusion in the follow-up, patients were evaluated for HBV and HCV markers, previous alcohol abuse, duration of cirrhosis, biochemical activity of liver disease, alpha-fetoprotein levels, and Child's stage of disease. The patients were followed up prospectively to define the incidence of HCC development, and these baseline parameters and patient age and sex were analyzed as risk factors by univariate and multivariate statistical tests.

Patients with Cirrhosis

Two hundred ninety consecutive patients with cirrhosis seen in our institute between January 1986 and December 1993 were included in this study. Enrollment criteria were: (1) histologic (82.5%) or clinical (17.5%) diagnosis of cirrhosis (clinical criteria for cirrhosis were the presence of ascites or of portal hypertension with laboratory evidence of chronic liver disease); (2) absence of any clinical or laboratory (alpha-fetoprotein level less than 400 ng/ml) or ultrasonographic sign of liver cancer at entry. The parameters evaluated in each patient at inclusion were: sex, age, known duration of cirrhosis (defined on the basis of the time interval between histologic or clinical diagnosis and inclusion in the study), serum alanine-aminotransferase (ALT) levels, serum alpha-fetoprotein levels (AFP), stage of cirrhosis according to Child's classification, serum HBV and HCV markers (hepatitis B surface antigen [HBsAg] and anti-HCV, as well as hepatitis B surface antibody anti-HBs and hepatitis B core antibody anti-HBc in patients without HBsAg), previous alcohol abuse (more than 80 g/day for male patients and more than 50 g/day for female patients).

At inclusion and during the subsequent visits, serum samples were taken from most patients and kept frozen. Follow-up was done by periodic (every 6 months) abdominal ultrasound examination. At each follow-up, patients also underwent clinical evaluation and serologic and biochemical testing. HCC was diagnosed by ultrasound assisted fine needle biopsy of focal lesions of the liver as they became detectable during follow-up. Microbiopsy specimens were fixed in 10% formalin and stained with hematoxylin and eosin. The presence of HCC was established according to internationally accepted criteria,[21] by an independent pathologist (A.C.), who was unaware of the clinical and laboratory data. Ultrasound examination was performed with a high resolution real-time instrument (AUC P40, Ansaldo, Hitachi Medical Corporation, Tokyo, Japan) with a 3.5 MHz convex transducer. Fine needle biopsy was done under sonographic guidance using 22-gauge thin needles (Ecojekt, modified Chiba needle, Hospital Service, Hakko Shoji, Japan).

Serologic Testing

HBsAg, anti-HBs, and anti-HBc were analyzed by commercially available kits. Anti-HCV was determined (partially by retrospective analysis of stored sera) by second generation enzyme linked immunosorbent assay (ELISA, Ortho Diagnostic System, Raritan, NJ), and all positive samples were analyzed by second generation recombinant immunoblotting assay (Chiron Corporation, Emeryville, CA) to confirm specificity and to define the pattern of antibody profile.

Statistical Analysis

Chi-square test and Fisher exact test were used, when appropriate, to compare the frequency of HCC development in relation to HBsAg and anti-HBV positivity and ALT behavior. Kaplan–Meier's product limit survival analysis was performed to evaluate the cumulative probability of HCC development in patients during follow-up. Univariate analysis by the Mantel–Cox log rank test was used to define the influence of stratifying variable. Multivariate analysis by Cox's proportional hazards model (stepwise option) was used to evaluate the independent roles of each variable (age, sex, duration of cirrhosis, AFP, Child's stage, alcohol abuse, HBsAg, and anti-HCV) for development of HCC, and P values were calculated by Wald's test. A P value of less than 0.05 for univariate analysis and less than 0.1 for multivariate analysis was considered statistically significant.

Results

Clinical and Serologic Characteristics of Patients with Cirrhosis at Entry

At entry, there were 180 male and 110 female patients; mean patient age was 57.9 years (SD ± 9.8 years; range,

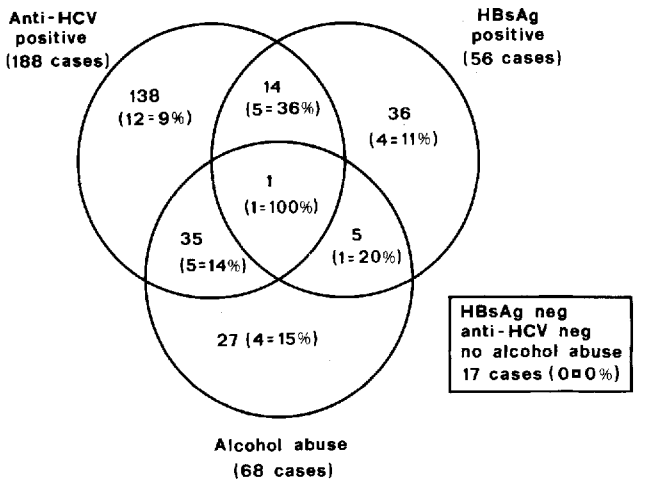

Figure 1. Number of cirrhotic patients with HCV or HBV infection or alcohol abuse or combinations of these risk factors and the number of those (in brackets) who had hepatocellular carcinoma (HCC) develop during follow-up.

24–81 years). The known duration of cirrhosis had a mean of 33.8 months (SD ± 33.3 months; range, 12–144 months). Serum ALT levels at entry were normal or less than 1.5 the upper limit of normal range in 39.0%, between ×1.5 and ×3 normal in 28.0%, and greater than ×3 normal in 33.0% of patients. During follow-up, serum ALT levels remained normal or less than ×1.5 normal in 31.2% of patients. In 21.9% of patients with initial elevated ALT (greater than ×1.5), there was a progressive and persistent normalization of ALT levels during follow-up, whereas in 46.9% of patients, ALT remained high or fluctuated during the entire follow-up period. Baseline AFP levels were less than 20 ng/ml in 212 (73.1%) of patients and greater than 20 ng/ml in the remaining 78 (26.9%) patients.

According to Child's classification 221 (76.2%) patients had Stage A disease, 61 (21.0%) had Stage B, and 8 (2.8%) had Stage C. Seventy-six (26.2%) patients had a history of alcohol abuse. Baseline sera were not available for analysis of viral markers in 17 patients. Among the 273 patients who could be tested, 56 (20.5%) were positive for HBsAg and 188 (68.9%) were positive for anti-HCV. The distribution of HBsAg, anti-HCV, and the presence of alcohol abuse in the 273 patients in whom these three data were available, and their associations, are shown in Figure 1. The remaining 17 patients tested negative for HBsAg and anti-HCV and had no history of heavy alcohol intake; 6 of these patients had primary biliary cirrhosis, whereas the remaining 11 had an autoimmune[5] or cryptogenic[6] disease.

Follow-up Outcome

During follow-up, 12 patients died with no clinical evidence of HCC, 4 underwent transplant and the explanted liver was HCC free, and 38 (13.1%) were lost to follow-up 24–71 months after the beginning of the study. After a mean follow-up period of 46.3 ± 21.4 months (range, 8–96 months), 32 (11.0%) patients had HCC develop (Fig. 2), with a calculated annual rate of approximately 3%.

HCC Development in Relation to Baseline Features

The frequency of HCC development during follow-up in relation to sex, serum AFP levels, stage of cirrhosis, hepatitis virus markers, and alcohol abuse at entry is shown in Table 1. Male patients had HCC develop more frequently than did female patients. Patients with baseline alpha-fetoprotein levels higher than 20 ng/ml and who were positive for HBsAg had HCC develop more frequently than did patients who were negative for HBsAg ($P < 0.05$; chi-square test).

Figure 1 shows how many patients had HCC develop during follow-up in relation to different combinations of three risk factors, i.e., HBsAg and anti-HCV positivity and alcohol abuse. Patients with dual HBsAg and anti-HCV positivity, with or without alcohol abuse, had the highest rate of HCC appearance, whereas the lowest rate (0%) was in patients who had none of the three risk factors. Patients who tested positive for HBsAg or for anti-HCV had similar frequency (11.1% and 9.7%, respectively) of tumor appearance; these rates increased when the patient also abused alcohol.

When the activity of liver disease was considered, on the basis of the ALT profiles seen during follow-up, no significant differences in frequency of tumor development were seen in relation to ALT levels at entry. However, HCC development was observed significantly more frequently in patients with a persistent elevation of ALT levels during follow-up than in those

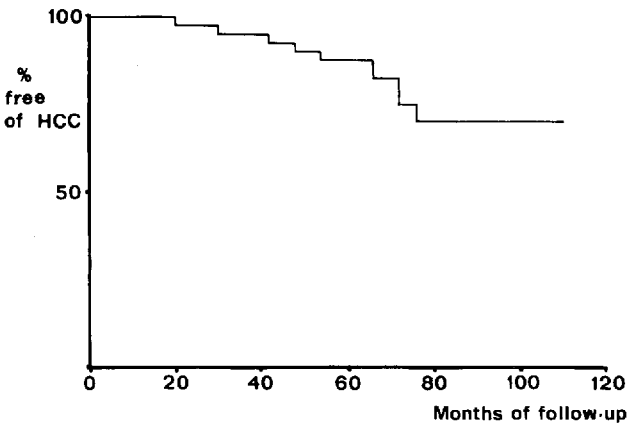

Figure 2. Cumulative probability of being free of HCC in patients with cirrhosis during follow-up (Kaplan–Meier method).

Table 1. Frequency of Hepatocellular Carcinoma Development During Follow-Up of 290 Cirrhotic Patients in Relation to Demographic Features, Base-Line Clinical and Laboratory Findings and Hepatitis Virus Markers

	No cases at risk	No developing HCC (%)
Sex		
males	180	24 (13.3)
females	110	8 (7.3)
Alpha-fetoprotein		
< 20 ng/ml	212	19 (8.9)
≥ 20 ng/ml	78	13 (16.6)
Child's stage		
A	221	26 (11.7)
B	61	6 (9.8)
C	8	0 —
HBsAg		
positive	56	11 (19.6)
negative	217	21 (9.6)
Anti-HCV		
positive	188	23 (12.2)
negative	85	9 (10.5)
Alcohol abuse		
present	76	11 (14.4)
absent	214	21 (9.8)

HCC: hepatocellular carcinoma; HBsAG: hepatitis B surface antigen; anti-HCV: hepatitis C virus antibodies.

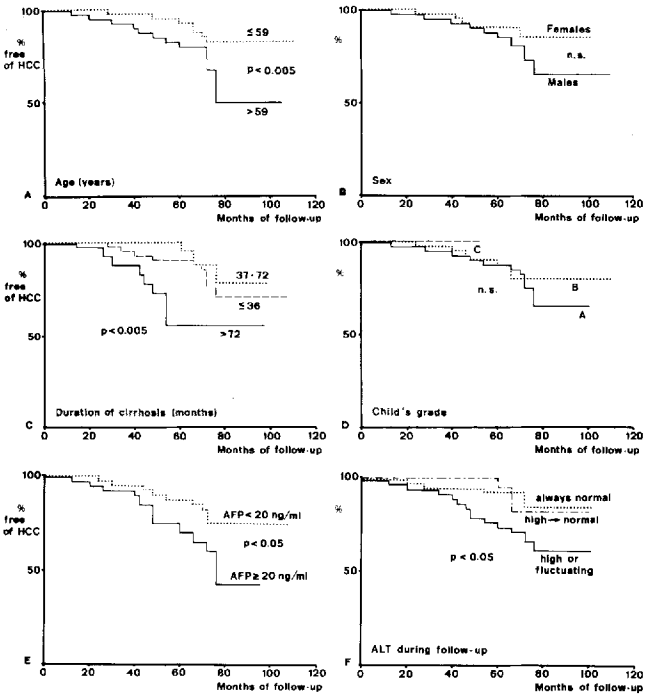

Figure 3. Cumulative probability of being free of HCC in patients with cirrhosis in relation to: (A) age; (B) sex; (C) duration of cirrhosis; (D) Child's grade; (E) serum AFP levels; and (F) ALT activity during follow-up (Kaplan–Meier method and log rank test). NS: not significant.

with persistently normal or nearly normal values ($P < 0.02$) or in those who had enzyme normalization ($P < 0.05$) (Table 2).

Univariate Analysis of Risk Factors for HCC Development in Cirrhosis

The following baseline features were considered in relation to the cumulative probability of HCC developing during follow-up: age, sex, duration and stage of cirrhosis, AFP, ALT, HBsAg, anti-HCV, alcohol abuse, and the association between the last three risk factors. By univariate analysis (Fig. 3) (Kaplan–Meier method and log rank test), the cumulative probability of HCC development was significantly higher in patients older than 59 years of age ($P < 0.005$), those with longlasting (more than 72 months) cirrhosis ($P < 0.005$), those with AFP levels greater than 20 ng/ml at study entry ($P < 0.05$), and those who maintained high or fluctuating ALT levels during follow-up ($P < 0.05$). No differences were seen in relation to stage of cirrhosis. Male patients had

Table 2. Alanine Transaminase Levels at Entry and During Follow-Up in Relation to Hepatocellular Carcinoma Development in 290 Patients With Cirrhosis

	No. cases at risk	No. developing HCC (%)	
ALT at entry			
Normal or below ×1.5	113	7 (6.1)	
From ×1.5 to ×3	81	12 (14.8)	
Above ×3	96	13 (13.5)	
ALT during follow-up			
Always normal	91	5 (5.5)	
Normalization during follow-up	63	4 (6.3)	$P < 0.02$
		$P < 0.05$	
Always high or fluctuating	136	23 (16.9)	

ALT: alanine transaminase; HCC: hepatocellular carcinoma.

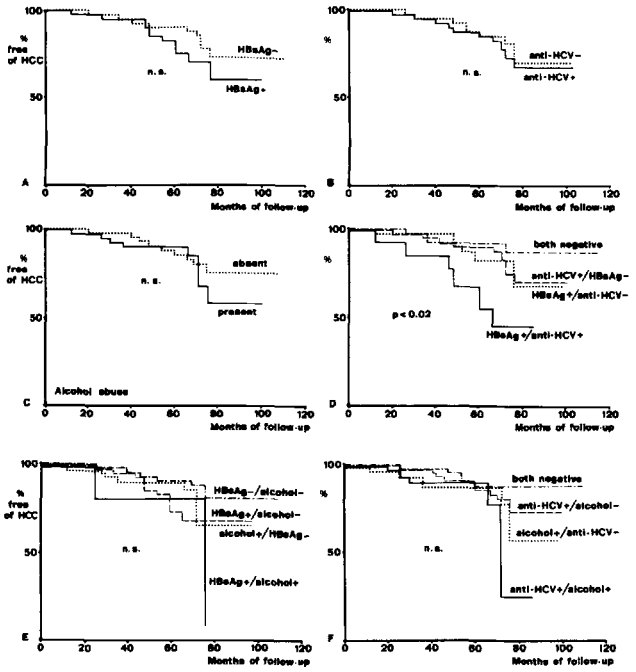

Figure 4. Cumulative probability of being free of HCC in patients with cirrhosis in relation to: (A) hepatitis B surface antigen positivity; (B) hepatitis C antibody positivity; (C) presence of alcohol abuse (more than 80 g/day in male patients and > 50 g/day in female patients); (D) presence of dual HBsAg and anti-HCV positivity, single HBsAg or anti-HCV positivity, negativity for both markers; (E) presence of HBsAg positivity and alcohol abuse, single HBsAg positivity or alcohol abuse, negativity for both factors; and (F) presence of anti-HCV positivity and alcohol abuse, single anti-HCV positivity or alcohol abuse, negativity for both risk factors (Kaplan–Meier method and log rank test). NS: not significant.

HCC develop more frequently than did female patients, but the difference did not reach statistical significance.

The same analysis made for viral markers and alcohol abuse (Fig. 4) confirmed that patients who had HBsAg positivity had a higher probability of HCC developing than did patients with HBsAg negativity, but the difference was not statistically significant ($P = 0.08$). No significant differences were seen in relation to anti-HCV positivity or presence of alcohol abuse. However, patients who tested positive at entry for HBsAg and anti-HCV had HCC develop significantly more frequently ($P < 0.02$) than did any other category of patients (Fig. 3), whereas the association of alcohol abuse with either HBsAg positivity or anti-HCV positivity did not increase significantly the cumulative risk for HCC development.

Development of HCC in Patients with HBsAg Negativity and with Antibodies to HBV

Because the data obtained in patients with dual HBsAg and anti-HCV positivity suggested that infection with both viruses could increase significantly the risk of HCC development in cirrhotic patients, we have analyzed the behavior of patients with anti-HCV positivity who had serologic evidence of past HBV infection and who tested positive for antibodies to HBV. However, the frequency of tumor development in these patients (8 of 70; 11.4%) was similar to that of patients with HBsAg negativity without dual positivity for anti-HBV (anti-HBs and anti-HBc) and anti-HCV (13 of 147; 8.8%).

Multivariate Analysis of Risk Factors for HCC Development in Cirrhosis

To identify the independent role of different variables, the multivariate Cox's proportional hazards model was used, in which sex, age, duration, and Child's stage of cirrhosis, alcohol abuse, baseline AFP levels, serum HBsAg and anti-HCV positivity, and combinations of risk factors were considered (Table 3). The factors that were identified as having an independent role for HCC development were male sex ($P < 0.02$), age ($P < 0.01$), dual positivity for HBsAg and anti-HCV at entry ($P < 0.02$), and presence of alcohol abuse ($P < 0.08$).

Discussion

The precise roles of HBV and HCV infection as risk factors for HCC have been debated. Most available data are based on prevalence and case–control studies.[7,22–26] In longitudinal surveys conducted in Japan,[27] Taiwan,[11] and the United Kingdom[8] before the identification of HCV, HBsAg carriage in patients with cirrhosis was

Table 3. Multivariate Analysis of Factors Associated With the Development of Hepatocellular Carcinoma in Cirrhotic Patients (Survival Analysis With Covariate by Cox's Model—Wald Test)

Variable	Coefficient	SE	EXP (coefficient)	*P*
Male sex	0.82	0.44	2.28	0.014
Age*	0.07	0.02	1.08	0.009
Duration*	NS	NS	NS	NS
AFP > 20 ng/ml	NS	NS	NS	NS
Child's stage	NS	NS	NS	NS
HBsAg pos.	NS	NS	NS	NS
Anti-HCV pos.	NS	NS	NS	NS
Alcohol abuse	0.73	0.40	2.08	0.079
HBsAg+/anti-HCV+	1.63	0.48	5.11	0.012
HBsAg+/alcohol	NS	NS	NS	NS
Anti-HCV+/alcohol	NS	NS	NS	NS

SE: standard error; EXP: exponential; AFP: alpha-fetoprotein; HBsAg: hepatitis B surface antigen; Anti-HCV: hepatitis C virus antibodies; NS: not significant.
* Continuous value.

proven to be a risk factor for HCC. In a prospective study of 447 Italian patients with cirrhosis, Colombo et al.[28] found no relation between etiology and HCC development. In a recent prospective Japanese study of 917 patients with chronic liver disease Tsukuma et al.[29] found that presence of cirrhosis and HBsAg and anti-HCV positivity were associated with a significantly higher risk of HCC development. In another prospective study of 795 Japanese cirrhotic patients studied more recently by Ikeda et al.,[30] it was emphasized that HCV-related cirrhosis might be more frequently involved in HCC development than is HBsAg-related cirrhosis. Our study is the first prospective study in which HBsAg and anti-HCV positivity have been analyzed in white cirrhotic patients as risk factors for HCC. All of our patients had cirrhosis, which did not allow us to analyze cirrhosis as a risk factor. However, the high incidence rate of HCC (approximately 3%/year) observed in our patients, was similar to that reported in other studies,[28,29] thus confirming the close association between cirrhosis and HCC.

In our study, patients with a more active liver disease, as shown by persistently high ALT levels, had a significantly greater risk of HCC development than did those with inactive cirrhosis. This may reflect the role of ongoing inflammation, cell necrosis, and regeneration in the carcinogenetic process, independently of etiologic agents.

In agreement with previous reports, male patients with cirrhosis had HCC develop more frequently than did female patients, and past history of heavy alcohol consumption also was an independent risk factor for HCC. The role of alcohol has been disputed, and it is interesting that its association with HCC development has been confirmed in the multivariate analysis in our patients, who come from a region of high alcohol consumption in the general population. Other factors found to predispose to HCC development in cirrhotic patients were advanced age and long-lasting disease; these factors were independent of the stage of cirrhosis.

With regard to the influence of HBV and HCV infection in promoting tumor development in our patients, HBsAg positivity at entry was associated with a slightly higher risk of HCC developing during follow-up, but HBsAg per se did not emerge as a risk factor for HCC by univariate or multivariate analysis. Hepatitis C virus infection was common among our population of cirrhotic patients (68.9%), and the appearance of HCC in patients with anti-HCV positivity was almost identical to that in patients with anti-HCV negativity, thus excluding that HCV infection per se could have a significant effect on tumor development. The most likely explanation of why HBV and HCV were not found in our study to be independent risk factors for HCC is that our study population contained an extremely high percentage of patients with at least one recognized risk factor, such as HBV or HCV infection or alcohol abuse, with an inadequate control population of patients with cirrhosis but without these risk factors. However, when HBV and HCV infection both were present, the risk of HCC appearance during follow-up was markedly increased and was confirmed as significant at the statistical level by multivariate analysis. In contrast, patients with a combination of alcohol abuse and HBsAg or anti-HCV positivity were not found to have increased risk for HCC when compared with patients with only a single risk factor.

Thus, in our series of white cirrhotic patients, who were observed in a geographic area where HBV infection is not an uncommon cause and HCV infection is an extremely common cause of chronic liver disease, the association of the two infections appeared to operate a strong oncogenic effect on liver cells. This was true only for patients with actual, chronic HBV infection because patients with HBsAg negativity and with only antibodies to HBV did not have an increased incidence of HCC when they also had positivity for anti-HCV. How dual HBV and HCV infection promotes tumor development remains unknown. Because HCC occurred mainly in patients with evidence of ongoing liver disease, as shown by persistent ALT level abnormalities, it may be suggested that HBV could act as the "initiating" factor by its capacity of disarranging cellular genes, whereas HCV may behave as the promoting factor, by causing persistent liver cell necrosis and regeneration. More specific interactions between the two viruses at the molecular levels also may be involved because phenomena of virus interference between HBV and HCV have been reported to occur in vitro and in vivo.[31] Our results indicated that patients with more active and long-lasting cirrhosis, and particularly those in whom dual HBV and HCV infection is documented, are at the highest risk of HCC and represent the categories of cirrhotic patients who should be followed up more closely for early diagnosis of tumor development.

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