Characteristics of Patients with Chronic Hepatitis C who Develop Hepatocellular Carcinoma after a Sustained Response to Interferon Therapy

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BACKGROUND. The objective of the current study was to determine the characteristic features of sustained responders who develop hepatocellular carcinoma after treatment with interferon for chronic hepatitis C.

METHODS. This study included 3626 patients with chronic hepatitis C who had received interferon monotherapy. Cox proportional hazards analysis was used to compare sustained responders who did and did not develop hepatocellular carcinoma, and nonsustained responders who developed hepatocellular carcinoma in a multicenter, retrospective cohort study.

RESULTS. Among 1197 sustained responders, 27 patients developed hepatocellular carcinoma (2.3%). Compared with sustained responders who did not develop hepatocellular carcinoma, patients who developed disease more often were male ($P = 0.0212$), were older ($P = 0.0068$), and had advanced-stage histologic disease before interferon therapy ($P = 0.0345$). Conversely, compared with patients with hepatocellular carcinoma who were not sustained responders, patients who were sustained responders tended to be older at the time of the initiation of interferon therapy ($P = 0.0552$) and at the time hepatocellular carcinoma was detected ($P = 0.0593$), and they also were predominantly male ($P = 0.0507$). The histologic staging and serum aminotransferase levels at the initiation of interferon therapy, the interval to the detection of tumor, and the tumor size showed no significant differences between the two groups.

CONCLUSIONS. Sustained responders in the group at high risk for developing hepatocellular carcinoma after interferon therapy were older, more often were male, and had more advanced histologic disease stage. Such patients should be followed carefully periodically for > 10 years after they complete interferon therapy. ***Cancer* 2004;101:1616–22.** © *2004 American Cancer Society.*

KEYWORDS: chronic hepatitis type C, hepatocellular carcinoma, interferon, sustained responder.

In Japan, chronic hepatitis C (CH-C) with advanced histologic staging often progresses to hepatocellular carcinoma (HCC),[1] although patients who are seropositive for antihepatitis C virus (anti-HCV) antibodies or for HCV RNA do not always progress to cirrhosis or HCC.[2,3] Risk factors for developing HCC in patients with CH-C are advanced histologic stage, irregular regeneration of hepatocytes, heavy drinking, higher serum alanine aminotransferase (ALT) levels or lower serum albumin levels, male gender, and older age.[1,4–7] Since 1992, patients with CH-C commonly have been treated with interferon α (IFN-α) or IFN-β, which are covered by public health insurance in Japan. Because IFN improves hepatic inflammation and inhibits the progression of hepatic fibrosis, it

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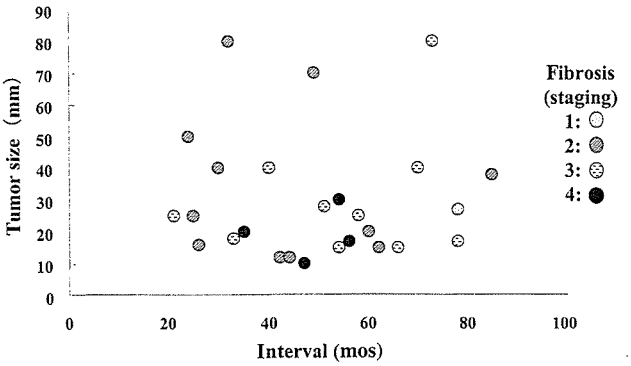

FIGURE 1. The interval from the completion of IFN therapy to the detection of SR HCC statistically did not correlate significantly with the tumor size or hepatic staging.

has been suggested that the incidence of HCC may be reduced by IFN treatment. In fact, IFN therapy reportedly was effective not only for improving liver biochemistry and eliminating HCV RNA but also for reducing the inflammation/fibrosis scores and lowering the risk of HCC, especially in sustained responders (SR patients).[8–14]

Although a significant decrease in the incidence of HCC has been observed in SR patients after IFN therapy,[9–14] HCC is detected in some of them.[15–25] The clinical features of SR patients who develop HCC (SR HCC patients) and the long-term incidence of HCC in SR patients remain unclear, and the optimal duration and frequency of follow-up have not been established. Therefore, we analyzed SR HCC patients to determine their characteristic features compared with SR patients who did not develop HCC (SR non-HCC patients) and non-SRs who developed HCC (non-SR HCC patients).

MATERIALS AND METHODS

Patients

For this study, 3626 patients with CH-C were enrolled (2344 males and 1282 females) who had received IFN therapy between January 1990 and November 2001. Data from these patients were collected from 6 institutions and related hospitals, including 1371 patients from Kyoto Prefectural University of Medicine, 1478 patients from Osaka University, 497 patients from Miyazaki Medical College, 130 patients from Nagoya University, 102 patients from Shinsyu University, and 48 patients from Yamaguchi University. All patients were seropositive for anti-HCV antibodies, positive for serum HCV RNA, and seronegative for hepatitis B virus surface antigen. We excluded patients who had coexisting liver diseases, such as autoimmune hepatitis or primary biliary cirrhosis, and confirmed that they did not abuse alcohol (daily alcohol intake > 60 g of ethanol). No patients were infected with human immunodeficiency virus (HIV). At the time of entry into this study, no patients showed evidence of HCC, as determined by ultrasonography (US) and/or computed tomography (CT) studies. In principle, patients underwent liver biopsy prior to IFN therapy, and the histologic diagnoses were reached according to the classification of Desmet et al.[26] The gender, mean age, and histologic disease stage at the initiation of IFN therapy are shown in Table 1.

TABLE 1
Characteristics of Patients with Chronic Hepatitis C who were Treated with Interferon[a]

Characteristic	Sustained responder	Nonsustained responder	*P* value[b]
No. patients	1197	2429	—
Male:female ratio	776:421	1568:861	0.8826
Age (yrs, mean ± SD)	49.4 ± 11.9	51.2 ± 10.6	< 0.0001
Histologic staging score: No. of patients (%)			
F1	385 (38.6)	522 (25.8)	
F2	322 (32.3)	613 (30.3)	< 0.0001
F3	262 (26.3)	782 (38.6)	
F4	29 (2.9)	109 (5.4)	
Not available	199	403	

SD: standard deviation; IFN: interferon.
[a] All data were determined before interferon therapy.
[b] *P* values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.

Natural IFN-α, recombinant IFN-α-2a, and recombinant IFN-α-2b were used in this study. In general, the IFN treatment protocol was within the range covered by public health insurance in Japan, namely, 3–10 MU of IFN-α for 24 weeks (daily for 2 weeks and 3 times per week for 22 weeks). In a few patients, administration of IFN-α was prolonged to 52 weeks. In some patients who suffered from severe side effects, the therapy period was shortened. In addition, patients for whom the total dose of IFN was < 200 MU were excluded from the study. Patients who had been treated with peginterferon or IFN/Ribavirin also were excluded. There was no difference noted with regard to the treatment protocol among the institutions and their related hospitals. We checked the laboratory findings at the end of IFN therapy and 6 months later. SR patients were defined as those who demonstrated continuous normal serum ALT levels for 6 months after finishing IFN therapy. The remaining patients were regarded as non-SR patients. The patient population included 1197 SR patients and 2429 non-SR patients.

We followed all patients for at least 1 year after the end of IFN therapy. The mean ± standard deviation

(SD) follow-up was 5.9 years ± 1.9 years. In SR patients, in general, we performed biochemical examinations, which sometimes included α-fetoprotein, every 3–12 months after confirming a sustained response. US and/or CT studies were performed at least once annually. However, because the incidence of HCC in non-SR patients—especially those with advanced-stage disease (fibrotic scores of F3 or F4)–was expected to be higher than that in SR patients, US and/or CT studies were performed every 3–6 months in non-SR patients. This strategy was similar in all of the institutions, and the frequency of radiographic examination was calculated to avoid unnecessary cost and not to miss HCC. However, some SR patients and non-SR patients who skipped or stopped visiting the outpatient clinic and some patients who were followed by their home physicians were not followed sufficiently. The diagnosis of HCC was based on appropriate radiologic findings (hepatic angiography, dynamic CT, magnetic resonance imaging).[27] When it was difficult to determine a final diagnosis with the radiologic findings, a histologic diagnosis was reached by tumor biopsy. In 17 of 27 SR HCC patients, a histologic diagnosis of HCC was obtained by the examination of resected hepatic tumors or biopsied tumor specimens. Patients who were diagnosed with HCC within 1 year after the end of IFN therapy were excluded from this study because of the possibility that a small but detectable HCC was missed before IFN therapy. Written informed consent to receive IFN therapy and to participate in this follow-up study was obtained from all patients, and the ethical committees of the participating institutions approved this study.

Statistical Analysis

Statistical analysis was performed using the SAS/PC statistical package (SAS Institute, Cary, NC). The Fischer exact probability test was used to compare the frequencies of gender. The Wilcoxon two-sample test was used to compare age, histologic staging, serum ALT level, interval between the end of IFN therapy and the detection of HCC, and the size of HCC. The independent risk factors for developing HCC in SR patients were examined by Cox proportional-hazards analysis; the variables were gender, age, histologic stage, and serum ALT level. Patients who had missing data were excluded from this analysis. Each variable was transformed into categorical data comprised of two-sample, ordinal numbers for multivariate analysis. P values were two-sided, and P values < 0.05 were considered statistically significant.

RESULTS

Characteristic Features of SR HCC Patients

During the observation of 3626 patients, HCC was detected in 259 patients; however, 19 patients were excluded, because HCC was detected within 1 year after they completed IFN therapy. The distribution of the remaining 240 HCC patients among the 6 institutions was as follows: 109 patients from Kyoto Prefectural University of Medicine (HCC incidence, 8.0%), 102 patients from Osaka University (HCC incidence, 6.9%), 3 patients from Miyazaki Medical College (HCC incidence, 0.6%), 15 patients from Nagoya University (HCC incidence, 11.5%), 8 patients from Shinsyu University (HCC incidence, 7.8%), and 3 patients from Yamaguchi University (HCC incidence, 6.3%). The incidence of HCC did not differ significantly among the institutions, except for Miyazaki Medical College. partly because hepatic fibrosis was less advanced in patients from this institution compared with patients from the other five institutions. Of 240 patients, 27 were SR patients, and 213 were non-SR patients. The ages of the 240 patients at the initiation of IFN therapy ranged from 37–77 years (mean age ± SD, 59.1 years ± 6.6 years) and varied from 39–83 years (63.6 years ± 6.8 years) at the time HCC was detected.

Among the 27 SR HCC patients, 5 patients consumed ≈ 50 g of ethanol daily. By evaluating liver specimens and biochemical examinations, including γ-glutamyl transferase, we excluded the possibility of alcoholic liver diseases in these patients. Serum HCV RNA was evaluated in the SR HCC patients by reverse transcriptase-polymerase chain reaction analysis. Twenty-six SR HCC patients were complete responders (seronegative for HCV RNA both at the end of IFN therapy and 6 months later), and 1 SR HCC patient was a biochemical responder (seropositive for HCV RNA at the end of IFN therapy). In 1 complete responder who developed HCC, serum HCV RNA became positive 12 months after completing IFN therapy.

No correlation could be found between the interval before HCC was detected, tumor size, or hepatic histologic stage among the SR HCC patients (Fig. 1). HCC that was detected long after discontinuing IFN therapy was not always large, and the patients with large HCC did not always show more advanced stage according to liver histology. The greatest dimensions of the 2 largest SR HCC tumors were 80 mm and were detected 32 months and 73 months after the end of IFN therapy. The greatest dimension of SR HCC found after the longest interval (85 months) was 38 mm.

Tumor tissue samples could be examined from 18 of 27 SR HCC patients. Two samples were categorized

TABLE 2
Comparisons between Sustained Responders with and without Hepatocellular Carcinoma[a]

Characteristic	SR HCC	SR non-HCC	*P* value[b]
No. of patients	27	1170	
Male:female ratio	25:2	751:419	0.0016
Age (yrs, mean ± SD)	60.7 ± 7.5	50.2 ± 12.4	< 0.0001
Serum ALT (IU/L, mean ± SD)	111.7 ± 67.7	122.6 ± 109.9	0.7267
Histologic staging score: No. of patients (%)			
F1	1 (3.7)	384 (39.6)	
F2	11 (40.7)	310 (32.0)	< 0.0001
F3	10 (37.0)	252 (26.0)	
F4	5 (18.5)	24 (2.5)	

SR: sustained responder; HCC: hepatocellular carcinoma; SD: standard deviation; ALT: alanine aminotransferase; IFN: interferon.

[a] All data were determined before interferon therapy.

[b] *P* values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.

TABLE 3
Factors Associated with the Development of Hepatocellular Carcinoma in Sustained Responders[a]

Characteristic	Risk ratio	95% CI	*P* value
Male vs. female	5.498	1.290–23.439	0.0212
Age	7.378	1.737–31.326	0.0068
Stage of liver disease	2.344	1.064–5.164	0.0345
Serum ALT	1.331	0.606–2.923	0.4768

95% CI: 95% confidence interval; ALT: alanine aminotransferase.

[a] All data were determined before interferon therapy. Statistical analysis was performed using the Cox proportional hazards test. The variable for age was set at < 50 years or ≥ 50 years, the variable for stage was set < F3 or ≥ F3, and the variable for the serum alanine aminotransferase level was set at < 88 UI/L or ≥ 88 UI/L. The variables age and serum alanine aminotransferase level were determined as median data. The variable for stage was set to obtain the largest hazard ratio.

as well differentiated HCC, 11 samples were moderately differentiated HCC, 2 samples were poorly differentiated HCC, and 2 samples were undifferentiated HCC. One sample was the necrotic tissue after transcatheter arterial embolization therapy (TAE). Nontumorous liver tissue samples from 18 patients were evaluated for their fibrosis scores in resected HCC or tumor biopsy specimens. Liver fibrosis scores improved in nine patients, did not change significantly in eight patients, and worsened in one patient.

Sixteen of 27 SR HCC patients underwent partial hepatectomy, and 10 patients were treated with TAE and/or percutaneous ethanol injection therapy. Because one patient changed his hospital after the diagnosis of HCC, we could not know his prognosis.

Comparison between SR HCC Patients and SR Non-HCC Patients

We compared 27 SR HCC patients with 1170 SR non-HCC patients. The SR HCC patients included 25 males (92.6%) and 2 females (7.4%), and the SR non-HCC patients included 751 males (63.5%) and 419 females (35.8%). At the time IFN therapy was initiated, the mean age of the SR HCC patients was 60.7 years ± 7.5 years (range, 37–70 years), whereas the mean age of the SR non-HCC patients was 50.2 years ± 12.4 years (range, 17–73 years). Thus, the SR HCC patients more often were male ($P = 0.0016$) and were older ($P < 0.0001$) compared with the SR non-HCC patients (Table 2).

The fibrotic scores in biopsied liver specimens before IFN therapy for the SR HCC patients included 1 F1 specimen (3.7%), 11 F2 specimens (40.7%), 10 F3 specimens (37.0%), and 5 F4 specimens (18.5%); and the fibrotic scores for the SR non-HCC patients included 384 F1 specimens (39.6%), 310 F2 specimens (32.0%), 252 F3 specimens (26.0%), and 24 F4 specimens (2.5%). The 2 female SR HCC patients both had F4 specimens. Among the total SR population, SR HCC patients had more advanced-stage disease ($P < 0.0001$). The mean serum ALT level at the initiation of IFN therapy was 111.7 IU/L ± 67.7 IU/L in the SR HCC patients and 122.6 IU/L ± 109.9 IU/L in the SR non-HCC patients (Table 2).

Cox proportional-hazards analysis of factors associated with the development of HCC in the SR patients was performed with four variables (gender, age, histologic stage, and serum ALT level). In this analysis, the hazard ratios for age, stage, and serum ALT level were calculated between the two groups. The age variable was set at < 50 years or ≥ 50 years, the fibrotic score (stage) variable was set at < F3 or ≥ F3, and the variable for serum ALT level was set at < 88 IU/L or ≥ 88 IU/L. The variables age and serum ALT level were determined as median data. We chose the variable for stage to obtain the greatest hazard ratio. The SR HCC patients more often were male ($P = 0.0212$, 95%CI, 1.290–23.439), were older ($P = 0.0098$, 95%CI, 1.737–31.326), and had advanced-stage disease according to liver histology ($P = 0.0345$; 95%CI, 1.064–5.164) before IFN therapy. Gender, age, and histologic stage before IFN therapy were considered independent risk factors for the development of HCC (Table 3).

Comparison between SR HCC Patients and Non-SR HCC Patients

We compared the clinical characteristics of the 27 SR HCC patients with the 213 non-SR HCC patients. The non-SR HCC patients included 161 males (75.6%) and 52 females (24.4%). The mean age of the non-SR HCC patients at the initiation of IFN therapy was 58.9 years ± 6.5 years (range, 40–77 years), and the mean age at

TABLE 4
Comparisons between Sustained Responders and Nonsustained Responders among Patients with Hepatocellular Carcinoma

Characteristic	SR	Non-SR	*P* value[a]
No. of patients	27	213	
Male:female ratio	25:2	161:52	0.0507
Age at the initiation of IFN (yrs, mean ± SD)	60.7 ± 7.5	58.9 ± 6.5	0.0552
Age at the detection of HCC (yrs, mean ± SD)	65.1 ± 7.8	63.4 ± 6.7	0.0593
Serum ALT (IU/L)[b]	111.7 ± 67.7	120.5 ± 56.4	0.2027
Histologic staging score: No. of patients (%)[b]			
F1	1 (3.7)	12 (5.6)	
F2	11 (40.7)	36 (16.9)	0.1861
F3	10 (37.0)	135 (63.4)	
F4	5 (18.5)	30 (14.1)	
Interval (mos, mean ± SD)[c]	49.3 ± 18.2	49.7 ± 24.8	0.7484
Tumor size (mm, mean ± SD)	31.2 ± 20.1	21.3 ± 9.9	0.1573

SR: sustained responder; IFN: interferon; SD: standard deviation; HCC: hepatocellular carcinoma; ALT: alanine aminotransferase.
[a]*P* values were calculated with the Fisher exact probability test and the Wilcoxon two-sample test.
[b]Data were determined before interferon therapy.
[c]The interval was between the completion of interferon therapy and the detection of hepatocellular carcinoma.

time HCC was detected was 63.2 years ± 6.7 years (range, 44–83 years). The mean serum ALT level in the non-SR HCC patients at the start of IFN therapy was 120.5 IU/L ± 56.4 IU/L. The fibrotic scores of biopsied liver specimens obtained from the non-SR HCC patients before IFN therapy included 12 F1 specimens (5.6%), 36 F2 specimens (16.9%), 135 F3 specimens (63.4%), and 30 F4 specimens (14.1%). Thus, concerning gender and age, the SR HCC patients tended to be predominantly male ($P = 0.0507$) and were older (both at the initiation of IFN therapy [$P = 0.0552$] and at the time HCC was detected [$P = 0.0593$]) compared with the non-SR HCC patients; however, the serum ALT levels and the histologic stage before IFN therapy among the SR HCC patients did not differ significantly compared with the non-SR HCC patients (Table 4).

The mean interval between the end of IFN therapy and the detection of HCC for the SR HCC patients was 49.3 months ± 18.2 months (range, 21–85 months), which was not significantly different from that for the non-SR HCC patients (49.7 months ± 24.8 months; range, 12–141 months). The mean greatest dimension of SR HCC was 31.2 mm ± 20.1 mm, which was slightly greater than, but not significantly different from, the mean greatest dimension of non-SR HCC (21.3 mm ± 9.9 mm) (Table 4).

DISCUSSION

In the current study, we compared the clinical characteristics of SR HCC patients with the characteristics of SR non-HCC patients to determine the characteristic features of SR HCC. The incidence of HCC among the 1197 SR patients was 2.3%, and the incidence among the 2429 non-SR patients was 8.8% during the mean follow-up of 5.9 years. In patients with CH-C, aging and advanced hepatic histologic stage reportedly are major risk factors for HCC development.[1,4] This was true for the SR population in our current investigation, because the risk ratio for developing HCC was > 7 times greater in older patients (≥ 50 years) and was more than twice as high in patients who had advanced histologic stage disease (fibrotic score ≥ F3) according to a Cox proportional-hazards analysis. Khan et al. also reported that male gender is an important risk factor for HCC development.[5] In the current study, males were more than five times more likely to develop HCC in the SR population. Thus, older male patients with advanced hepatic fibrosis were considered to be a high-risk group for developing HCC among the SR population (Table 3).

Conversely, compared with the non-SR HCC patients, the SR HCC patients were older at the initiation of IFN therapy ($P = 0.0552$) and at the detection of HCC ($P = 0.0593$), and they were predominantly male ($P = 0.0507$). Although these characteristics may not have differed significantly in the current study, a study of even larger size may show that this indeed is a trend. The histologic staging, the serum ALT level at the initiation of IFN therapy, the interval for the detection of HCC, and the tumor size did not differ significantly between the two groups. The tumor size in SR HCC patients was slightly greater compared with the tumor size in non-SR HCC patients, most likely because of the extended interval of screening for HCC after patients attained a sustained response to IFN therapy (Table 4).

Some previous articles reported that HCV RNA may survive in the hepatic tissues of SR HCC patients[28–30] and may be involved in the carcinogenesis or growth of HCC. Although we could not demonstrate the presence of HCV RNA in tumors and surrounding hepatic tissues from SR HCC patients, eradication of HCV from these tissues, along with the nontumorous hepatic tissues, was confirmed in several previous studies,[15–21] suggesting that the persistence of HCV is not essential for the growth of HCC in SR patients.

To ascertain the time of HCC occurrence, several studies were performed that examined the doubling time (DT) of HCC. Two studies from Japan reported that the DT of HCC measuring < 3 cm in greatest dimension was 93.0 days ± 57.4 days or 195.0 days ± 171.0 days.[31,32] Barbara et al. reported that the DT of HCC measuring < 5 cm in greatest dimension was 204.2 days ± 135 days.[33] Recently, Toyoda et al. re-

ported similar results, assuming that the greatest dimension of occult HCC was 5 mm before IFN therapy.[34] We calculated the growth interval between a single HCC cell and an HCC measuring 1 cm in greatest dimension on the assumption that the DT of HCC was 90 days and concluded that the growth interval may be > 6 years.[8] Because smaller and well differentiated HCCs have a longer DT, the growth interval to reach 1 cm in greatest dimension may be much longer than 6 years. Therefore, it is probable that small HCC may have existed in the liver prior to IFN therapy in the current SR HCC patients.[35]

It cannot be determined with certainty how long SR patients should be followed after they complete IFN therapy. Judging from the results obtained in the current study, we recommend that, when SR patients are male, age > 50 years old, and have F3 or F4 histologic stage, they should be checked by US or CT at least twice per year for > 10 years. Other SR patients with less advanced disease should be checked at least once per year.

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Hepatitis B virus-related insertional mutagenesis in chronic hepatitis B patients as an early drastic genetic change leading to hepatocarcinogenesis

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Growing evidence demonstrates that hepatitis B virus (HBV) integration and resulting insertional mutagenesis play an important role in cell growth or maintenance in hepatocellular carcinomas (HCCs). To determine if HBV integration occurs and affects cellular genes at such a stage of infection, we analysed viral–host junctions in chronic hepatitis tissues without HCC using PCR amplification with primers specific to human *Alu*-repeat and HBV. We obtained 42 independent viral–host junctions from six patients examined and identified chromosomal locations for 20 of the 42 junctions. In six clones, each integration apparently affected a single gene. These six candidate genes included one known tumor suppressor gene, three human homologs of drosophila genes that are critical for organ development, one putative oncogene and one recently found chemokine. Our data, together with previously reported HBV integrants in HCCs, suggested preferential HBV integration into chromosome 3 ($P=0.022$). Our virus-tagging approach provided (a) firm evidence of HBV integration in hepatocytes at an early stage of chronic infection and (b) revealed cellular genes possibly affected by HBV integration and potentially involved in early steps of the process leading to carcinogenesis.
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Introduction

Epidemiologic data provide compelling evidence (Beasley *et al.*, 1981; Yu *et al.*, 2000) for a role of hepatitis B virus (HBV) in the development of hepatocellular carcinoma (HCC). HBV is thought to be one of the DNA tumor viruses, such as human papillomavirus (HPV) and Epstein–Barr virus (Nevins and Vogt, 1996). HBV also shares with oncogenic retroviruses, a unique replication strategy through reverse transcription and a characteristic lifecycle that includes integration into the host genome (Bréchot *et al.*, 2000). Despite extensive research, the precise mechanism whereby HBV infection contributes to hepatocarcinogenesis remains unclear. One complication is that human tumorigenesis is a multistep process and several viral mechanisms are possibly involved in each step, including cis- and trans-activation of cellular genes by viral proteins, antiapoptotic action, induction of genomic instability and insertional mutagenesis (Nevins and Vogt, 1996).

Several groups have independently reported HBV integration into the human telomerase reverse transcriptase (hTERT) gene in HCCs, as well as in hepatoma derived cell lines, proving that viral insertion activates hTERT expression and, possibly, maintenance of telomere length (Gozuacik *et al.*, 2001; Horikawa and Barrett, 2001; Ferber *et al.*, 2003a). Insertional mutagenesis is an important mechanism common to both RNA and DNA virus-related oncogenesis. HPV integration into the hTERT gene has recently been reported (Ferber *et al.*, 2003a) and others have reported clonal T-cell proliferation induced by integration of retrovirus into a human proto-oncogene (Hacein-Bey-Abina *et al.*, 2003). Furthermore, the integration of woodchuck hepatitis virus (WHV), an animal virus resembling HBV, often occurs in c-Myc or N-Myc genes. Direct transforming activity of WHV/Myc integrants is thought to lead to the development of animal HCCs (Hsu *et al.*, 1988; Fourel *et al.*, 1990). Other reports describe genes with important roles in cell growth, such as retinoic acid receptor beta, cyclin A2, mevalonate kinase, sarco/endoplasmic reticulum calcium ATPase 1 and human minichromosome maintenance protein 8, undergoing deregulation by HBV insertion (for reviews, see Bréchot *et al.*, 2000; Paterlini-Bréchot *et al.*, 2003). Cumulatively, these data strongly suggest that HBV insertional mutagenesis plays an important role in the human oncogenic process.

We previously reported that HBV integration occurs early during HBV infection, even after acute self-limiting hepatitis (Murakami *et al.*, 2004). Insertional mutagenesis may represent the first drastic genetic change during a long-lasting chronic viral carrier state, preceding the development of HCC by a few decades. Furthermore, we may be able to investigate HBV

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integration with little influence of genomic recombination and chromosomal losses or gains during the early phase of chronic infection.

There have been few investigations on HBV integration in chronic hepatitis tissues. A few reports have described host sequences adjacent to viral integration in chronic hepatitis (Yaginuma *et al*., 1987; Takada *et al*., 1990). This paucity of data reflects limited human genome information as well as the lack of a suitable cloning method. We investigated integrated HBV–host junctions in liver tissues with chronic hepatitis B without HCC using HBV-*Alu* PCR (Minami *et al*., 1995). We definitively identified host nucleotide sequences and chromosomal locations of HBV integration sites in chronic hepatitis tissues. This virus-tagging approach also identified genes possibly affected by HBV integrations at an early stage of chronic HBV infection. Some of these genes are aberrantly expressed depending on the hepatoma cell lines or HCC and surrounding nontumor tissues, suggesting a role in hepatocarcinogenesis. Our study also provided additional data on the chromosomal location of 20 HBV integrants. These data suggested preferential HBV integration on chromosome 3 in contrast to the previous contention that HBV integration is a random event (Tokino and Matsubara, 1991; Bréchot *et al*., 2000).

Results

Amplification and identification of viral–host junctions using HBV-Alu PCR and sequencing

HBV-*Alu* PCR was performed using liver DNA from six patients with chronic hepatitis type B. Electrophoresis and subsequent hybridization of the PCR product with an HBV probe showed smearing signals in all six patients, suggesting multiple polyclonal HBV integrations in chronically HBV infected liver (Figure 1). The PCR products were inserted into a plasmid vector, a total of 160 clones that hybridized to an HBV probe were selected and their nucleic acid sequences were determined. Of these, 45 clones consisted of only HBV sequences but the remaining 115 clones contained viral–host junctions as intended. There were duplications of clones and, as a result, we obtained a total of 42 different viral–host junctions.

Chromosomal location of isolated viral–host junctions

A database homology search of host sequences on Genbank and UCSC revealed the chromosomal locations of viral–host junctions in 20 of 42 clones (Table 1). We could not determine the chromosomal locations in the remaining 22 clones because they consisted of (a) extremely short cellular sequences or (b) only *Alu*-repeats. These inserts contained an *Alu* primer sequence, unidentified short sequences, probably of host origin, adjacent HBV sequences and an HBV primer sequence. They presumably reflect viral integrations into *Alu*-repeats or *Alu*-clusters.

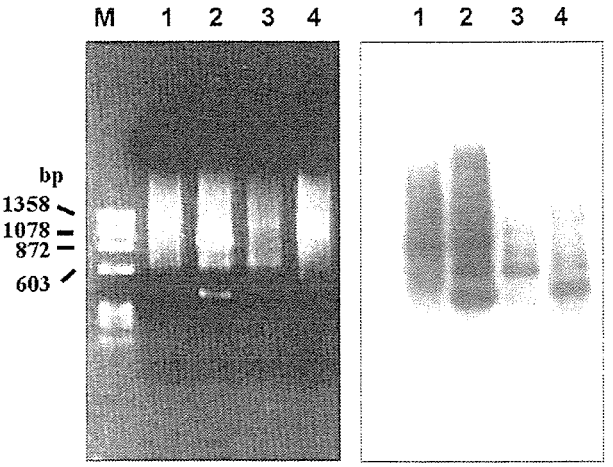

Figure 1 1.0% agarose gel electrophoresis of HBV-*Alu* PCR product and corresponding Southern hybridization with a total HBV probe of two representative cases. Smearing signals indicate polyclonal HBV integrations in chronic hepatitis tissues. Lanes 1 and 3: PCR with a HBx primer and a 5′-*Alu* primer. Lanes 2 and 4: PCR with a HBx primer and a 3′-*Alu* primer

HBV integration has been considered a random event and until now there has been no known chromosomal preference (Bréchot *et al*., 2000). Assuming that HBV integration is random and the chromosomal location of the viral–host junction depends on the size of each chromosome, we calculated the expected rate of HBV integration into each chromosome. We found three integrations in chromosome 3, a value two-fold higher than expected (1.34), but not statistically significant ($P=0.15$). Two other groups have recently reported consecutive analyses of HBV integration in HCCs and identified chromosomal localizations of 15 and 17 viral–host junctions (Gozuacik *et al*., 2001; Wang *et al*., 2004). We performed statistical analysis using 52 viral–host junctions observed either in our laboratory or reported by others (Table 2). This analysis of data from three independent laboratories demonstrated that integrations in chromosome 3 were always higher than anticipated and the overall frequency (8/52) was statistically significant ($P=0.022$). There were no significant preferences in the other chromosomes.

Genes possibly affected by HBV integration

HBV integration can affect human gene expression by interrupting an open reading frame or by activating genomic transcription through viral enhancer activity (Nevins and Vogt, 1996; Bréchot *et al*., 2000). As the gene-poor regions greater than 500 kb are termed gene deserts (Nobrega *et al*., 2003), we listed human genes located within 250 kb from or interrupted by HBV integration in the human genome databases.

Of the 20 integrants (Table 1), 10 were found within intronic sequences of human genes with confirmed protein production (Genbank and/or UCSC) and two were situated in intronic sequences of genes with hypothetical protein products (Genbank). Three integrants were located within 'gene deserts' and no genes were apparently

Table 1 Clones containing viral–host junctions

		No. of clone	*Length of cellular sequence (bp)*	*Ch. location*	*Interrupted gene*	*Near gene 1*	*Near gene 2*
TK	1	17	5	NI			
	2	4	681	11p12			
	3	4	114	16q13.3	AXIN1 int2		
	4	3	391	7q21.11			
	5	3	183	7q22.3	SRPK2 int17	LHFPL3; 8.6 kb DN	MLL5; 98 kb UP
	6	2	4	NI			
	7	1	817	15q13.3+2q36.3	KLF13 int1	LOC283711; 47 kb UP	
	8	1	76	6p22.3			
	9	1	78	NI			
	10	1	9	NI			
	11	1	12	NI			
	12	1	6	NI			
	13	1	11	NI			
HS	1	6	508	2q31.2	PDE11A int17	FLJ13946; 150 kb UP	
	2	3	8	NI			
	3	1	373	3p22.3		STAC; 114 kb UP	
	4	1	549	8q21.11	KCNB2 int5	TERF1; 168 kb UP	
	5	1	359	3q13.12	BBX int3		
	6	1	64	NI			
	7	1	6	NI			
	8	1	20	NI			
	9	1	1	NI			
	10	1	27	NI			
	11	1	376	4p12		OCIA; 6 kb UP	
TN	1	8	150	1p36.23		PARK7; 22 kb DN	MIG6; 4 kb DN
	2	2	127	3p14.1	AL713702 int		
	3	1	82	19q13.42		CDC42EP5; 1.5 kb UP	LAIR2; 28 kb UP
	4	1	473	5p15.2	CTNND2 int20		
	5	1	11	NI			
OR	1	1	189	17q24	PITPNC1 int14	FALZ; 148 kb UP	
	2	1	11	NI			
IW	1	17	6	NI			
	2	8	472	1p35.3	EYA3 int14		
	3	2	305	11q21	BC026191 int1	SRP46; 28 kb DN	SEST3; 75 kb DN
KM	1	4	8	NI			
	2	3	3	NI			
	3	3	6	NI			
	4	1	642	18q22.2		SOCS4; 61 kb DN	
	5	1	109	NI			
	6	1	167	5q34	ODZ2 int7		
	7	1	7	NI			
	8	1	49	NI			
Total		115					

No. = number; NI = not identified; int = intron and number; DN = from 3′ end of the gene; UP = from 5′ end of the gene

affected. In the other five clones, integrations occurred within 1.5 to 168 kb of the viral–host junctions. Therefore, we listed 27 cellular genes that might be affected by HBV integration from 17 of 20 clones (Table 1).

We observed HBV integrations close to multiple genes in eight of the clones, making it difficult to determine which genes are actually affected. We were also prudent in identifying HBV integrations situated upstream or downstream of the genes. Therefore, we chose genes whose open reading frames were interrupted by HBV integration. In six clones only a single gene was interrupted and there was apparently no other gene within 250 kb of the viral–host junction. These genes are candidates for genes affected by HBV and, as a result, they are possibly related to cell proliferation and survival. They include axis inhibitor 1 (AXIN1), homolog of *bobby sox* (BBX), AL713702 (hypothetical protein), catenin delta-2 (CTNND2), eyes absent 3 (EYA3), and homolog of odd Oz 2 (ODZ2). In order to characterize these six genes we examined their expression in hepatic cell lines and HCC tissues.

mRNA expression of cellular genes interrupted by HBV integration in cell lines, HCC and surrounding nontumorous tissues

With the exception of AXIN1 in human liver, the function and expression of the genes interrupted by

Table 2 Chromosomal location of HBV–host junctions identified in this study, by Gozuacik *et al.* and by Wang *et al.*

		Frequency					
Chr.	*Size (Mb)*	*This study*	*Gozuacik*	*Wang*	*Total*	*Expected freq. to XX*	*Expected freq. to XY*
1	246.1	2	1	1	4	4.24	4.31
2	243.6	1	3		4	4.19	4.27
3	199.3	3	2	3	8	3.43	3.49
4	191.7	1		1	2	3.30	3.36
5	181.0	2	1	2	5	3.12	3.17
6	170.9	1		1	2	2.94	2.99
7	158.5	2		1	3	2.73	2.78
8	146.3	1		2	3	2.52	2.56
9	136.4		3		3	2.35	2.39
10	135.0			1	1	2.32	2.37
11	134.5	2		1	3	2.32	2.36
12	132.1		1		1	2.27	2.31
13	113.0			1	1	1.95	1.98
14	105.3		1		1	1.81	1.84
15	100.3	1			1	1.73	1.76
16	90.0	1	1		2	1.55	1.58
17	81.9	1			1	1.41	1.43
18	76.1	1	1	1	3	1.31	1.33
19	63.8	1		1	2	1.10	1.12
20	63.7		1	1	2	1.10	1.12
21	47.0					0.81	0.82
22	49.4					0.85	0.87
X	153.7					2.65	1.35
Y	50.3					0.00	0.44
Total		20	15	17	52		
46XX	6039.2						
46XY	5935.8						

Chr = chromosome number; freq = frequency

HBV integration have not been fully investigated. Therefore, we semiquantitatively analysed mRNA expression of AXIN1, BBX, AL713702, CTNND2, EYA3, and ODZ2 in five human hepatoma-derived cell lines. These six genes were expressed, although not abundantly, in some or all of these cells (Figure 2). It is noteworthy that three of these genes, ODZ2, CTNND2, and AL713702, showed marked differences in mRNA expression levels dependent on the cell lines. CTNND2 was not expressed in HLE or HLF cells, whereas AL713702 was not expressed in HLE, HLF, and HepG2 cells, even after two rounds of PCR using nested primers (data not shown). ODZ2 was scarcely expressed in HepG2 cells, but was abundant in HLE and HLF cells.

In order to further characterize the expression patterns of these six genes, we analysed mRNA expression levels in seven human HCCs and corresponding nontumor tissues (Figure 3). EYA3 and BBX showed relatively homogenous expression levels in tumor and nontumor tissues. In contrast, AXIN1, ODZ2, and AL713702 demonstrated aberrant expression patterns. AXIN1 was not expressed in one tumor tissue and predominantly expressed in nontumor tissues in three of seven cases. In accordance with aberrant expression in hepatoma cell lines, ODZ2 was not expressed in one tumor tissue and showed lower expression in tumor tissues in four of seven cases. AL713702 was expressed in only one tumor tissue and three nontumor tissues. CTNND2 was expressed in all tissues and showed predominant expression in three tumor tissues, but showed lower expression in one nontumor tissue.

Discussion

We and others previously developed a PCR-based method to rapidly and specifically amplify HBV–host junctions (Minami *et al.*, 1995). This technique, coupled with the availability of the human genomic database, permitted the identification of as many HBV integration-related genes as have been reported in the past two decades (Bréchot *et al.*, 2000; Gozuacik *et al.*, 2001; Paterlini-Bréchot *et al.*, 2003). We here employed this HBV-*Alu* PCR because of its advantage in amplifying viral–host junctions that coexist with free viral sequences. Attempts to amplify viral–host junctions are often disrupted by free HBV-derived amplicons. This is particularly evident in chronic hepatitis tissues since free viral loads are usually greater in chronic hepatitis than in HCC (personal observation). In this study, 115 viral–host junctions and no more than 45 clones consisting only of HBV sequences were identified by screening 160 HBV-containing clones.

Our data, together with those of two other groups, have identified preferential HBV integrations into chromosome 3. We, as well as Gozuacik *et al.*, employed an HBV-*Alu* PCR method to identify viral–host junctions, but Wang *et al.* used a conventional cloning

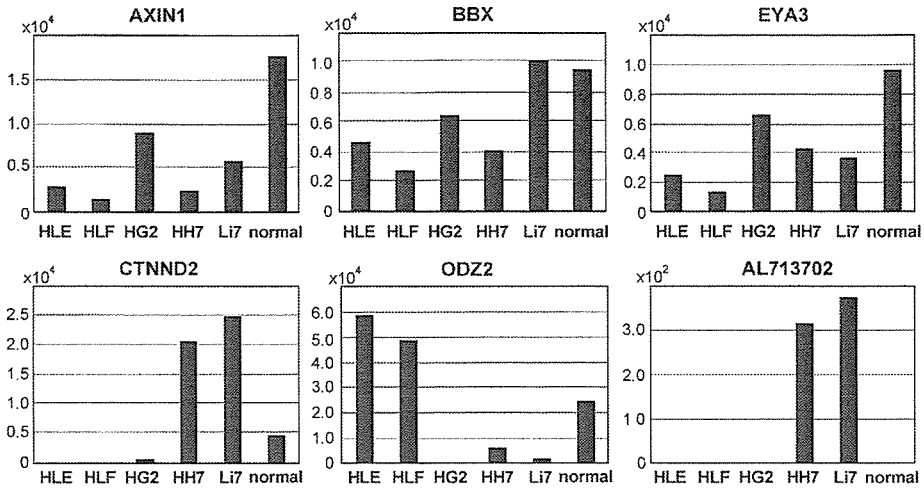

Figure 2 mRNA expression levels of six genes interrupted by HBV integration in hepatoma-derived cell lines. Copy number of mRNA is standardized and expressed as compared to 1.0×10^6 copies of beta-actin mRNA expression level. HG2, HepG2; HH7, Huh7; normal, data from normal liver

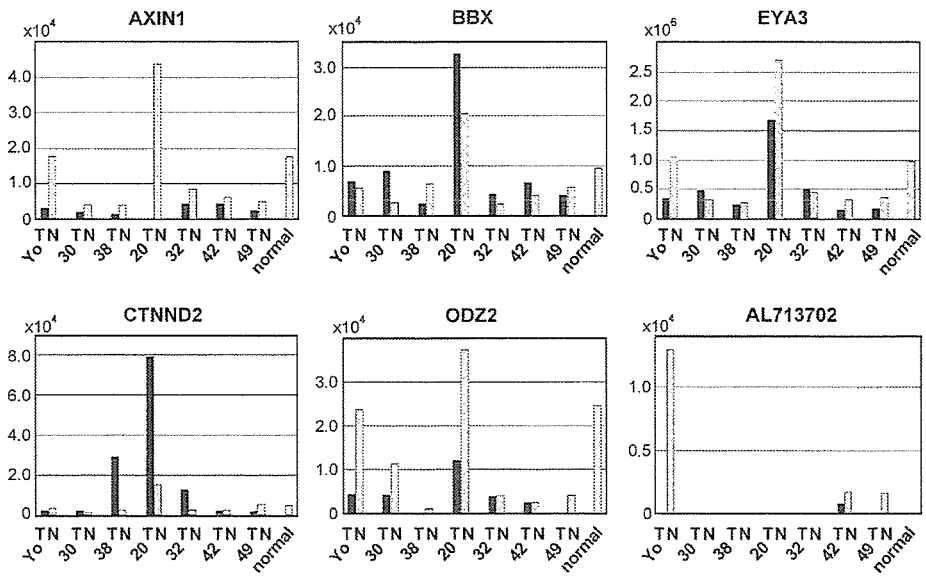

Figure 3 mRNA expression levels of six genes interrupted by HBV integration in HCC (T) and surrounding nontumor tissues (N). Copy number of mRNA is standardized and expressed as compared to 1.0×10^6 copies of beta-actin mRNA expression level in each tissue

procedure. Therefore, the identities of chromosomal preferences are independent of a cloning strategy bias. We also performed a database search for integrated HBV sequences in Genbank and identified 17 chromosomal locations in hepatoma-derived cell lines, HCCs, and chronic hepatitis tissues (data not shown). Integration in chromosome 3 was found in two of the 17 cases. Although these reports may contain intentional bias, the overall frequency of HBV integrations found in chromosome 3, including those in the database search, yielded a statistically significant ratio of 0.145 ($P = 0.016$). We listed the integration sites in chromosome 3 that have been reported in the literature (Table 3). There were no common genes affected by HBV, but 3p14 and 3q25, which correspond to common fragile sites, FRA3B and FRA3D, respectively, may be preferential targets. It remains to be resolved whether or not this chromosomal preference is related to a viral integration process or to genomic recombination events during hepatocarcinogenesis or both.

Viral integration events often show preferential or common sites. For example, WHV preferentially integrates into Myc oncogene families (Hsu *et al*., 1988; Fourel *et al*., 1990), whereas HPV integration in cervical carcinoma frequently occurs near the c-Myc locus (Ferber *et al*., 2003b) and recurrently in the hTERT gene (Ferber *et al*., 2003a). Retroviral tagging has identified several common genes affected by integration in mouse hematopoietic tumors (Shen *et al*., 2003). As for HBV, common integration into hTERT has recently

Table 3 HBV integrations at chromosome 3 found in the literature and this study

	Location	*Near gene*	*Note*
Gozuacik	3p26	Inositol 1,4,5-triphosphate receptor type 1 (IP3R type 1)	
Dejean	3p24.2	Retinoic acid receptor beta (RARB)	Genbank X04014
Wang	3p23	Not indicated	Rearranged
This study	3p22.33	SRC homology 3 and cystein-rich domain (STAC)	
Wang	3p14–3p21	Not indicated	Rearranged
This study	3p14.1	TAFA-1	
		Centromere	
Gozuacik	3q11.2	Alpha 2,3 sialyltransferase (ST3GAL VI)	
This study	3q13.12	Human homologue of bobby sox (BBX)	
Koshy	3q22	Mitochondrial ribosomal protein L3 (MRPL3)	Genbank K01659
Wang	3q25	Not indicated	Rearranged
Paterlini-Bréchot	3q25.3	IL-1R-associated kinase 2 (IRAK2)	

been reported (Gozuacik *et al*., 2001; Horikawa and Barrett, 2001; Ferber *et al*., 2003a; Paterlini-Bréchot *et al*., 2003). This observation, together with our finding of preferential HBV integration into chromosome 3, strongly suggests that HBV integration is not a random event and that there may be selective pressure. As HBV integration is not essential to its proliferative lifecycle, such selection would enhance viral survival in host cells. This mechanism could be related to the stimulation of host cell cycle, inhibition of cellular apoptosis, cell growth, and escape from the host immune system, leading to cellular proliferation in multistep tumorigenesis.

It has also been reported that HPV integration occurs in chromosomal fragile sites (Thorland *et al*., 2003). Common fragile sites are thought to be highly unstable and preferential sites for translocations, deletions, intrachromosomal gene amplification, and integration of plasmid DNA and tumor viruses, such as SV40 (Smith *et al*., 1998). They are considered to be related to carcinogenesis owing to such genomic alterations. In our study, HBV integrations were found within common fragile sites in four of 20 (20%) clones (data not shown). However, most of the common fragile sites are not precisely mapped and additional studies are needed to define the relationship between integrated sequences and common fragile sites by molecular mapping.

All of the viral sequences that we identified were situated in either intronic or intergenic sequences, but not in the exons. Viral insertional mutagenesis can affect cellular gene expression by regulating enhancer and promoter activities or by altering the exon–intron junction, possibly forming chimeric transcripts or disrupting transcription. Human intergenic sequences can regulate transcription in genes residing as far away as 1 Mb (Lettice *et al*., 2002). Retroviral integrations apparently affect expression of genes located over hundreds of kilobases from the integration site (Fourel *et al*., 1994). Although it is difficult to definitively identify genes affected by viral insertion, we have listed genes within 250 kb of HBV integrations. Nevertheless, in six cases, integrations occurred within the gene coding regions and no other gene existed within 250 kb. Therefore, these genes are excellent targets for insertional mutagenesis.

Among these six genes, AXIN1 is well characterized in the mouse during development and in human cancer. AXIN1 is a regulatory protein of a Wnt signaling pathway, which is crucial for the vertebral dorsal–ventral axis formation (Zeng *et al*., 1997) and is also related to colorectal and hepatic carcinogenesis in humans. Satoh *et al*. (2000) reported the simultaneous occurrence of AXIN1 mutation and the loss of heterozygosity in the responsible region in some human HCCs. On the basis of these data, AXIN1 is believed to function as a tumor suppressor.

EYA3, ODZ2, and BBX are human homolog of drosophila genes. EYA3 is a human homolog of the drosophila *eyes absent* gene, which is essential for eye development. In humans, EYA3 gene exhibits phosphatase activity and uses this enzymatic function to switch transcriptional activation of cofactors (Li *et al*., 2003). ODZ2 is a human homolog of the drosophila *tenascin major* (ten-m) gene, an example of a pair-rule gene, that is, every odd-numbered body segment is deleted in ten-m mutant drosophila embryos. In mammals it encodes a transmembrane protein, putatively acting as a transcriptional regulator (Bagutti *et al*., 2003). BBX is a human homolog of the drosophila *bobby sox* gene located in a *flightless* region and encoding a product with transcription factor activity (Maleszka *et al*., 1998). All three human homologs of drosophila genes are essential to the developments of drosophila, but their functions in humans are still unknown.

CTNND2 is a component of the adherens junction complex and its overexpression alters cell morphology and motility (Lu *et al*., 1999). It is expressed primarily in the central nervous system (Paffenholz and Franke, 1997), but it is also found in cancerous tissues in the prostate (Burger *et al*., 2002).

AL713702 is a spliced mRNA with a hypothetical protein product. However, very recently, Tom Tang *et al* showed that AL713702 encodes a chemokine-like protein and they named it TAFA-1. TAFA-1 is almost exclusively expressed in the brain, but its function remains unresolved (Tom Tang *et al*., 2004).

With the exception of AXIN1, the expression of these six genes in the liver, has not been studied extensively. Our study showed that all of these genes are expressed in hepatocytes at various levels and frequencies. Consistent with previous reports, AXIN1, a tumor suppressor protein, is markedly suppressed in three tumor tissues in comparison to corresponding nontumor tissues. Both

EYA3 and BBX exhibit homogenous expression in cell lines and HCC tissues. Inasmuch as these two proteins are presumably transcriptional regulators and are expressed in the liver, additional study is needed to elucidate their roles in normal liver, chronic hepatitis and HCC tissues. ODZ2 and CTNND2 exhibit aberrant expression depending on the cell lines; for example, ODZ2 expression was higher in nontumorous tissues than in tumors in four of the seven cases, suggesting a tumor suppressive role. It is important to note that ODZ2 also colocalized with a tumor suppressor protein, PML, in the nucleus (Bagutti *et al.*, 2003). In contrast, CTNND2 expression was much higher in tumors than in nontumorous tissues in three of the seven cases. This expression pattern is consistent with a report on CTNND2 expression in prostate cancers (Burger *et al.*, 2002) and suggests a tumor-promoting role of CTNND2. AL713702/TAFA-1 was expressed in two of five cell lines and in three of seven HCC cases, preferentially in nontumorous tissues.

In summary, six genes interrupted by HBV integration included one known tumor suppressor gene, three human homologs of drosophila genes that are essential for development, one putative oncogene, and one newly found chemokine. Our observations suggest that HBV-related insertional mutagenesis in chronic infected livers may be important in regulating cell growth and in the maintenance of viral proliferation in host cells. Interestingly, two genes, TAFA-1 and CTNND2, are exclusively expressed in the central nervous system in humans, but we identified their expression in HCCs, in surrounding cirrhotic liver and in hepatoma-derived cell lines. Their role in hepatocytes undergoing carcinogenic or inflammatory transition needs further investigation.

We listed 27 genes located within 250 kb of and possibly affected by HBV integrations, including several genes related to cell growth and survival. SRPK2 is a serine/threonine kinase expressed in mouse brain, lung, and testis. It phosphorylates the HBV core protein and is a possible molecular target for inhibiting HBV replication (Daub *et al.*, 2002). TERF2 is a key component of vertebrate telomeres and plays a protective role in cellular senescence (van Steensel *et al.*, 1998). OCIA is a novel protein found by screening cDNA libraries of ovarian cancer (Luo *et al.*, 2001). However, which gene is really affected is difficult to determine. In fact, we found that cells with HBV integration in the EYA3 (clone IW 2, Table 1) yielded 46 copies per 5000 cells whereas those with integration in the 11q21 (clone IW 3, Table 1) yielded 71 copies per 5000 cells (data not shown), suggesting that the extent of clonal expansion was comparable between these two integrated clones. It is also noteworthy that noncoding intergenic sequences sometimes regulate other gene expressions (Nobrega *et al.*, 2003). Sequences that appear unimportant at the moment may have critical roles in cell growth or viral proliferation. The accumulation of viral-tagging information will narrow the genomic region repeatedly affected and lead to the identity of affected genes and regions.

In conclusion, our results provide the first extensive description of HBV integration in chronic hepatitis tissues. These integrations are potentially susceptible to deregulate gene expression and lead to cell death or cell survival and uncontrolled growth. Our data have also suggested the utility of the viral-tagging approach to identify cellular genes related to cell growth and survival. Additional studies are needed to determine the function of the genes identified in this study and to elucidate the mechanisms regulating viral survival in host cells and leading to carcinogenesis.

Materials and methods

Liver tissues and DNA preparation

Liver tissues with chronic active hepatitis were obtained by needle biopsy from six patients diagnosed with chronic HBV infection. HCC and surrounding nontumor tissues were surgically obtained from seven patients, including four with HCV infection, two with HBV infection, and one with both HBV and HCV. These procedures were performed during the patients' clinical management. All of the patients provided written informed consent and the Ethics Committee of Kyoto Prefectural University of Medicine approved all aspects of the study. DNA was extracted using a G'NOME DNA isolation kit (BIO 101, Joshua Way, CA, USA) according to the manufacturer's instructions.

Cell lines and RNA preparation

Cell lines, HLE, HLF, HepG2, Huh7, and Li7, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA from normal liver was purchased from BD Biosciences (CA, USA).

Amplification of viral–host junction

Viral–host junctions were amplified by using primers specific to human *Alu* repeat and to HBV X region as previously reported (Murakami *et al.*, 2004). The amplified products were electrophoretically separated on 1.0% agarose gel, transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia) and the HBV-specific products were visualized by hybridization using a digoxigenin-labeled total HBV probe (DIG DNA Labeling and Detection kit, Roche Diagnostics).

Cloning and sequencing of PCR products

The PCR products were blunted by T4 DNA polymerase treatment and ligated into a plasmid pCAPs, using a PCR Cloning kit (Roche Diagnostics). Plasmid DNA was obtained by using a standard protocol. Insert DNA was separated on 1.0% agarose after enzyme digestion, blotted on a Nylon membrane and screened for HBV sequence by hybridization. HBV positive clones were sequenced by a 377 Prism DNA sequencer (Applied Biosystems Inc.).

Identification of chromosomal location of viral–host junction

The sequences were searched for homologies to HBV and pCAPs using the BLAST2 program on the NCBI homepage (http//www.ncbi.nih.gov/BLAST/). Sequences other than HBV and pCAPs were examined by the BLAST and MegaBLAST programs on the NCBI homepage and by the BLAT program on the UCSC genome browser

(http//www.genome.ucsc.edu/cgi-bin/hgBlat, July 2003 freeze) to search for homologies to the human genome.

RT–PCR and quantification of RNA expression

In total, 1 μg of RNA from each cell line or liver tissue was transcribed into cDNA using an AMV reverse transcriptase and a random nine-mer primer. One fortieth of the cDNA was subjected to a semiquantitative 50 cycle RT–PCR using a LightCycler and a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as per the Touch-Down PCR protocol. In order to deny possible amplification from pseudogenes, nontranscribed RNA was included and the amplified PCR product for each primer set was confirmed by direct sequencing. The specificity and quality of amplified PCR products were verified by electrophoresis on 1.5% agarose. The quantity of each cDNA was standardized using beta-actin; the detection limit was defined as 1.0×10^2 copies to 1.0×10^6 beta-actin expression level by using a LightCycler Control kit and a human beta-actin primer set (Roche Diagnostics). Each quantification was performed in duplicate. The primer sequences are listed (Table 4).

Statistical analysis

A binomial test was employed to analyse the chromosomal frequency of HBV integration. Values of $P<0.05$ were considered to be statistically significant.

Table 4 Primer sequences used for RT–PCR

Gene	*Direction*	*Sequence (5′ to 3′)*
Beta-actin	Sense	CAAGAGATGGCCACGGCTGCT
	Antisense	TCCTTCTGCATCCTGTCGGCA
AXIN1	Sense	CAGGCCACTATGGAGGAAAA
	Antisense	AGGGACAGGGTGTCTGCAT
BBX	Sense	CTCTCCGGGTTGCATGTACT
	Antisense	TGCCACTGAAGACACTTTCG
ODZ2	Sense	CTCTATGACCCCCTGACCAA
	Antisense	GACCTGCTTTCTCTCGGATG
CTNND2	Sense	AAAGGGATCCAGATGCTGTG
	Antisense	AATCACTTCGTGCAGTGTGC
EYA3	Sense	CCAGCATCTCAAACCAGGAT
	Antisense	TCTTGGGAAGAAGTGGCATC
AL713702 (TAFA-1)	Sense	GGAGGGACGTGTGAAGTGAT
	Antisense	CTTGGGTGAATTCTCGTGGT

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A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase

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***Background/Aims*: Long-term follow-up study was performed to identify the candidates for antiviral therapy for hepatitis C virus (HCV) infection among carriers with persistently normal aminotransferase (ALT $\leq$30 U/L) levels (PNAL).**

***Methods*: One hundred and twenty-nine HCV carriers with PNAL who underwent liver biopsy and had platelet count over 150,000/μl were entered and 69 were followed for over 5 years. Thirty-five patients underwent serial liver biopsies. Serum ferritin and thioredoxin levels were also determined.**

***Results*: Seventeen patients had normal liver histology, 10 had moderate chronic hepatitis and the remainder 102 had mild hepatitis. Serum ferritin and thioredoxin levels were normal. The mean follow-up period for the 69 patients was 8.5 years. Of these 69 patients, 10 had persistently normal ALT levels (group A), 39 had transient elevation of ALT (group B), and 20 changed to symptomatic chronic hepatitis (group C). The rate of progression of fibrosis for groups A, B, and C were 0.05, 0.04, and 0.08, respectively. Hepatocellular carcinoma was not diagnosed in any of the patients.**

***Conclusions*: Around 90% of HCV carriers with PNAL have normal to mild liver histology. This long-term follow-up study demonstrated that 30% of such carriers became candidates for antiviral therapy within 5 years.**

***Keywords*: Hepatitis C virus; Chronic hepatitis C; Asymptomatic HCV carrier; Normal serum ALT; Interferon**

1. Introduction

An estimated 170 million individuals are infected with hepatitis C virus (HCV) worldwide and chronic hepatitis C has recently become the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) in many countries including Japan. Most HCC develop in patients with advanced staged chronic hepatitis or cirrhosis, and rarely from mild chronic hepatitis type C.

It is thought that type C liver cirrhosis and HCC develop over 20–35 years following HCV infection [1], however, around 25% of patients with chronic HCV infection have normal serum aminotransferase (ALT) levels [2,3]. We reported previously that asymptomatic HCV carriers were predominant among females and that most of them had histologically minimal to mild chronic hepatitis [4]. In that paper, we defined asymptomatic HCV carriers as persistently HCV RNA positive patients with normal serum ALT levels ($\leq$30 U/L) over 1 year. However, it has been reported that HCV carriers with normal serum ALT level had more advanced liver histology compared to HCV carriers with elevated serum ALT [5]. This discrepancy might be attributed to differences in the definition of the normal range of serum ALT used by various centers, however, it is very important to clarify whether HCV carriers with persistently normal ALT level (PNAL) are candidates for antiviral therapy.

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The current normal limit of serum ALT is 40 U/L, however, a recent report from an Italian group demonstrated that the healthy ranges for serum ALT were 30 U/L for men and 19 U/L for women, respectively [6], which are lower than the current values that have been used over the past 15 years. This criterion of normal serum ALT might be reasonable because a few cirrhotic patients have from 30 to 40 U/L of ALT [7].

In Japan, the number of HCC patients with HCV infection has increased since 1975. Antiviral treatment for chronic hepatitis C resulted in the inhibition of hepatic inflammation and progression of hepatic fibrosis and as a consequence the inhibition of the development of HCC [8–13]. Thus, inhibition of HCC is a very important issue in the treatment of patients with chronic hepatitis C. It remains controversial whether asymptomatic HCV carriers are candidates for antiviral therapy because of the low efficacy of treatment and flare-ups post treatment. However, taking into consideration the recent progress in antiviral therapy for chronic hepatitis C patients, the National Institute of Health Consensus Development Conference reported that patients with hepatitis C with normal serum ALT levels are candidates for interferon and ribavirin therapy [14]. Recently, a multicenter, randomized, controlled study for the treatment of patients with chronic hepatitis and persistently normal ALT levels with pegylated interferon alpha and ribavirin for 48 weeks led to eradication in 40% of patients infected with genotype 1b patients [15], which is similar to the results for symptomatic chronic hepatitis C patients [16,17]. However, most HCV carriers with PNAL have minimal to mild liver histology and their prognosis might be very good. Thus, there is some doubt, whether they are candidates for antiviral treatment to inhibit the progression of liver disease and hepatocarcinogenesis.

Recently, it has been reported that oxidative stress is an important factor in the development of HCV-related HCC [18–22] and the HCV core protein may generate oxidative stress via mitochondrial injury [23,24]. It is also demonstrated that iron overload generates oxidative stress, resulting in hepatic injury, and DNA damage and consequently this becomes an important factor for hepatocarcinogenesis [22,25,26].

We report here the biochemical and histological results of 8.5 years of follow-up of HCV carriers with PNAL. The data were analyzed according to the definitions of normal range ($\leq$30 U/L) of serum ALT and platelet count (PLT) over 150,000 μl/ml. We also analyzed the status of oxidative stress using serum ferritin and thioredoxin levels. These results demonstrate the importance of the normal range of serum ALT, oxidative stress and follow-up study to decide the indication for antiviral therapy of HCV carriers with PNAL.

2. Patients and methods

2.1. Eligibility and definition

This study was conducted from January 1990 to August 2004.

HCV carriers with persistently normal ALT levels (PNAL) were defined as those patients who were HCV RNA positive by reverse transcriptase polymerase chain reaction (RT-PCR), had normal serum ALT levels ($\leq$30 U/L) over a 12-months period and on least three different occasions and platelet count of over 15×10^4 μl/ml. Patients positive for hepatitis B surface antigen (HBsAg), previous interferon (IFN) treatment, a history of heavy alcohol abuse, anti-nuclear antibody (ANA) and anti-smooth muscle antibody (ASMA) positivity, patients with overt Diabetes mellitus and obesity (body mass index; over 30 kg/m^2) were excluded from this study.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and approved by the Ethics Committee of Kyoto Prefectural University of Medicine. Informed consent was obtained from every patient.

2.2. Quantification and determination of HCV RNA and genotyping

Frozen-stored sera from 129 individuals were tested. Serum HCV RNA levels was determined using the AMPLICOR GT HCV MONITOR (Roche Diagnostic Systems, Tokyo, Japan). The detection range of this assay was between 0.5 and 850 KIU/ml, and each sample was measured again after dilution with distilled water. HCV genotypes 1 and 2 were determined by a serologic genotyping assay [27]. Genotypes 1 and 2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds et al. [28].

2.3. Study design

Of the 129 patients who underwent liver biopsy, 69 patients enrolled in this study and followed over 5 years (8 males, 61 females). These patients received blood tests every 4 months for an initial 2 years and then received blood tests every 6 months when they remained still normal ALT. αfetoprotein (AFP) was measured every years in all patients, and all patients underwent ultrasonography every year to detect HCC.

All patients submitted to a liver biopsy using a Menghini needle guided by ultrasonography prior to entry. Formalin-fixed liver specimens were stained with hematoxylin and eosin for morphological evaluation, with Masson's trichrome stain for assessment of fibrosis, and with Perls' Prussian blue stain (from February 1998) for assessment of iron loading. Histological follow-up studies were carried out for 35 patients 3.4–13.4 years (mean: 6.8 years) after the first biopsy.

The histological findings of HCV carriers with PNAL were interpreted and scored according to the classification proposed by Desmet et al. [29] and Ishak et al. [30]. Steatosis is defined having fat droplets in over 10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0 to 4+, based on the scoring system of MacSween et al. [31].

Fasting blood samples were collected in the morning. Serum ALT, blood glucose level, serum ferritin, platelet count (PLT), serum HCV RNA level and HCV genotype were examined in the laboratory of our university hospital, using the standard analytical method; the ULA ALT value was 30 U/L. Serum thioredoxin (TRX) levels were measured with a sensitive sandwich ELISA kit (Fujirebio, Inc., Tokyo, Japan) as described previously [26,32] and of the 129 patients 47 were available for this assay. Blood chemistry was done every 4–6 months during the follow-up period.

2.4. Statistical analysis

Data values are expressed as medians with interquartile ranges. We compared continuous variables using the Mann–Whitney *U*-test. The Kruskal–Wallis test was used for multiple group comparisons, and Spearman correction coefficients were used to examine the relationship between groups. Frequency analysis was performed with the χ^2 test, and Fisher's exact test. *P* values of less than 0.05 were considered significant.

3. Results

3.1. Demographic and clinical features

The demographic and clinical features of the 129 HCV carriers with PNAL are shown in Table 1. Twenty-four were male and 105 were female. No significant differences were noted in age, serum ALT, PLT, and follow-up period between males and females. Serum ferritin levels were 76.1±53.4 ng/ml in male and 60.0±43.3 ng/ml in female. Serum HCV RNA levels were significantly ($P=0.0012$) higher in G1 compared with G2 (648.7±622.5 KIU/ml vs 356.2±628.8 KIU/ml (Table 1).

Characteristics of the 69 patients followed over 5 years are also shown in Table 1. Their mean follow-up period was 8.5±2.4 years.

Of the 105 female patients, 44 had serum ALT levels ≦19 U/L and 61 had serum ALT levels of 20–30 U/L at entry. There were no significant differences in their ages, platelet count, serum ferritin levels, serum HCV RNA levels, or BMI (Table 2).

Serum thioredoxin (TRX) levels in these patients were within the normal range, and significantly lower than those of patients with chronic hepatitis and cirrhosis (Table 3).

Table 1
Characteristics of 129 HCV carriers with persistently normal ALT who underwent liver biopsy

	$N=129$	Followed over 5 years ($N=69$)
Follow-up period (years)	5.7±3.6	8.5±2.4
Age (years)	48 (21–77)	45 (29–71)
Male ($N=24$)	49.8±16.4	42.3±14.9
Female ($N=105$)	47.2±12.5	46.63±11.6
Sex (M/F)	24/105	8/61
ALT (U/L)	8–30	9–30
Male ($N=24$)	22.5±5.7	21.1±5.4
Female ($N=105$)	21.6±4.8	22.3±5.1
PLT ($\times 10^4$/ml)	15–31	15–31
Male ($N=24$)	20.3±4.4	20.9±5.3
Female ($N=105$)	21.8±4.4	21.2±4.0
Ferritin (ng/ml)	5–225	5–225
Male	76.2±53.5	84.6±59.2
Female	60.0±43.3	66.6±52.5
HCV RNA (KIU/ml)	6–3350	22–2100
G1 ($N=58$)	648.9±622.57*	595.1±561.1** ($N=32$)
G2 ($N=45$)	356.2±628.8	211.0±219.2 ($N=27$)
Mixed and unclassified	6–1994	
BMI (kg/m^2)	16–27	16–27
Male	22.2±1.7	21.9±1.9
Female	21.3±2.2	21.0±2.4

Values were expressed as mean±SD. P values were calculated by Mann–Whitney U-analysis with correction for tie. *$P=0.0012$ (G1 vs G2); **$P=0.0006$ (G1 vs G2).

Table 2
Baseline of female patients between HCV carriers having ≦19 U/L of ALT and HCV carriers showing 20–30 U/L of ALT

	ALT≦19 (U/L)	20<ALT≦30 (U/L)	P value
Number of patient	44	61	
Age (y.o)	44.9±12.5	48.8±12.2	
ALT (U/L)	16.0±2.4	24.3±2.9	<0.0001
PLT ($\times 10^4$/μl)	22.0±4.4	21.6±4.3	
HCV RNA (KIU/ml)	400.2±555.1	500.7±541.1	0.3896
BMI (kg/m^2)	21.2±2.3	21.4±2.2	

Values were expressed as mean±SD. P values were calculated by Mann–Whitney U-analysis with correction for tie.

3.2. Liver histology

The results of liver histology for the first biopsy are described in Table 4. Normal liver histology was noted in 17 (14%) subjects, 102 (79%) showed minimal to mild chronic hepatitis, 10 (8%) had moderate chronic hepatitis.

Steatosis was seen in nine patients (7%) and iron loading was noted in 6/50 (12%).

3.3. Follow-up study of laboratory data

Of the 69 patients followed over 5 years (mean±SD: 8.5±2.4 years), 10 (14%) had continuously normal ALT (group A), 39 (57%) showed transient elevation of ALT (group B), and 20 (29%) changed to chronic hepatitis with continuously abnormal serum ALT (group C) (Table 5). Of the 61 female patients, eight were group A, 34 were group B, and 19 were group C. There were no significant differences in age, ferritin levels, serum HCV RNA levels, or BMI among the three groups. However, serum ALT levels were significantly lower in group A compared with group B and C (Table 6). The number of patients having ALT levels≦19 IU/L in these three groups were seven (7/8:87.5%) in group A, 12 (12/34:35.3%) in group B, and three (3/19:15.8%) in group C.

Table 3
Serum thioredoxin (TRX) levels in 47 HCV carriers with PNAL at liver biopsy

	Serum thioredoxin (ng/ml)
HCV carriers with PANL ($n=47$)	27.7 [9.1–38.5]
Chronic hepatitis ($n=124$)	34.5 [8.6–135.6][a]+
Liver cirrhosis ($n=24$)	42.5 [21.4–97.2][a]++
Control ($n=15$)	24.9 [1.3–50.7][a]

*$P=0.0012$ when compared with G2. The overall significance of differences between four groups according to non-parametric Kruskal–Wallis analysis of variance was $P<0.001$. Therefore, the significance of differences between groups was determined by Scheffe's method: +$P<0.01$; ++$P<0.001$, compared to HCV carriers with PNAL.

[a] These data were reported in J Hepatol 2000; 33: 616–622.

Table 4
Liver histology of 129 carriers at the first biopsy

Grade	Stage of liver fibrosis				Total number of patients
	F0	F1	F2	F3	
A0	17 (11)	3 (1)	0	0	20 (12)
A1	24 (21)	75 (62)	2 (2)	0	101 (85)
A2	0	6 (5)	2 (2)	0	8 (7)
A3	0	0	0	0	0
Total	41 (32)	84 (68)	4 (4)	0	129 (104)

Numbers of female patients are given in parentheses.

The stage of liver fibrosis in the 22 female patients with ALT levels≦19 IU/L at entry were F0 ($N=10$) or F1 ($N=12$). The frequency of stage F0 liver histology was slightly higher in group A and B patients compared with group C. However, there were no significant differences among the three groups.

Seven patients from group C had ALT levels over 100 U/L during the follow-up period and received antiviral therapy (five received interferon monotherapy and two received interferon plus ribavirin therapy), and five had a sustained virological response.

3.4. Follow-up study of liver histology

Thirty-five patients submitted to repeat biopsies and five of them a third biopsy. Of the 35 patients, 5 were in group A, 16 in B, and 14 in C. The intervals between the first biopsy and the last biopsy in these three groups were 7.3±2.1 years (group A), 6.8±2.0 years (group B), and 6.1±2.3 years (group C). The changes in stage of live fibrosis are shown in Fig. 1 (group A), 2 (group B), and 3 (group C). Progression of fibrosis stage was noted in 2 of 5 in group A, 5 of 16 in group B, and 6 of 14 in group C, as shown in Figs. 1–3. The median rates of fibrosis progression per year for these three groups were 0.05, 0.04, and 0.08 fibrosis unit, respectively. There were no significant differences in the rate of fibrosis progression per year between group A and B, B and C, and A and C (A vs B; $P=0.6643$, B vs C; $P=0.0699$, A vs C; $P=0.3512$).

Of the 32 female patients who received serial biopsies, 10 had ALT levels≦19 U/L at entry, in four of whom had F0 stage progress to F1. One F0 and five F1 patients showed no changes in their stages during the follow-up periods.

Table 5
Changes of serum ALT in 69 patients followed over 5 years

	No. of patients
Persistently normal (group A)	10 (14%)
Transient elevation (group B)	39 (57%)
Continuous elevation (group C)	20 (29%)

Group A, continuously normal serum ALT during the follow-up period. Group B, serum ALT transiently over 31 U/L during the follow-up period. Group C, serum ALT became continuously abnormal during the follow-up period.

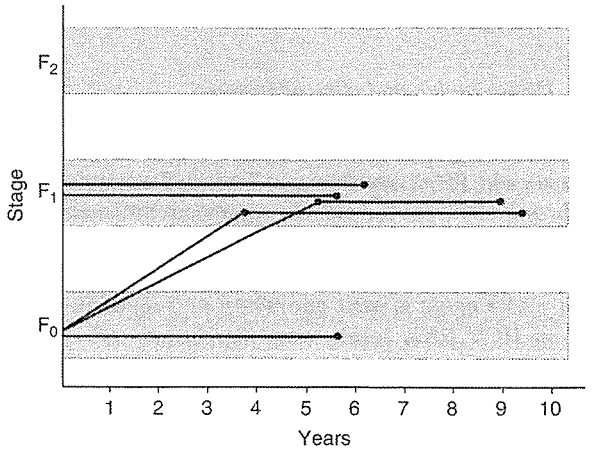

Fig. 1. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels remained normal during the follow-up period. Five patients with persistently normal serum aminotransferase levels submitted to repeat biopsies and the stage of liver fibrosis progressed from F0 to F1 in two patients after 3.4 and 5 years.

3.5. Follow-up study of AFP and ultrasonography

Three patients in group C showed transient elevation of AFP over 20 ng/ml. No patients in groups A or B had elevations of serum AFP during their follow-up periods. HCC was not detected in any patients by ultrasonography and/or computed tomography. AFP titers in those three patients did not increase further.

4. Discussion

The present study demonstrated several characteristics of HCV carriers with persistently normal ALT levels (PNAL).

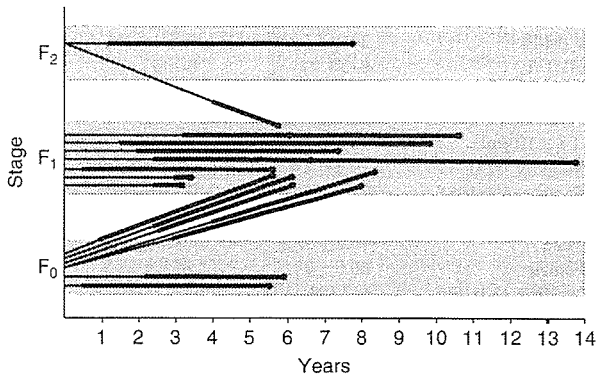

Fig. 2. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels were transiently elevated during the follow-up period. Sixteen patients with transient elevation of serum aminotransferase levels submitted to repeat biopsies and the stage of liver fibrosis progressed from F0 to F1 after 5.3–8.1 years in five patients. One patient showed the regression of the stage of liver fibrosis from F2 to F1 after 5.5 years. The left side edge of the large bar indicates the initial recording of abnormal serum aminotransferase during follow-up period.