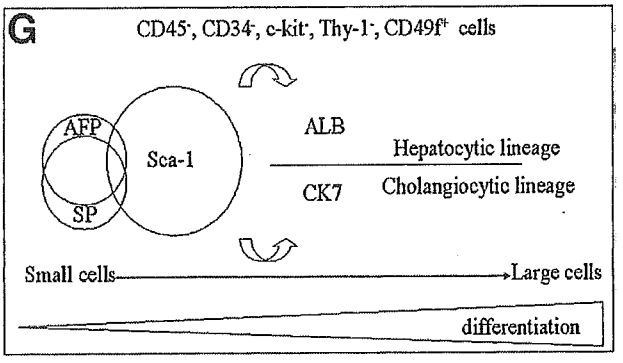

Figure 11. (Cont'd.)

fully established highly replicative monolayer colony formation. Furthermore, we succeeded in subculturing these colonies and maintaining colony formation. A series of these culture systems maintained cell–cell contact consistently. The main population (MP) of growing cells comprised those committed to a hepatocytic or cholangiocytic lineage. However, using our culture system, we additionally identified SCCs (10%–30% of total cells) containing AFP-positive immature cells at day 21 after plating. Thereafter, immature cells were stably expanded. Because cultured cells were heterogeneous containing cells at various differentiation stages, further analysis was performed.

Flow cytometric analysis of the heterogeneous expanded cells showed a subpopulation of $CD45^-$, $CD34^-$, $Thy\text{-}1^-$, $c\text{-}kit^-$, $CD49f^+$ cells. Novel candidate Sca-1–positive cells and SP cells suitable for purifying hepatic stem/progenitor cells were additionally identified (Table 1).

Initially, we compared expanded cells with previously reported oval cells.[26–28] Our cells did not express CD34, Thy-1, or c-kit, which are known rat and human oval cell markers. With regard to mouse oval cells, Petersen et al[33] recently reported that oval cells were enriched in the $Sca\text{-}1^+$, $CD45^+$, and $CD34^+$ fraction from damaged adult mouse livers. In our study, however, Sca-1–positive cells were immunonegative for CD45 and CD34. Thus, it is difficult to ascertain whether the cells Petersen et al and we describe are same cells. Sca-1 has been used to purify mouse hematopoietic stem cells in bone marrow[34] and progenitor cells of mammary glands[35] and heart.[36] Our immunocytochemistry data showed that Sca-1–positive cells were mainly found near the small cells expressing AFP and that approximately 1%–3% of Sca-1–positive cells were also immunopositive for AFP. Our Sca-1–positive cells have the ability to form large colonies and to produce bidirectionally lineage-committed cells; however, when SP, Sca-1-positive cells were compared with MP, Sca-1–positive cells, the former had a greater ability to form large colonies and contained more AFP-positive cells than the latter.

It is interesting to note that 5%–20% of our long-term cultures consisted of SP cells. This is the first report of an SP fraction in long-term–cultured hepatic cells. SP cells are characteristic of other stem cell types, such as those of hematopoiesis[37] and muscle.[38] Under our culture conditions, SP cells were small, contained a large number of AFP-positive cells, and had ability to form large colonies that contained immature and bidirectional lineage-committed cells. Using the 2-acetylaminofluorene/partial hepatectomy model, Shimano et al[39] reported that SP cells were induced and that oval cells express ATP-binding cassette transporter (ABCG2/BCRP1), which has been

Table 1. Phenotypic Properties of Our Expanded Cells

Cellular classification	Marker	Our expanded cells	Reference
Hepatic stem/progenitor cell markers	AFP	+	6, 19, 31, 32, 33
	CD34	–	26
	Thy-1	–	27
	c-kit	–	28
	Sca-1	+ (5%–20%)	33
	SP	+ (15%)	39
Hepatocytic lineage cell markers	ALB	+	7, 8, 9
	α1 antitrypsin	+	7, 8, 9
	G-6-P	+	7, 8, 9
Mature hepatocytic cell markers	TAT	–	20
	TO	–	7, 8, 9
	CPSI	+ (<1%)	5
Cholangiocytic lineage cell markers	CK7	+	6
	CK19	+	7, 8, 9
	BGP	+	7, 8, 9
Hematopoietic lineage cell marker	CD45	–	7, 8, 9
α_6-Integrin subunit	CD49f	+	7, 8, 9, 20

G-6-P, glucose-6 phosphatase.

identified in SP cells. This study suggested that oval cells have an SP phenotype. Our results support these observations and indicate that SP cells include hepatic stem/progenitor cells. We conclude that both Sca-1–positive cells and SP cells contain hepatic stem/progenitor cells with extensive growth potential. In view of the finding that the overall size of SP cells was smaller than that of Sca-1–positive cells and that SP cells had a greater ability to form large colonies and contained more AFP-positive cells than Sca-1–positive cells, we conclude that the SP characteristic is a more immature marker than Sca-1 expression (Figure 11*G*).

Generally, small cells are regarded as immature cells,[24] and at day 21 after plating, we identified SCCs in the colony centers. Lázaro et al[32] also observed small cells that they called blastlike cells in their human fetal liver cultures. Both cell types have a high nuclear–cytoplasmic ratio. However, the locations of cells expressing AFP and cell-surface markers are different. SCCs included AFP-positive cells and were expanded extensively with our culture system, but blastlike cells were AFP-negative with AFP-positive cells on the periphery and could not be expanded extensively for long periods. With regard to cell-surface markers, our cells were $CD34^-$, $Thy\text{-}1^-$, but the blastlike cells were $CD34^+$, $Thy\text{-}1^+$. It is possible that these differences reflect different species and culture conditions.

Our culture system facilitates easy enrichment and extensive expansion of hepatic stem/progenitor cells reproducibly without the need for feeder layers,[40,41] which can become the source of artifacts.[41] This system enables the maintenance of cultures over the long-term and the identification of new cell characteristics. By altering culture conditions, we could not only expand and cryopreserve immature cells, but also induce morphologically mature hepatocytes and mature hepatocytic markers. Expanded cells also have bidirectional differentiation potential and can partially improve liver function in mice with severe liver damage. Furthermore, because our culture system uses serum-free conditions, it can be used to directly evaluate the role of growth factors and supplements. This culture system is thus very useful for investigating the mechanisms of hepatic stem/progenitor cells and discovering a novel strategy for cell therapies.

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Original Paper

Effect of Interferon on Incidence of Hepatocellular Carcinoma in Patients with Chronic Hepatitis C

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KEY WORDS:
HCC; IFN; Chronic hepatitis C; HCV

ABBREVIATIONS:
Interferon (IFN); Hepatocellular Carcinoma (HCC); Alanine Aminotransferase (ALT); Complete Response (CR); No Response (NR); Biochemical Response (BR); Hepatitis B Virus (HBV); Hepatitis C Virus (HCV); Enzyme-Linked Immunosorbent Assay (ELISA); branched DNA (bDNA)

ABSTRACT

Background/Aims: The aim of this study was to evaluate whether IFN prevents the development of HCC in patients with chronic hepatitis C.

Methodology: 103 patients with chronic hepatitis C received IFN and 30 control patients were enrolled in this study.

Results: In 33 patients (32.0%) who received IFN, alanine aminotransferase (ALT) decreased to normal range and HCV RNA became negative (complete response: CR). In 7 patients (6.7%), ALT decreased to less than 50 IU/L or stayed within the normal range, but HCV RNA remained positive (biochemical response: BR). In 63 patients (61.1%) and 30 control patients, ALT did not change and HCV RNA remained positive (no response: NR). HCC developed in 5 (4.9%) of the 103 patients who received IFN and 7 (23.3%) of the control patients ($p<0.01$). In 5 patients who developed HCC, the response to IFN was NR and no HCC developed in patients with CR or BR. In addition, 5-year cumulative rate of development of HCC in 63 IFN NR patients and in control was 7.9% and 23.3% ($p<0.05$).

Conclusions: IFN decreased the development of HCC in not only patients with CR or BR but also patients with NR.

INTRODUCTION

In epidemiological surveys of liver cirrhosis and hepatocellular carcinoma (HCC), hepatitis B virus (HBV) and hepatitis C virus (HCV) are major causes of liver cirrhosis, and are thought to be carcinogenic in the liver (1-4). In Japan, the proportion of HBV-positive patients with HCC is decreasing, whereas there is an increasing association of HCC in HCV-positive cirrhosis (3-5). Therefore, in Japan, HCV infection seems to be a more important factor associated with HCC. Although the relation between HCV and HCC is intimate, the carcinogenic mechanism of HCV in the liver is largely unknown (6,7). HCV is a positive-strand RNA virus and cannot integrate its genome into the host DNA. Therefore HCV may be indirectly oncogenic, exactly through inflammation, necrosis and regeneration of hepatocytes. Interferon (IFN) has been demonstrated to have antiviral, immunomodulatory and antitumoral effect (8). In several randomized controlled surveys, the efficacy of IFN to patients with chronic hepatitis C has been reported (9-16). These surveys showed that IFN treatment decreases serum alanine aminotransferase (ALT) concentrations, showing resolution of hepatic inflammation and improving liver histology. INF treatment eradicated HCV RNA in about one-third of patients, and the patients who responded to IFN treatment with CR (ALT decreased to normal range and HCV RNA became negative) were unlikely to develop liver cirrhosis or HCC (17-19). However, it is not known whether IFN treatment reduces the risk of progression to HCC in patients who respond to IFN therapy with NR (ALT did not change and HCV RNA remained positive). We determined the rate of development of HCC, evaluated whether IFN prevents the development of HCC and assessed the risk factors for the development of HCC in 103 consecutive patients with chronic hepatitis C who received IFN treatment.

METHODOLOGY

Patients

Between January 1992 and July 1996, 152 patients with chronic hepatitis C were referred to our hospital. All patients were eligible for study if they had no evidence of type B hepatitis, autoimmune hepatitis or HCC judged by serological markers (HBsAg, antinuclear-antibody, AFP and des-gamma-carboxy prothrombin), ultrasonography, computed tomography or a combination of these methods. None of the patients had been treated with IFN previously. The serum level of HCV RNA was measured within 6 months before the initiation of IFN or symptomatic treatment.

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The diagnosis of chronic hepatitis C was based on the results of liver function tests, positive reaction for HCV antibody (anti-HCV), positive reaction for HCV RNA and the liver biopsy. Anti-HCV and HCV RNA were detected in all patients. Histological diagnosis was made according to the established criteria (20). Nineteen patients were excluded because they did not fulfill one or more of the eligible criteria. Thus, the remaining 133 patients were randomly assigned to treatment; 103 and 30 were IFN and symptomatic treatment (control patients), respectively. These 103 patients treated with IFN consisted of 50 males and 53 females. The mean age of these patients at the beginning of treatment was 52.2 years. The number of patients with a history of blood transfusion was 47 (46%). Concerning alcohol consumption, 61 (59%) were non-drinkers, 31 (30%) were social drinkers (habitual alcohol intake of less than 60g/day), and 11 (24%) were heavy drinkers (habitual alcohol intake of equal to or more than 60g/day). **Table 1** shows the details of clinical, virological and histological characteristics of 103 IFN treatment patients and 30 control patients with chronic hepatitis C. There were no significant differences in the clinical features between 103 IFN treated and 30 control patients. They had a follow-up period of more than five years (7.8±1.8 years). That period was defined as the interval from the beginning of the first treatment to the date of the patient's last visit to our hospital or Aug 2001. Patients treated with IFN were randomly selected and they were included in this study if they had received at least $200x10^6$ IU IFN in total. All procedures were approved by the ethics committee of The Nippon Dental University.

TABLE 1 Clinical, Virological and Histological Characteristics of 103 IFN Treated Patients and 30 Control Patients with Chronic Hepatitis C

Clinical characteristics	IFN treatment patients (N=103)	Control patients (N=30)
Age (M±SD)	52.2±14.0 (16-71)	53.5±11.7 (23-70)
Sex (M/F)	50/53	13/17
Transfusion (+/-/unclear)	47/50/6	14/11/5
Alcohol (++/+/-)	11/31/61	9/15/6
ALT (IU/L, M±SD)	123±104 (820-54)	142±136 (482-45)
PLT ($x10^4$, M±SD)	19.0±5.10 (28.9-7.1)	16.5±4.3 (25.8-8.9)
Virological characteristics		
HCV serotype (I/II/unclear)	61/20/22	9/4/17
Serum levels of RNA (high/low/unclear)	50/31/22	7/6/17
Histological characteristics		
F1/F2/F3/unclear	30/38/11/24	6/10/8/6
A1/A2/A3/unclear	30/36/13/24	5/11/9/5
Total dose of IFN (MU, M ±SD) (IFN-α 40; α2a 15; α2b 25; β 23)	426±154 (880-200)	

Viral Markers

Anti-HCV was detected by the second generation HCV enzyme-linked immunosorbent assay (ELISA) (Ortho Diagnostics, Tokyo, Japan). The presence or absence of HCV RNA was determined by RT nested PCR using primers based on the sequences of the 5'-UTR of the HCV genome, as described previously (21). The serum level of HCV RNA was measured by use of a quantitative branched DNA (bDNA) signal amplification assay (version 1.0; Chiron, Emeryville, U.S.A). Assay results are expressed as the number of copies of HCV genome equivalents/mL. The data are shown in Meq (mega equivalents)/mL. The lower limit of the assay was 0.5Meq/mL. HCV serotyping was analyzed by the immunoserological typing methods with a commercial kit (Kokusai Diagnostic Corporation, Kobe, Japan) and serological group 1 indicated genotype 1a or 1b and group 2 indicated genotype 2a or 2b.

Interferon (IFN)

Patients were treated with natural IFN-α (Sumitomo Pharmaceutical, Tokyo, Japan), recombinant IFN-α2a (Nippon Roche K.K. Tokyo, Japan), recombinant IFN-α 2b (Schering-Plough, Osaka, Japan), or natural IFN-β (Mochida Pharmaceutical, Tokyo, Japan). IFN-α, recombinant IFN-α2a or recombinant IFN-α2b was administered intramuscularly in a dose of $3\text{-}10x10^6$ IU daily for 2-4 weeks and then three times a week for a total of 14-28 weeks (combined schedule). Natural IFN-β was administered by drip infusion at a dose of $3\text{-}6x10^6$ IU daily for 6-8 weeks (daily schedule). The total dose ranged from 200 to $880x10^6$ IU. The patients were examined at weekly intervals during the first month, and then once a month thereafter. Complete responders (CR) were defined as patients in whom HCV RNA was not detected in serum for at least 6 months after the end of treatment and ALT decreased to normal range. Biochemical responders (BR) were defined as ALT decreased to less than 50 IU/L or stayed in the normal range but HCV RNA remained positive. Non-responders (NR) were defined as HCV RNA remained positive and ALT did not decreased to less than 50 IU/L or did not stayed in the normal range.

Liver Biopsy

Liver needle biopsy was performed in all patients before the beginning of IFN treatment. Liver histology was evaluated according to Inuyama's classification which is the histological classification for diagnosis of chronic hepatitis and predicting the progression of the disease (20). The grades of fibrosis are classified from F0 to F3 with varying degrees of fibrosis. Cirrhosis of the liver is expressed as F4. Based on the degree of infiltration of lymphocytes and necrosis of hepatocytes, the activities are classified from A1 to A3. In addition, A0 is defined as having no necro-inflammatory activity.

Staging and grading criteria are as follows:

F0, no fibrosis;	A0, no necro-inflammatory reaction;
Fl, fibrous portal expansion;	A1, mild necro-inflammatory reaction;

F2, bridging fibrosis; (portal-portal or portal-central linkage)	A2, moderate necro-inflammatory reaction;
F3, bridging fibrosis with lobular distortion; (disorganization)	A3, severe necro-inflammatory reaction;
F4, cirrhosis.	

Statistics

Student's *t*-test was used to determine differences between the groups for continuous variables. The chi-square test including Fisher's exact test was used to evaluate the homogeneity of groups. Data were expressed as the mean±standard error (SE). The observation period began at enrollment in the study and ended at the time of Aug 2000. Development ratio of HCC was calculated from a period between the enrollment in the study and appearance of HCC. Development ratio of HCC was calculated by the Kaplan-Meier method, and 103 patients who received IFN and 30 control patients were compared using the Wilcoxon test. Cox proportional-hazard regression analysis was performed to estimate the relative ratio for each potential risk factor for the development of HCC. *P*-values of less than 0.05 were considered significant.

RESULTS

Therapeutic Effect of IFN

In 33 patients (32.0%) given IFN, ALT decreased to normal range and HCV RNA became negative (CR). In 7 patients (6.7%) given IFN, ALT decreased to less than 50 IU/L or stayed within the normal range, but HCV RNA remained positive (BR). In 63 patients (61.1%) given IFN, ALT did not change and HCV RNA remained positive (NR) **(Table 2)**. In 30 control patients, ALT did not change and HCV RNA remained positive.

TABLE 2 Numbers of Patients Progressed to Hepatocellular Carcinoma (HCC)

	IFN treatment patients			Control patients		
	HCC (-)	HCC (+)	Total	HCC (-)	HCC (+)	Total
	98	5 (4.9%)	103	23	7 (23.3%)	30
CR	33	0	33			
BR	7	0	7			
NR	58	5 (7.9%)	63			

TABLE 3 Clinical, Virological and Histological Comparisons between Patients with and without Progression to HCC in 63 IFN Non-responders

	Progressing to HCC	Not progressing to HCC
No.	5	58
Age (M±SD)	60.0±3.3	52.1±15.7 ($p<0.01$)
Sex (M/F)	3/2	27/31
Transfusion (+/-/unclear)	2/2/1	23/32/3
Alcohol (++/+/-)	0/2/3	5/16/37
ALT (IU/L, M±SD)	98±29	119±114
PLT (x10^4, M±SD)	10.4±2.5	16.9±5.5 ($p<0.01$)
HCV serotype (I/II/unclear)	3/0/2	36/10/12
Serum levels of RNA (high/low/unclear)	3/0/2	35/13/10
F1/F2/F3/unclear	0/0/5/0	13/22/9/14 ($p<0.001$)
A1/A2/A3/unclear	0/2/3/0	13/24/7/14
Total dose of IFN (MU, M±SD)	339±47	475±194 ($p<0.05$)

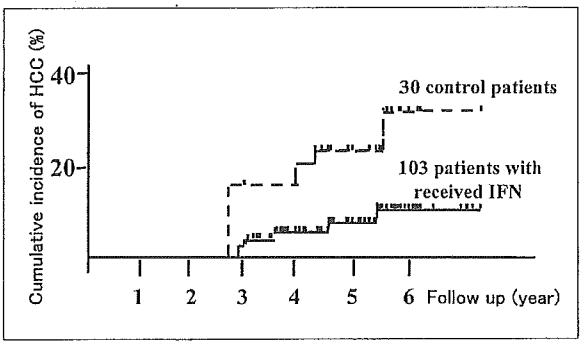

FIGURE 1 The three-year cumulative rates of HCC development in 103 patients with INF treatment and 30 control patients without INF treatment were 1.0 and 13.3%, respectively. The five-year rates in those patients were 4.9 and 23.3%, respectively. The development rate was significantly lower in patients received IFN than in control patients ($p<0.01$).

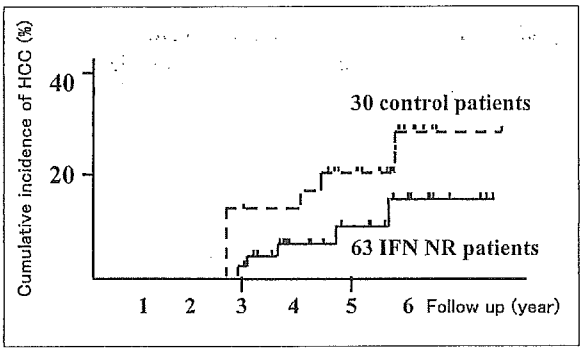

FIGURE 2 The three-year cumulative rates of HCC development in 63 IFN NR patients and 30 control patients were 1.6 and 13.3%, respectively. The five-year rates in those patients were 7.9 and 23.3%, respectively. The development rate was significantly lower in 63 IFN NR patients than in control patients ($p<0.05$).

Crude Development Ratio of HCC

The mean follow-up period was 7.8±1.8 (mean ±SD) years for 103 patients who received IFN and 30 control patients. During the follow-up period, HCC developed in 5 of the 103 patients who received IFN (4.9%, 3 males and 2 females) as judged by ultrasonography, computed tomography, angiography and liver biopsy if indicated **(Table 3)**. The median age of these five patients who progressed to HCC was 60.0 years old (55-64). Of 5 patients, 4 received natural IFN-α and one received natural IFN-β and the median cumulative dosage was 339x10^6 IU (256-360) IFN. The median interval of development of HCC from the beginning of the IFN treatment was about 4.9 years (3.2-7.4) and the mean tumor size at diagnosis of HCC was 23.2mm in diameter (12-48). In these five patients, response of IFN was all NR and the histological findings demonstrated severe fibrosis (F3) **(Table**

3). However, 40 patients with response of CR or BR did not progress to HCC. HCC developed in 7 (23.3%) of 30 control patients during the follow-up period **(Table 2)**. The three-year cumulative rates of HCC development in 103 patients with INF treatment and 30 control patients without INF treatment were 1.0 and 13.3%, respectively. The five-year rates in those patients were 4.9 and 23.3%, respectively **(Figure 1)**. The development rate was significantly lower in patients who received IFN than in control patients ($p<0.01$).

Comparison of Development Ratio of HCC between 63 IFN NR Patients and 30 Control Patients

In 63 IFN NR patients, HCC developed in 5 (7.9%). On the other hand, in 30 control patients, HCC developed in 7 (23.3%) **(Table 2)**. The three-year cumulative rates of HCC development in 63 IFN NR patients and 30 control patients were 1.6 and 13.3%, respectively. The five-year rates in those patients were 7.9 and 23.3%, respectively **(Figure 2)**. The development rate was significantly lower in 63 IFN NR patients than in control patients ($p<0.05$). These results suggest that IFN treatment might play an important role in decreasing the development to HCC even in IFN NR patients.

DISCUSSION

Chronic hepatitis C often progresses to liver cirrhosis and HCC after a long period of infection. Accordingly, chronic HCV infection, together with chronic HBV infection, has been thought to play an important role in development of HCC. However, the mechanism of malignant transformation in chronic HCV infection has not yet been clarified. Since HCV is an RNA virus without reverse transcriptase activity, integration into host DNA is rare (22) and there are no reports that a gene related to HCV causes transactivation. These findings are different from the theory that HBV can be oncogenic and that integrated HBV DNA sequences have been found not only in HBV carriers but also in anti-HBc-positive patients without HBsAg (23). In multistepwise carcinogenesis, the accumulation of genetic alterations was proposed as one of the mechanisms of malignant transformation (24). In patients with chronic active hepatitis, hepatocytes are continuously damaged and regenerated. Thus, the rate of cell turnover might influence carcinogenesis during chronic HCV infection. So, if chronic HCV infection ceases, carcinogenesis might be prevented or reduced. IFN inhibits viral replication and reduces hepatic necrosis, inflammation and fibrosis. Furthermore, it also has an antiproliferative effect (8). Therefore, if IFN treatment is effective with subsequent outcome of BR or CR, IFN might prevent carcinogenesis or reduce the incidence of HCC. The aims of our study were to elucidate the rate of development to HCC in chronic hepatitis C and to evaluate whether IFN prevents the development to HCC. In our trial, 40 patients with chronic hepatitis C, in whom treatment with IFN was effective (CR and BR), have not progressed to HCC during the follow-up period (7.8±1.8 years) after IFN treatment. Reichard *et al.* reported that nearly all had a good response in sustained responders to IFN, not only biochemical and virological but also histological normalization or near normalization. They concluded that in sustained responders to IFN, the long-term prognosis was excellent (18). Similar observations have been reported and those data indicated a marked decrease in HCC development among patients treated with IFN (8-11).

In the present study, the incidence of HCC in the patients, in whom treatment was ineffective (NR) but the histological staging of hepatic fibrosis was mild (F1 or F2), was significantly lower than that in the controls. However, HCC developed in patients with NR with severe hepatic fibrosis (F3). Ikeda *et al.* reported that in HCV-related hepatitis, the incidence of HCC steadily increased as long as hepatic necrosis, inflammation and viral replication continued (13). These findings may indicate that when inflammation, necrosis and degeneration of the hepatocytes might come to an end or be reduced with IFN treatment and when the incidence of genetic abnormalities accumulating during cell turnover might be lower, the incidence of progression to HCC might decrease.

Another aim of our study was designed to assess risk factors for the development to HCC in 63 NR patients who received IFN treatment. In 63 NR, clinical, virological and histological comparisons between patients with and without progression to HCC were investigated. There were statistically significant differences of mean age ($p<0.01$), platelet counts ($p<0.01$) and total administration dose of IFN ($p<0.05$) in two groups. Furthermore, in all patients who progressed to HCC, the histological findings demonstrated severe fibrosis (F3) ($p<0.001$) as compared with the patients without HCC. Many risk factors of progression to HCC have been reported in patients with chronic hepatitis C (4,11,13,17). Ikeda *et al.* reported that the best predicting factor of hepatic carcinogenesis in chronic HCV infection was histological staging of hepatic fibrosis. In addition, GGT, a history of blood transfusion, albumin, and alcohol consumption were also regarded as significant risk factors (13). However, our study cannot address these points except for histological staging of hepatic fibrosis among IFN NR patients. Moreover, the possibility of unrecognized selection bias of patients enrolled at a single referral center, the small number of patients analyzed and the inability to investigate other factors of hepatic carcinogenesis such as aflatoxin exposure, obesity and hepatic iron limit the generalization of our preliminary findings. Therefore, we need further analysis based on a larger population.

In summary, IFN treatment prevents progression of HCC not only in patients with CR or BR but also in patients with IFN NR. However, it would be impossible for IFN to decrease the development of HCC in patients with severe fibrosis, in whom the response to IFN is NR. In the near future, additional population-

based prospective longitudinal trials should be studied to investigate the effects of longer courses of chronic hepatitis C patients with IFN treatment on the development to HCC.

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Overexpressed Id-1 Is Associated with a High Risk of Hepatocellular Carcinoma Development in Patients with Cirrhosis without Transcriptional Repression of p16

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BACKGROUND. Inhibitor of differentiation/DNA binding protein 1 (Id-1) plays a pivotal role in the regulation of cell proliferation and carcinogenesis via inhibiting basic helix-loop-helix (HLH) transcription factors. Recently, Id-1 was found to repress p16 in tumorous tissue specimens including hepatocellular carcinoma (HCC), but its relevance in precancerous liver tissues is unknown.

METHODS. Id-1 expression in the liver tissue specimens of 112 patients with cirrhosis without HCC was studied by immunohistochemical analysis. Correlations were investigated between Id-1 expression and clinicopathologic features, the status of p16, and the risk of HCC occurrence.

RESULTS. A high expression of Id-1 was observed in 42 patients (38%). The level of Id-1 expression was not associated with clinical standard parameters or the status of p16 in cirrhotic tissue specimens. The cumulative incidence of HCC development was significantly higher in a group of patients with high Id-1 expression ($P = 0.0008$). Multivariate analysis revealed that increased Id-1 expression is an independent significant factor for the risk of HCC development in patients with cirrhosis (relative risk = 2.75, $P = 0.003$).

CONCLUSIONS. The results of the current study suggested that increased expression of Id-1 may play an important role in the early step of hepatocarcinogenesis, and might serve as a useful marker for determining patients with cirrhosis with a high risk of HCC occurrence. ***Cancer* 2005;104:1037–44.**

KEYWORDS: hepatocellular carcinoma, cirrhosis, Id-1, p16.

Hepatocellular carcinoma (HCC) is one of the most frequently found malignancies worldwide, which mainly arises from cirrhosis caused by chronic hepatitis B virus/hepatitis C virus infection (HBV/HCV), alcohol abuse, and hemochromatosis. Many clinicians have suggested that cell proliferation status may be closely involved in the mechanistic nature of hepatocarcinogenesis. Follow-up studies using a bromodeoxyuridine assay, silver staining of nucleolar organizer region proteins (AgNOR), and immunostaining for Ki-67 and proliferating cell nuclear antigen (PCNA) have shown that a high rate of hepatocellular proliferation was closely associated with the increased risk of HCC development in patients with cirrhosis.[1–8] This suggests that assessment of cell proliferation in liver biopsy samples might help to determine patients with cirrhosis who require careful surveillance of HCC.

Because DNA is prone to exposure of mutagens during cell rep-

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lication, the relation between increased hepatocellular proliferation and carcinogenesis is plausible. Nevertheless, the regulatory mechanism of cell proliferation in hepatocarcinogenesis is unknown. Several clinical studies have reported no significant relation between serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase and PCNA labeling index (LI) in liver tissue specimens from patients with cirrhosis,[4,7,8] suggesting that inflammatory stimuli in the diseased liver cannot be fully attributable to the increased proliferation of hepatocytes.

Recent studies have shown that inhibitor of differentiation/DNA binding (Id) proteins, which act as dominant negative inhibitors of basic helix-loop-helix (HLH) transcription factors,[9] play a pivotal role in the regulation of cellular proliferation.[10] Among the members of the Id family, Id-1 is overexpressed in many types of tumor tissue specimens and may play a crucial role in carcinogenesis.[11,12] Because Id-1 is overexpressed in tissue specimens from dysplastic lesions in the pancreas,[13] it is highly likely that Id-1 is implicated in the early step of carcinogenesis. More recently, Id-1 has been found to oppose an Ets-mediated activation of tumor suppressor p16,[14] which is inactivated in approximately one-half the patients with HCC.[15–17] The biologic role of Id-1 in hepatocarcinogenesis is intriguing, but no studies have examined the correlation between the status of Id-1 and HCC development in patients with cirrhosis.

To investigate the role of Id-1 in an early step of hepatocarcinogenesis, we examined the status of Id-1 expression in patients with cirrhosis by immunohistochemical staining and compared expression with clinical variables. Moreover, to determine whether overexpressed Id-1 represses p16 in cirrhosis, the correlation of Id-1 and p16 expression was investigated by immunohistochemical staining and methylation-specific polymerase chain reaction (PCR). Finally, to investigate the clinical significance of Id-1 in cirrhosis with regard to hepatocarcinogenesis, the relation between the status of Id-1 and the risk of HCC occurrence was analyzed by a retrospective follow-up study.

MATERIALS AND METHODS

Patients

The records of 112 patients who were histologically diagnosed with cirrhosis were retrieved from our files of liver biopsy performed over the past 20 years at Niigata University Graduate School of Medical and Dental Science (Niigata City, Japan). The cohort comprised 36 patients with HBV-associated cirrhosis, 54 patients with HCV-associated cirrhosis, and 22 patients with alcohol-induced cirrhosis. There were 91 men and 21 women with a mean age of 63 ± 9 years (range, 43–87 years). At the time of biopsy, none of the patients showed evidence of dysplastic nodules or HCC in the livers as assessed by ultrasonography (US), computed tomography (CT) scan, or magnetic resonance imaging (MRI) scan. Patients who had any episodes of HCC treatment before the time of biopsy were excluded from analysis, as were patients who received interferon therapy after the biopsy was performed.

As a rule, patients with cirrhosis had been regularly followed up at 1–3-month intervals with monitoring of serum α-fetoprotein (AFP) levels, and every 3–6 months with US, CT, or MRI scans. When the serum levels of AFP or characteristics of the US/CT scan/MRI scan imaging pattern changed during the follow-up, the occurrence of HCC was evaluated by US-guided biopsy or CT scan during arterioportography. All the patients in the study were followed for ≥ 6 years or until the development of HCC, and the follow-up period was set as the time from the biopsy to HCC occurrence or to the last observation. Informed consent was obtained from all patients to participate in the study, which was approved by the institutional guidelines of Niigata University Graduate School of Medicine and Dental Science.

Immunohistochemical Analysis

All tissue samples were immediately fixed in neutral-buffered formalin for 3 days after the biopsy procedure was performed, and embedded in paraffin. For immunohistochemical staining of Id-1, deparaffinized thin-sliced sections were microwaved in 10 mM citrate buffer (pH 6.0) for antigen retrieval. They were then treated with 1% hydrogen peroxidase for 30 minutes to block endogenous peroxidase, followed by a 10% normal goat serum block. Tissue sections were incubated overnight at 4 °C with anti–Id-1 rabbit polyclonal antibody (1:50) (C-20; Santa Cruz Biotechnology, Santa Cruz, CA), which is known to successfully detect Id-1 in paraffin-embedded human tissue sections and not cross-react with Id-2, Id-3, or Id-4.[13] Color development was carried out using the vector Elite ABC kit (Vector Laboratories, Burlingame, CA) with 3,3′-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, MO) and the reacted sections were counterstained with hematoxylin. Preabsorption of the primary antibody with specific blocking peptide of Id-1 (Santa Cruz) was verified to abolish the immunoreactivity. Substitution of the primary antibody with normal immunoglobulin of the same species was used for negative controls. Tissue samples from four normal livers served as normal controls. Smooth muscle cells of vessels known to express Id-1[18] were regarded as an

internal positive control. Cytoplasmic staining of the cells was considered positive because Id-1 lacks a nuclear localization signal.[18–20] To confirm the reproduction of the staining intensity, each tissue sample was stained in duplicate, and immunostaining was assessed by counting 200 cells in the region of interest by 2 observers without knowledge of the clinical characteristics of the samples. Scoring of immunostaining for Id-1 expression was assessed by the percentage of positive cells and the staining intensity, which was based on experience from previous studies.[19,20] The percentage of positive cells was divided into 4 groups: 2 points, 11–50 % of the positive cells; 3 points, 51–80% of the positive cells; and 4 points, > 80% of the positive cells. The intensity of the immunostaining signals was categorized and adjusted to the internal positive control as follows: 1 point, weak immunoreactivity; 2 points, moderate immunoreactivity; and 3 points, strong immunoreactivity. The sum of the points for the percentage score and the intensity score were calculated, and specimens were categorized into 4 groups according to the total score: negative, < 10% of the immunoreactive cells regardless of the intensity; weak expressor, 2–3 points; moderate expressor, 4–5 points; and strong expressor, 6–7 points. We found that this scoring method minimized subjective grading of the immunostaining.

For evaluating the cell proliferation status of the tissue samples, the LI of PCNA was assessed by using antihuman PCNA monoclonal antibody (1:50) (PC10; Dako A/S, Carpinteria, CA). The percentage of immunoreactive cells for PCNA was calculated by counting ≥ 200 cells at high magnification (× 400). For immunostaining of p16, deparaffinized tissue sections were reacted with a rabbit polyclonal anti-p16 antibody (1:400) directed against the entire region of the human p16 protein (PharMingen, San Diego, CA) overnight at 4 °C. The following procedure of p16 immunostaining was the same as that for Id-1. According to previous reports,[15–17] nuclear staining is considered to be a positive reaction. The degree of staining was graded as follows: weak expression, negative staining or < 50% of the immunoreactive cells; strong expression, positive staining in > 51% of the cells.

Methylation-Specific Polymerase Chain Reaction

Methylation-specific PCR (MSP) was performed to investigate the methylation status of the *p16* gene in cirrhotic livers. Genomic DNA was extracted from deparaffinized tissue sections from the biopsy liver sample.[16] DNA was modified with sodium bisulfite, and aliquots (50 ng) were amplified by PCR using primers specific for the unmethylated (5′-TTATTAGAGGGTGGGGTGGATTGT-3′, 5′-CAACCCCAAACCACAACCATAA-3′) or methylated (5′-TTATTAGAGGGTGGGGCGGATCGC-3′, 5′-GACCCCGAACCGCGACCGTAA-3′) *p16* gene as previously described.[21] The PCR products (10 μL) were resolved by electrophoresis on agarose gels containing ethidium bromide and visualized with ultraviolet illumination.

Statistical Analysis

Because the number of the patients in each group was disproportionate, the patients with negative and weak Id-1 expressors were assembled into a group of low Id-1 expressors, and moderate and strong Id-1 expressors were put into a group of high Id-1 expressors for statistical analysis. The association between the status of Id-1 and clinicopathologic parameters was examined using chi-square analysis. When appropriate, a Mann–Whitney test was used to test for statistical differences between the groups. Prospective curves of the HCC-free period in individuals were calculated by the Kaplan–Meier method[22] and the statistical significance between groups was determined by a log-rank test. The Cox proportional hazards model on Stat View 5.0 software (SAS Institute, Cary, NC) was used to evaluate the possible correlation of Id-1 status with the risk of HCC development in each of the patient groups. All reported *P* values are 2 sided and the data were considered statistically significant when $P < 0.05$.

RESULTS

Immunohistochemical Analysis of Id-1

It had previously been verified that the immunostaining pattern of the Id-1 antibody used in the current study corresponds to the gene expression in human tissue specimens.[23] In normal control liver tissue specimens, immunostaining for Id-1 was undetectable or weakly expressed in a few hepatocytes (Fig. 1A). However, many patients with cirrhosis showed positive expression of Id-1 with diverse staining intensity. In our study sample, Id-1 staining was negative in 5 patients (4%), weak in 65 patients (58%), moderate in 32 patients (29%), and strong in 10 patients (9%) (Fig. 1B–F). When the patients were divided into 2 groups of low Id-1 expressor (negative or weak Id-1 expressors; $n = 70$) and high Id-1 expressor (moderate or strong Id-1 expressors; $n = 42$), there was no significant difference in clinical variables such as age, gender, blood platelet count, and serum ALT and AFP levels (Table 1). The mean PCNA LI tended to be higher in the group of high Id-1 expressors than in low Id-1 expressors (4.0 ± 2.2% vs. 3.1 ± 1.4%, $P = 0.071$; Mann–Whitney test) (Table 1).

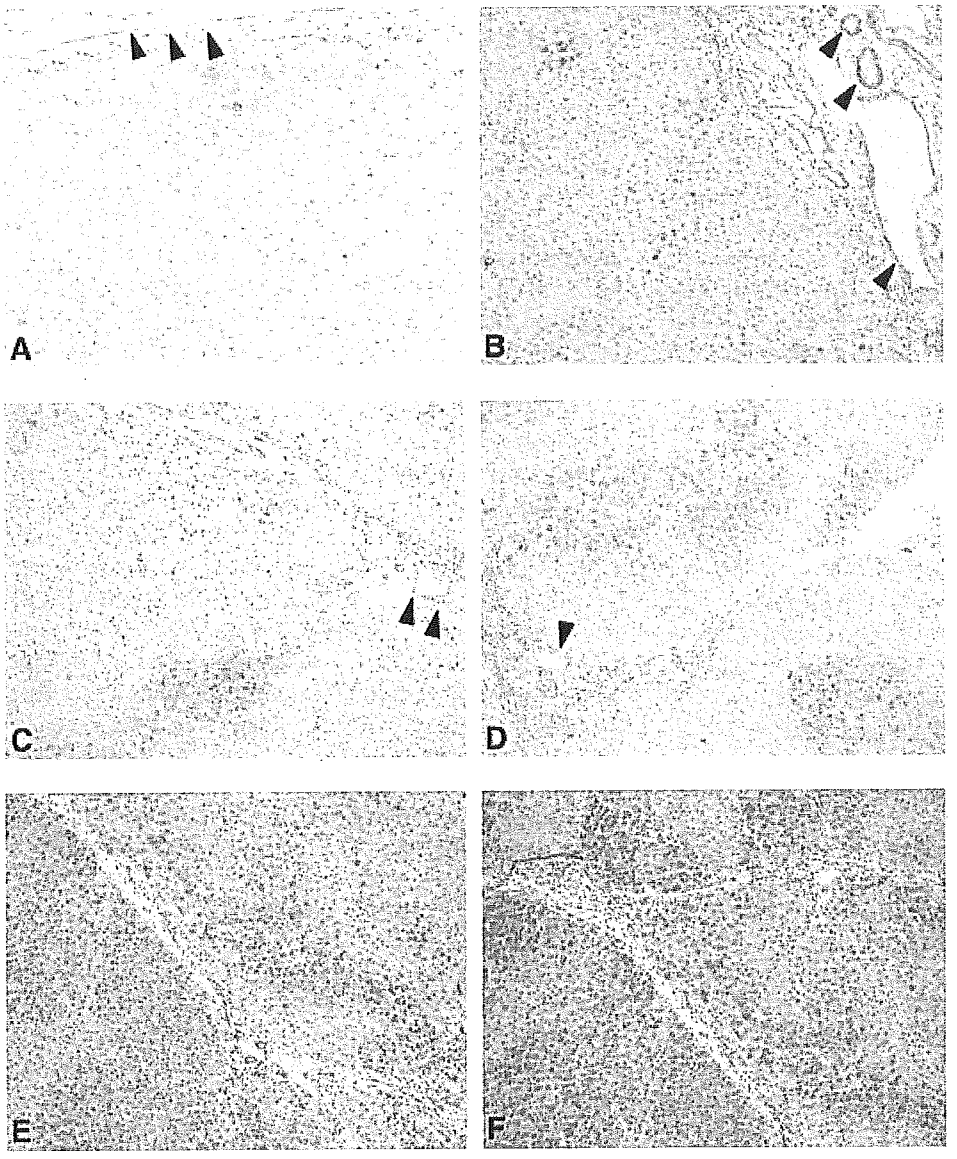

FIGURE 1. Representative immunohistochemical staining of inhibitor of differentiation/DNA binding protein 1 (Id-1) in liver tissue specimens. (A) Normal liver tissue specimen with weak Id-1 expression. (B) Negative Id-1 expressor, showing positive staining in < 10% of the hepatocytes. (C) Weak Id-1 expressor. (D) Moderate Id-1 expressor. (E, F) Strong Id-1 expressors with strong Id-1 expression in > 80% of the cells. Arrowheads indicate smooth muscle cells in the vasculature as internal positive controls. (Original magnification × 40.)

Relation between the Status of p16 and Id-1

The relation between p16 expression and Id-1 status was evaluated by immunohistochemical analysis using serial sections of the same tissue sample. Three (3%) of the 112 patients showed weak immunohistochemical expression for p16, but all remaining patients showed strong p16 expression irrespective of the status of Id-1 (Fig. 2A). The Mann–Whitney *U* test showed no relation between the level of p16 and Id-1 expression in patients with cirrhosis ($P = 0.650$). MSP detected hypermethylation of the *p16* gene in 4 of the 112 patients with cirrhosis (Fig. 2B). Of these, immunostaining for p16 was weak in 3 patients and strong in 1 patient, and the methylation status was strongly correlated with the immunostaining intensity for p16 protein (chi-square analysis: $P < 0.0001$).

Correlation of Id-1 Status with Hepatocellular Carcinoma Development in Patients with Cirrhosis

The median follow-up period of patients with cirrhosis was 80 months (range, 18–121 months). In 112 patients with cirrhosis, 21 patients (19%) developed HCC within 5 years. Of these, 7 were weak, 10 were moderate, and 4 were strong expressors (Table 1). The 5-year HCC-free rates as assessed by the Kaplan–Meier method were 87% and 63% in low and high Id-1 expressors, respectively, and the cumulative incidence of HCC development was significantly higher in high

TABLE 1
Comparison of Clinicopathologic Findings of Cirrhosis between High and Low Id-1 Expression

	Id-1 low			Id-1 high			
Variables	Negative	Weak	Total	Moderate	Strong	Total	*P* value
No. of patients	5	65	70	32	10	42	—
Mean age (yrs)	63 ± 10	63 ± 9	63 ± 9	63 ± 9	61 ± 9	62 ± 9	0.767
Males	4	52	56	27	8	35	0.804
HBsAg level	1	23	24	10	2	12	0.53
Anti-HCV level	3	31	34	12	8	20	0.922
ALT level (IU/L)	71 ± 21	84 ± 47	83 ± 46	76 ± 34	84 ± 31	78 ± 33	0.531
Platelet count ($\times 10^4$/mL)	10.1 ± 4.5	9.3 ± 3.3	9.4 ± 3.4	8.4 ± 3.8	8.4 ± 5.0	8.4 ± 4.1	0.194
AFP level (ng/mL)	35 ± 20	26 ± 27	27 ± 27	18 ± 11	23 ± 25	19 ± 16	0.103
PCNA LI (%)	2.0 ± 0.7	3.2 ± 1.4	3.1 ± 1.4	3.8 ± 2.2	4.8 ± 2.3	4.0 ± 2.2	0.071
HCC occurrence during 5 yrs	0	7	7	10	4	14	—

Id-1: inhibitor of differentiation/DNA binding protein 1; yrs: years; HBsAG: hepatitis B surface antigen; HCV: hepatitis C virus; ALT: alanine aminotransferase; AFP: α-fetoprotein; PCNA: proliferating cell nuclear antigen; LI: labeling index; HCC: hepatocellular carcinoma.
[a] *P* value: Id-1 low vs. high expressors.

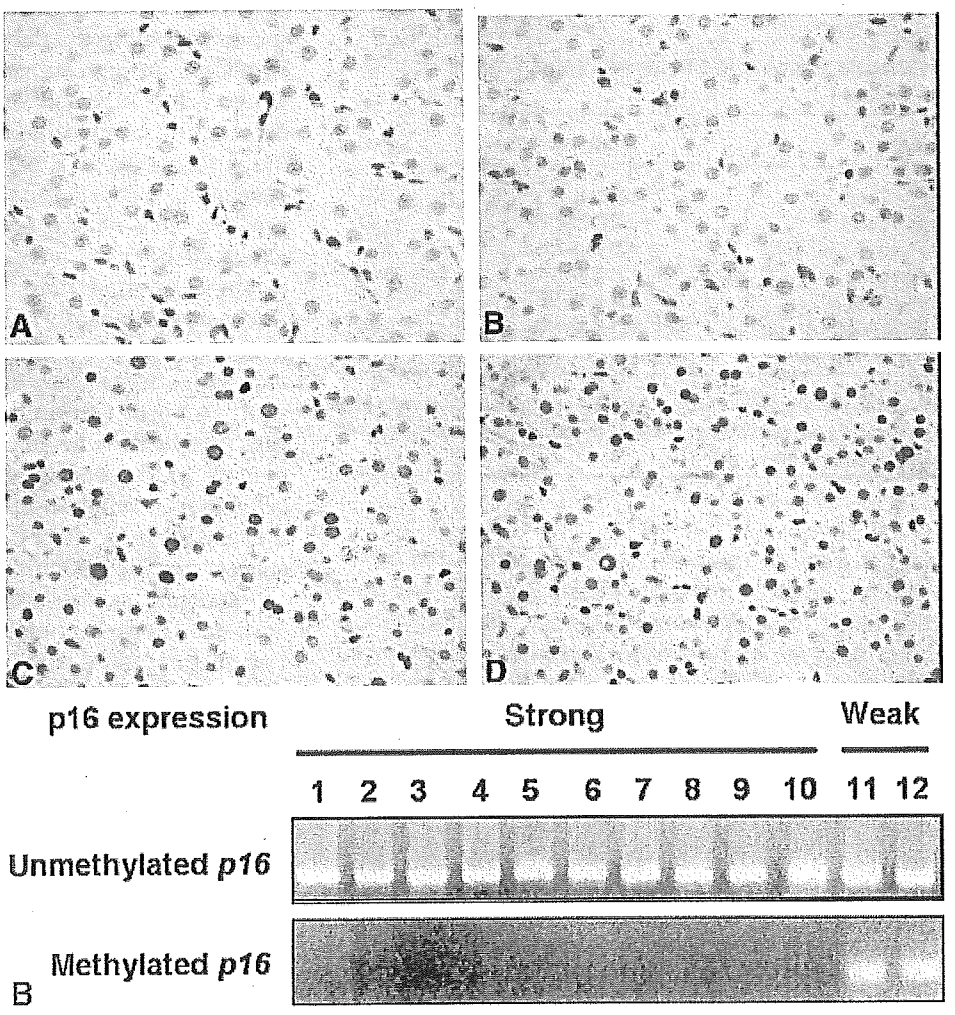

FIGURE 2. Status of p16 in cirrhosis. (A) Immunohistochemical staining for p16. (a,b) Weak p16 expression. Stromal cells lining the hepatic sinusoid can be regarded as internal positive controls. (c,d) Strong p16 expression. (original magnification × 200.) (B) Methylation-specific polymerase chain reaction (PCR) for the *p16* gene. The numbers depict representative cases of cirrhotic liver samples. Upper and lower columns indicate PCR products of unmethylated and methylated p16-specific primer sets, respectively.

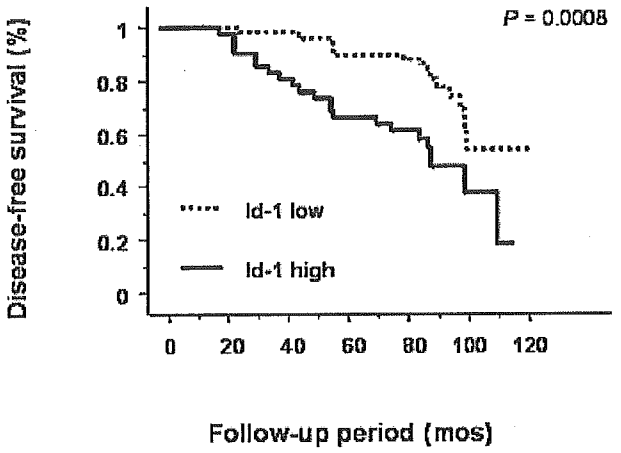

FIGURE 3. Cumulative hepatocellular carcinoma (HCC)-free survival in low and high expressors of inhibitor of differentiation/DNA binding protein 1 (Id-1). High Id-1 expressors (n = 42), designated Id-1 high, had a significantly increased rate of HCC occurrence than low Id-1 expressors (n = 70), designated Id-1 low (P = 0.0008; log-rank test).

TABLE 2
Multivariate Analysis of Id-1 Status and Clinical Variables for HCC Development

Variables	Relative risk (95% CI)	P value
Age (> 55 yrs)	1.13 (0.46–2.76)	0.797
Gender (male)	1.62 (0.76–3.48)	0.214
ALT level (7 > 100 IU/L)	0.99 (0.46–2.15)	0.985
Platelet count (< 10 × 10^4/mL)	1.31 (0.64–2.72)	0.454
AFP (> 20 ng/mL)	1.24 (0.62–2.49)	0.544
PCNA LI (> 5%)	0.76 (0.37–1.58)	0.465
Id-1 (high expressor)	2.75 (1.41–5.39)	0.003

Id-1: inhibitor of differentiation/DNA binding protein 1; HCC: hepatocellular carcinoma; 95% CI: 95% confidence interval; yrs: years; ALT: alanine aminotransferase; AFP: α-fetoprotein; PCNA: proliferating cell nuclear antigen; LI: labeling index.

Id-1 expressors than in low Id-1 expressors (P = 0.0008) (Fig. 3). Multivariate analysis of HCC-free survival using the Cox proportional hazard model indicated that high Id-1 expressors were recognized as an independent significant factor for the risk of HCC development in patients with cirrhosis (relative risk [RR] = 2.75, P = 0.003). The RRs of most of the clinical parameters were > 1.00, but were not statistically significant (Table 2).

DISCUSSION

In the current study, we examined Id-1 expression in 112 patients with cirrhosis and demonstrated that Id-1 expression is preferentially increased in patients with a high risk of HCC development. Immunohistochemical analysis showed that immunostaining for Id-1 was negative in 5 patients (4%) and positive in 107 patients (96%). The staining intensity was diverse among the 107 patients, with weak expression in 65 patients, moderate expression in 32 patients, and strong expression in 10 patients, possibly because the time of liver biopsy was heterogeneous among the patients in terms of the process of HCC development. However, we detected no significant differences in standard clinical parameters (age, serum ALT level, platelet count, and AFP levels) between the low and high Id-1 expressor groups.

We found that the mean PCNA LI of hepatocytes tended to be higher in the high Id-1 expressor group than in low Id-1 expressors, although statistical significance was not detected. Previous studies of AgNOR, Ki-67, and PCNA immunostaining have reported that increased hepatocellular proliferation was associated with the malignant property of cirrhotic livers,[1–8] supporting the idea that cell cycle progression may be closely involved in the early step of hepatocarcinogenesis. Unfortunately, however, there has been little consensus regarding the clinical significance of cell cycle regulators in precancerous liver tissue specimens. Kang et al.[24] reported that weak p53 expression was detected by immunohistochemical staining in 4 of 26 dysplastic nodules in patients with cirrhosis, whereas Choi et al.[25] reported that p53, cyclin D1, and cyclin E proteins were not expressed in dysplastic nodules in either cirrhotic or normal livers. Wagayama et al.[26] recently reported that the cumulative incidence of HCC was significantly higher in patients with cirrhosis with increased p21 expression. However, in that study, only 25 patients were evaluated. Therefore, another study comprising more patients may be required. Because cirrhosis is in a condition of perpetual inflammation, which consequently affects the turnover from cell death to renewal, obtaining accurate evidence of the relation between cell cycle regulators and carcinogenesis may be difficult.

Recently, several studies have shown that Id-1, a dominant negative regulator of basic HLH transcription factors, plays a crucial role in the mechanistic nature of increased cell proliferation during carcinogenesis.[9–12] Currently, Id-1 has been found to initiate DNA synthesis by inducing cell cycle G_1-S transition[27] and extending the cell lifespan through inactivation of the RB/p16 pathway.[28–30] Because Id-1 antagonizes the expression of differentiation-associated genes (e.g., *p21*, *p15*, and *p16*) via inhibiting DNA binding of bHLH or other activated proteins at the promoter regions,[31,32] overexpressed Id-1 may readily affect cell cycle machinery. Most importantly, the expression level of Id-1 has been indicated as a prognostic marker

in several types of early-stage cancers. Schindl et al.[33] reported that increased levels of Id-1 expression significantly influence prognosis in patients with cervical carcinoma Stage 1b. In addition, Schoppmann et al.[34] reported that overexpression of Id-1 represented a strong independent prognostic marker in patients with lymph node-negative breast carcinoma. The biologic significance of Id-1 in the early step of carcinogenesis is supported by the findings of Maruyama et al.,[13] who showed that Id-1 expression is significantly elevated in dysplastic and atypical papillary ducts in the pancreas as well as in cancer cells. In the current study, we found a significant increase in the cumulative incidence of HCC in the group of high Id-1 expressors during the long follow-up period (P = 0.0008). We also showed that Id-1 is an independent significant factor for the risk of HCC (RR = 2.75, P = 0.003), suggesting that Id-1 may become a useful marker for ascertaining cirrhosis patients who are more likely to develop HCC in the near term.

Investigating the signaling pathway elicited by Id-1 is an intriguing possibility to gain insight into the mechanism of early hepatocarcinogenesis. One of the most plausible candidates is p16, which is repressed by Id-1[28–30] and is reduced in approximately one-half of the patients with HCC.[15–17] To investigate whether p16 is repressed by Id-1 in precancerous liver tissue specimens, we evaluated the expression of p16 and compared it with the status of Id-1 in each of the patients with cirrhosis. Immunohistochemical analysis showed that 109 of the 112 patients in the current study had expression of p16 in > 50% of the hepatocytes, irrespective of the levels of Id-1 expression. There was no significant relation between the level of p16 and Id-1 expression, indicating that transcriptional repression of p16 by Id-1 in cirrhotic livers is unlikely. Kaneto et al.[35] reported that methylation of the *p16* gene promoter was detected in 5 of 17 patients with cirrhosis and in 4 of 17 patients with chronic hepatitis with HBV/HCV infections, suggesting that the *p16* gene is methylated in some patients with chronic liver injury as well as in some patients with HCC. In our study, although the methylated *p16* gene was detected in only 4 of 112 patients with cirrhosis, the methylation status of p16 showed a strong correlation with reduced immunohistochemical staining (P < 0.0001). This evidence strongly indicates that functional loss of p16 in cirrhotic livers is mainly caused by DNA methylation, not by Id-1–mediated signaling. However, our finding is distinct from the recent study by Lee et al.,[36] who showed a close inverse relation between the levels of Id-1 and p16 mRNA in human HCC tissue specimens. We surmise that Id-1 plays a critical role in early hepatocarcinogenesis, independent from the p16/RB signaling pathway, and in the later stage it promotes cancer aggressiveness via repression of p16 expression in HCC.

Cirrhosis is a terminal state of chronic liver injury, and many researchers have cautioned that the long-term inflammation per se may be a risk factor for the development of HCC. Recently, NF-κB has been found to link the mechanistic nature between inflammation and tumorigenesis in murine colitis-associated cancer and cholangitis-associated hepatoma.[37,38] This finding seems intriguing, because Id-1 is 1 of the main upstream regulators of NF-κB activity.[39] To address whether the Id-1/NF- κB signaling pathway plays a crucial role in the molecular link between chronic hepatic injury and HCC, we are now investigating the status of NF-κB in cirrhotic livers with overexpressed Id-1.

To date, the prognosis of patients with HCC is still poor, and identifying useful molecular markers for predicting HCC occurrence in cirrhosis is needed. However, there have been no established indicators responsible for future HCC occurrence in cirrhotic livers. Our study suggests that Id-1 may be a significant molecular marker for the risk of HCC development in precancerous liver tissue specimens. For more precisely determining individuals with a high risk of hepatocarcinogenesis, repeat liver biopsy to determine changes in Id-1 expression as well as to investigate the biologic role of Id-1 in cirrhosis appears important.

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HEPATOLOGY

Comparative study of genotype B and C hepatitis B virus-induced chronic hepatitis in relation to the basic core promoter and precore mutations

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Abstract

Background: The clinicopathological profiles and outcome of chronic hepatitis B can differ by hepatitis B virus (HBV) genotypes. In Japan, genotype B and C are two major HBV genotypes. The basic core promoter and precore mutations are other known viral factors for disease activity, although the relationship between HBV genotypes and these mutations is not fully understood.

Methods: The HBV genotypes in 90 patients with chronic hepatitis B were determined using an ELISA. Obtained data were correlated with clinicopathological parameters, basic core promoter, precore and the nucleotide 1858 mutations of the HBV genome.

Results: Among 90 cases, 20 (22.2%) had genotype B and 70 (77.8%) had genotype C HBV. Genotype B patients were older than genotype C patients (44.0 ± 13.9 vs 34.7 ± 11.0 $P = 0.0022$). The HBeAg was more prevalent in genotype C than B patients ($P = 0.0008$) while anti-HBe was more common in genotype B than C patients ($P = 0.0002$). Serum aspartate aspartate aminotransferase/alanine aminotransferase levels (B: 220.7 ± 612.8/257.0 ± 498.0 IU/L vs C: 111.3 ± 122.8/201.6 ± 229.4 IU/L, $P = 0.16$/0.48) and HBV viral loads in blood (B: 6.1 ± 3.1 log genome equivalent [LGE]/mL vs C: 6.7 ± 2.3 LGE/mL, $P = 0.42$) were equivalent. The seroconversion from HBeAg to anti-HBe occurred significantly earlier in genotype B than C patients (62 ± 53 months vs 136 ± 54 months, $P = 0.0028$) during the mean observation period of 149 ± 82 months even under various therapeutic modalities. The categories III and IV of the histological activity index in genotype C were higher (III: $P < 0.005$, IV: $P < 0.05$, $n = 68$) than that in B patients whereas category II was higher in genotype B than C patients ($P < 0.05$). The double mutation (1762T/1764A) in the basic core promoter was more frequently found in genotype C than in B HBV ($P = 0.0068$), whereas the precore mutation (1896A) was more common in genotype B than C HBV ($P = 0.0233$). The incidence of 1858C that was complementary to the precore mutation site in the stem-loop structure ∈, was equally rare in both genotype B and C HBV.

Conclusions: Genotype B patients were older, had earlier HBeAg seroconversion and exhibited more severe lobular necroinflammation, less portal inflammation and fibrosis than genotype C patients. This genotypic difference is related to the basic core promoter and precore mutations irrespective of 1858C.

Key words: basic core promoter, chronic hepatitis B, genotype, HAI score, HBV, precore mutation.

INTRODUCTION

Hepatitis B virus (HBV) eventually causes acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma in infected humans. The carrier rate of HBV is especially high in Asian and African countries. In Japan, vertical transmission of HBV from HBeAg-positive carrier mothers to their newborn babies during the

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perinatal period is a major cause of persistent HBV infection.[1] An asymptomatic period with positive HBeAg and normal liver function tests usually lasts during several decades of life in HBV carriers. Seroconversion from HBeAg to anti-HBe eventually occurs in some HBV carriers with a temporal burst of aspartate aminotransferase (AST)/alanine aminotransferase (ALT), while others continuously show positive HBeAg with or without elevation of AST/ALT.[2] This seroconversion results in normalization of liver function tests thereafter in most HBV carriers, whereas ALT abnormalities continue in some cases.[3] Seroconversion usually precedes the emergence of stop codon mutation at the precore (PC) nucleotide 1896 from G to A (1896A), resulting in a markedly reduced HBeAg secretion into blood.[4] Instead, stop codon mutation usually occurs as an escape phenomenon from anti-HBe antibodies and the host cytotoxic T lymphocytes, resulting in more severe flare up of ALT.[5] The primary infection by PC mutants eventually causes fulminant hepatitis in some individuals.[6–9] Other mutations at the basic core promoter region (BCP) also affect the efficiency of viral encapsidation and replication, and the disease activity.[10] The double mutation, A to T at nucleotide 1762 in association with T to A at 1764 (1762T/1764A) is reported to be the most common BCP mutation.[11]

Eight HBV genotypes (A–H) have been found according to the definition that more than 8% of the nucleotides in the entire HBV genome are replaced to become another genotype.[12–15] It is widely accepted that there is a worldwide geographic distribution in HBV genotypes.[16,17] The genotypes A and D are the two major genotypes prevailing in European countries,[18] while B and C are the prevailing genotypes in Southeast Asia including Japan.[19] The clinicopathological differences, including outcome of HBV-related chronic liver diseases in each part of the world, can be well explained by this geographically heterogeneous distribution of HBV genotypes. In fact, the genotype/serotype and viral load of hepatitis C virus (HCV) are well-known determinants for the efficacy of interferon in chronic hepatitis C.[20]

Several methods for HBV genotyping have been developed. These are whole or partial sequencing of the HBV genome,[12] multiplex polymerase chain reaction (PCR) using genotype-specific primers,[21] and restriction fragment-length polymorphism (RFLP) analysis of amplified PCR products of the S-gene,[22] although all aforementioned procedures seemed somewhat cumbersome and complicated for routine clinical use. Recently, Usuda *et al.* developed a new ELISA system for determining HBV genotypes and demonstrated its validity.[23] This system is based on the colorimetric detection of four genotype-specific epitopes present in the Pre-S2 region of the HBV genome by monoclonal antibodies. We determined HBV genotypes by using this ELISA system to elucidate the relationship between HBV genotypes and the clinicopathological profiles and outcome in patients with chronic hepatitis B. Moreover, we correlated the obtained data with the BCP, PC and 1858C mutations of the HBV genome to determine the relationship between HBV genotypes and these mutations.

METHODS

Patients

Ninety patients with chronic hepatitis B who visited the outpatient clinic at Niigata University Hospital periodically for the treatment and follow up of their liver function tests from January 1999 to December 2001, were enrolled into the study. Sera were collected at their initial consultation and stored at −20°C until use. They consisted of 18 women and 72 men and their mean age was 36.7 ± 12.2 years. Informed consent was obtained from all patients before serum collection and liver biopsy. The present study was fully approved by the Institutional Committee for Clinical Research on Human Subjects. Collected sera were used for determining HBsAg, anti-HBs, HBeAg, anti-HBe, HBV genotypes, HBV viral load in blood, AST/ALT levels and peripheral blood counts in each patient. The sera were also used for determining anti-HCV and anti-HIV. The patients positive for anti-HCV were excluded. There were no patients positive for anti-HIV. Anti-HDV was not routinely determined because of the paucity of HDV-positive patients in Niigata City, which is located on the main island of Japan.[24] Chronic alcoholic patients who consumed >80 g of ethanol daily were excluded. The patients with metabolic liver diseases including hereditary hemochromatosis, Wilson's disease, α_1-antitrypsin deficiency were all excluded. The DNA for direct sequencing was extracted from sera in 31 cases by the NaI method[25] as detailed later. The extracted DNA was subjected to the determination of partial nucleotide sequences in the core promoter and precore region of the HBV genome.

To fully evaluate the exact time when the seroconversion from HBeAg to anti-HBe occurred during their periodical visit to the clinic, two pair-matched groups by gender and age consisting of 12 genotype B patients and 12 genotype C patients who were positive for HBeAg at their initial consultation and who showed the seroconversion thereafter until the end of the observation period, were retrospectively analyzed. Seven patients with genotype B HBV were from the present study and another five patients with genotype B were newly added from those in the outpatient clinic at Niigata University Hospital. All 12 patients with genotype C HBV were from the present study. The mean observation period was 149 ± 82 months (minimum: 12 months, maximum: 270 months). All given treatments for each patient were recorded and described. The date at which HBeAg became negative while anti-HBe became and remained positive at least for the following 1 year was recorded case by case as the exact time-point for HBeAg seroconversion.

Hepatitis b virus and viral markers

HBsAg and anti-HBs were both detected by LumiPulse IV (Fuji Rebio, Tokyo, Japan), which was based on the chemiluminescent capture of specific antigen–antibody reactions. HBeAg and anti-HBe were both determined by Dainapack AX IMX (Dainabott, Tokyo, Japan). The

HBV viral load was quantified by the transcription-mediated amplification and hybridization protection assay (TMA-HPA; DNA Probe Chugai, Chugai Pharmaceutical, Japan).[26] The lower measurable limit of this method is 5×10^3 copies/mL. Anti-HCV and anti-HIV were determined using LumiPulse II Ortho HCV (Ortho Clinical Diagnostics, Tokyo, Japan) and LumiPulse II Ortho HIV-1/2, respectively, according to the manufacturer's instructions.

Determination of hepatitis B virus genotypes

The HBV genotypes were determined using the HBV Genotype ELISA Kit (1A64, Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions.[23] Briefly, blood HBsAg was captured by immobilized antibodies that specifically recognized a common S-region determinant, a. The captured antigen was then reacted with genotype-specific horseradish peroxidase-labeled monoclonal antibodies b, m, k, s and u. After washing, an enzyme substrate, tetramethylbenzidine, was added to the reaction and the reactivity was colorimetrically quantified at a 450-nm absorbance in a microtiter plate reader. The specific reaction pattern for each sample was evaluated and the genotype determined according to standard positive patterns for each HBV genotype.

Liver histology

The histological findings in liver biopsy specimens were semiquantified by using the histological activity index (HAI) by Knodell *et al.*[27] After informed consent was obtained from each patient prior to echo-guided or laparoscopy-assisted liver biopsy, liver specimens were able to be obtained from 68 out of 90 patients. The specimens were neutral buffered, formalin fixed, embedded in paraffin, and routinely stained after sectioning by silver impregnation, hematoxylin–eosin, Mallory–Azan, and observed in a light microscope. Category (I, II, III, IV, I + II + III) and the total score of HAI were determined by an independent hepatologist (TT) without knowing the clinical or biochemical data of each patient.

Direct nucleotide sequencing of the basic core promoter and precore regions of the HBV genome

The BCP and PC regions of the HBV genome were directly sequenced. The DNA was extracted from patient sera using the DNA Extractor Kit (Wako Pure Chemical, Tokyo, Japan). This kit is based on the NaI method by Ishizawa *et al.*[25] Extracted DNA was used as templates for the PCR reaction by a pair of outer primers, P1 and P2 flanking both the BCP and PC region.[28,29] The nucleotide sequence of P1 (nucleotide 1604–1623) was 5′-TCGCATGGAGACCACCG TGA-3′, and that of P2 (nucleotide 2076–2060) was 5′-ATAGCTTGCCTGAGTGC-3′. The amplified product served as templates for the second PCR using a pair of inner primers, P3 and P4. The nucleotide sequence of P3 (nucleotide 1653–1672) was 5′-CATAAGAG GACTCTTGGACT-3′, and that of P4 (nucleotide 1974–1957) was 5′-GGAAAGAAGTCAGAAGGC-3′. The nested-amplified PCR products were run on 3% agarose gels where these products made specific bands of expected sizes by ethidium bromide staining. These specific bands were cut and subjected to DNA purification by using GeneClean II (Bio 101, Vista, CA, USA). Purified DNA were subjected to cycle sequencing using BigDye Terminator Cycle Sequencing FS Ready Reaction Kit Version 2.0 (PE Biosystems Japan, Tokyo, Japan). Cycle-sequenced products were purified using Centri-Sep columns (Princeton Separations, NJ, USA) and applied to an ABI 310 Capillary Autosequencer (Applied Biosystems, CA, USA). The obtained nucleotide sequence was analyzed using DNasis version 3.0 (Hitachi Software Engineering, Yokohama, Japan) to detect any mutation of interest in this region.

Statistical analyses

When the two pair-matched groups comprising 12 genotype B and 12 genotype C patients with chronic hepatitis were compared for occurrence of the seroconversion from HBeAg to anti-HBe, the paired *t*-test was employed. The χ^2-test with Yates correction was used for correlation of HBV genotypes and HBeAg/anti-HBe status, and non-parametric Mann–Whitney *U*-test was employed for comparison of correlation of HBV genotypes and each category of the HAI scores. When the mutations in BCP (1762T/1764A), 1858C, and PC (1896A) regions were correlated with HBV genotypes, the χ^2-test was used.

RESULTS

Among 90 cases with chronic hepatitis B, 20 cases (22.2%) were determined as genotype B and 70 cases (77.8%) were determined as C. Gender, age, HBV viral load, AST and ALT levels at initial consultation are shown in Table 1. There was no statistically significant difference in gender between genotype B and C patients, while the genotype B patients were slightly but significantly older than genotype C patients. The mean HBV viral load was comparable between genotype B and C patients. The mean AST/ALT levels were also equivalent between these two groups (AST, $P = 0.16$; ALT, $P = 0.48$, respectively).

The relationship between HBV genotype and HBeAg/anti-HBe status is shown in the lower portion of Table 1. Definitely strong correlations between positive HBeAg and genotype C ($P = 0.0008$) and between positive anti-HBe and genotype B ($P = 0.0002$) were noted.

When the two pair-matched groups of chronic hepatitis B patients comprising 12 genotype B and 12 genotype C patients who were all positive for HBeAg at

Table 1 Genotype B and C patients with chronic hepatitis B

	Genotype B ($n = 20$)	Genotype C ($n = 70$)	P
Male/Femaleo	17/3	55/15	0.753
Age (years)	44.0 ± 13.9	34.7 ± 11.0	0.002
Viral load (LGE/mL)	6.14 ± 3.05	6.65 ± 2.30	0.416
AST (IU/L)	220.7 ± 612.8	111.3 ± 122.8	0.160
ALT (IU/L)	257.0 ± 498.0	201.6 ± 229.4	0.480
HBeAg positive/total	7/20	54/70	0.0008
Anti-HBe positive/total	14/20	15/70	0.0002

AST, aspartate aminotransferase; ALT, alanine aminotransferase.

initial consultation and who became positive for anti-HBe were compared (Fig. 1), the seroconversion from HBeAg to anti-HBe occurred apparently earlier in genotype B patients than genotype C patients (genotype B, 62 ± 53 months; genotype C, 136 ± 54 months; $P = 0.0028$). They all had various treatment sessions including interferon, glycyrrhizin injection, ursodeoxycholic acid and a Japanese herbal medicine, syo-saiko-to. The genotype C patients apparently had more therapeutic sessions than genotype B patients (Fig. 1), in contrast to the statistically significant delay in the HBeAg seroconversion. However, this difference was not able to be statistically analyzed because the amount, the mode of administration and the duration of therapeutic sessions were too heterogeneous for comparison.

The semiquantified HAI scores in the liver specimens of genotype B and C patients are shown in Table 2. The scores other than for category II were in general higher in genotype C patients than genotype B patients. The scores for categories III and IV were significantly higher ($P = 0.003$, $P = 0.0322$, respectively) in genotype C than in B patients. In contrast, the score for category II was slightly but significantly higher ($P = 0.0371$) in genotype B than in C patients.

The partial nucleotide sequence (nucleotide 1760–1932, 173 bp) containing the BCP/PC region of the HBV genome in the nested PCR products (nucleotide 1653–1974, 322 bp) in 31 patients with chronic hepatitis B are shown in Fig. 2 (genotype B HBV strains) and in Fig. 3 (genotype C HBV strains), respectively. The three mutation sites of interest in this region, the double mutation of BCP (1762T/1764A), 1858C and the PC mutation (1896A) are indicated by arrows in both Figures. The incidences of these three mutations in genotype B and C patients are summarized in Table 3. The incidence of the BCP double mutation (1762T/1764A) was significantly higher in genotype C than in genotype B HBV strains ($P = 0.0068$). In contrast, the PC mutation (1896A) was more frequently found in genotype B than in genotype C HBV strains ($P = 0.0233$). The incidence of 1858C, the complementary nucleotide site for the PC nucleotide (1896) in the stem-loop structure of the encapsidation signal ∈, was only one out of 11 (9.1%) genotype B strains and zero out of 20 (0%) genotype C HBV strains. Other than these three mutations of interest, both 1775A and 1915T were preserved in all genotype B patients, although these two sites were replaced by other nucleotides in the reference sequence (HPBADW1) for genotype B HBV. As to the relationship between the incidence of the BCP, PC mutations and HBeAg/anti-HBe status, the BCP mutation and positive HBeAg were not significantly correlated ($P = 0.46$), whereas the PC mutation and positive HBeAg were inversely correlated ($P = 0.037$) in all examined cases.

Table 2 HBV genotypes and HAI in 68 patients with chronic hepatitis B

HAI score	Genotype B ($n = 10$)	Genotype C ($n = 58$)	P
I	2.0 ± 1.8	3.2 ± 1.6	0.0626
II	2.6 ± 1.4	1.7 ± 1.1	0.0371
III	2.0 ± 1.1	2.9 ± 0.7	0.0030
IV	1.8 ± 1.0	2.5 ± 0.9	0.0322
I + II + III	6.6 ± 3.3	7.8 ± 2.8	0.3098
Total	8.4 ± 4.1	10.3 ± 3.3	0.1667

HBV, hepatitis B virus; HAI, histological activity index.

DISCUSSION

Genotypic differences of hepatitis viruses in terms of clinicopathological profiles, outcome, hepatocarcinogenesis, and responses to therapy help to determine treatment strategy for virus-related liver diseases. Genotype/serotype and viral load in blood are reported to be two major factors for predicting the efficacy of interferon in the case of chronic hepatitis C.[18] Eight HBV genotypes have been found, although clinical usefulness of genotyping in HBV-related liver diseases is not fully evaluated. In the present study therefore, we tried to find the genotypic differences in chronic hepatitis B in particular, and to correlate the obtained data with the nucleotide mutations of interest in the HBV genome that had already been well characterized and analyzed. We also correlated HBV genotypes with HBeAg/anti-HBe status and histological activity.

The significantly higher age and rate of negative HBeAg in genotype B patients when compared to those in genotype C patients were in accordance with the results recently obtained in a large-cohort study by