

Table 4 Serum AFP levels, tumor sizes and number of HCC foci of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			P-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/General-HCC patients	PBC-series/General-HCC patients	AIH-series/PBC-series
Serum-AFP	Total: 37	Total: 47	Total: 15831			
AFP - (< 15 ng/mL)	15 (40.5)	13 (27.7)	5756 (36.4)	<i>P</i> = 0.597	<i>P</i> = 0.216	<i>P</i> = 0.214
AFP + (≥ 15 ng/mL)	22 (59.5)	34 (72.3)	10075 (63.6)			
Serum-AFP (ng/mL)	Total: 37	Total: 47	Total: 15831			
(mean ± SD)	(2340.21 ± 8823.45)	(854.18 ± 2263.83)	NA			
< 15	15 (40.5)	13 (27.6)	5756 (36.4)			
15-199	12 (32.5)	16 (34.0)	5786 (36.5)	NA	NA	<i>P</i> = 0.106
200-399	1 (2.7)	6 (12.8)	902 (5.7)			(<i>P</i> < 0.001 ^b)
400-999	3 (8.1)	2 (4.3)	907 (5.7)			
≥ 1000	6 (16.2)	10 (21.3)	2480 (15.7)			
Maximum tumor size of HCC	Total: 36	Total: 48	Total: 15788			
(mean ± SD) (cm)	(3.75 ± 2.42)	(3.51 ± 1.69)	NA	NA	NA	<i>P</i> = 0.744
< 2	14 (38.9)	11 (22.9)	5123 (32.4)			(<i>P</i> = 0.028 ^a)
2.1-5.0	16 (44.4)	29 (60.4)	7434 (47.1)			
≥ 5.1	6 (16.7)	8 (16.7)	3231 (20.5)			
Number of HCC foci	Total: 38	Total: 49	Total: 16187			
(mean ± SD)	(1.58 ± 2.05)	(1.74 ± 1.97)	NA	NA	NA	<i>P</i> = 0.418
Single	33 (86.8)	38 (77.6)	9365 (57.9)			(<i>P</i> = 0.805)
Double	2 (5.3)	7 (14.3)	2850 (17.6)			
Multiple	3 (7.9)	4 (8.1)	3972 (24.5)			

The *P*-value in the first row was calculated from the χ^2 test and Fisher's exact test. The *P*-value in the following row was calculated from the Mann-Whitney *U*-test and the *P*-value below, indicated in parentheses, was calculated from the *F*-test. ^a*P* < 0.05, ^b*P* < 0.01, Statistically significant. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; AFP: α -fetoprotein; SD: Standard deviation; NA: Not available.

the most commonly selected forms of treatment were chemolipiodolization (CL) and transcatheter arterial embolization (TAE); other options included percutaneous ethanol injection therapy (PEIT) and radiofrequency ablation (RFA). Differences in the choice of therapeutic procedures were noted as follows, although no comparisons reached statistical significance following the Bonferroni correction: (1) The rate of CL or TAE among HCC patients with AIH was greater than the rate of operations among general HCC patients (*P* = 0.048), (2) The rate of CL or TAE among HCC patients with AIH was greater than the rate of PEIT and RFA among general HCC patients (*P* = 0.018), and (3) The rate of CL or TAE in HCC patients with PBC was greater than the rate of PEIT and RFA among general HCC patients (*P* = 0.027). Additionally, the frequency with which HCC patients with PBC chose to forgo treatment was significantly higher than the frequency with which general HCC patients chose to undergo PEIT or RFA (*P* = 0.003). Although not statistically significant, the frequency with which HCC patients with AIH refused therapeutic interventions was also higher than the frequency of PEIT or RFA in the general HCC population (*P* = 0.038, Table 5). Ideally, data on survival by treatment modality should be presented. However, the number of patients receiving each treatment modality who were able to be followed up to death was small. Hence, the mean period from HCC development to death was calculated from patient survival following all treatment options. Future prospective studies are needed to further analyze mean survival for each

treatment alternative.

Across all three patient groups, we found that liver failure was the leading cause of death, followed by rupture of HCC. Among general HCC patients, neoplastic death was most common (1487/2700, 55.1%), although differences between causes of death did not reach statistical significance. Comparisons between patient groups showed that: (1) The rate of neoplastic death in general HCC patients was higher than the rate of variceal rupture in HCC patients with AIH (*P* = 0.050), (2) The rate of neoplastic death in general HCC patients was higher than the rate of gastrointestinal bleeding in HCC patients with AIH (*P* = 0.013), and (3) The rate of neoplastic death in general HCC patients was greater than the rate of variceal rupture in HCC patients with PBC (*P* = 0.050, Table 5).

DISCUSSION

While autoimmune liver disease is more common among women than men in Japan, HCC in our group of patients with autoimmune liver disease was more common in men than women (Table 2). Men with AIH had a 1.6-fold greater risk of HCC than women, while men with PBC had a 3.2-fold greater risk of HCC than women with PBC. Moreover, when we followed AIH and PBC patients during HCC surveillance, we noted that the rate of HCC development was higher in male patients with autoimmune liver disease than in female patients with autoimmune liver disease.

Cirrhosis was found in only 18/31 (58.1%) of HCC

Table 5 Therapy and outcome of reported cases of hepatocellular carcinoma associated with autoimmune hepatitis and primary biliary cirrhosis, compared to cases of general hepatocellular carcinoma in Japan

Clinical status	Compiled numbers (%)			P-values		
	HCC patients with AIH (AIH-series)	HCC patients with PBC (PBC-series)	General-HCC patients	AIH-series/ General-HCC patients	PBC-series/ General-HCC patients	AIH-series/ PBC-series
Therapy choices for HCC	Total: 38	Total: 47	Total: 17005	Operation vs CL or TAE $P = 0.048^1$	RFA, PEIT vs CL or TAE $P = 0.027^1$	
CL or TAE	18 (47.4)	17 (36.2)	4636 (27.2)	RFA, PEIT, MCT vs CL or TAE $P = 0.018^1$	RFA, PEIT vs No therapy $P = 0.003^2$	
Operation	8 (21.0)	16 (34.0)	5268 (31.0)	RFA, PEIT, MCT vs No therapy $P = 0.038^1$		
RFA, PEIT, MCT	6 (15.8)	6 (12.8)	4890 (28.8)			
Chemotherapy	0 (0)	0 (0)	765 (4.5)			
Others	0 (0)	0 (0)	122 (0.7)			
No therapy	6 (15.8)	8 (17.0)	1324 (7.8)			
Clinical outcome	Total: 37	Total: 49	Total: 16646			
Alive	20 (54.1)	31 (63.3)	13946 (83.8)	$P < 0.001^b$	$P < 0.001^b$	$P = 0.389$
Dead	17 (45.9)	18 (36.7)	2700 (16.2)			
Cause of death	Total: 16	Total: 18	Total: 2700	Neoplastic death vs variceal rupture $P = 0.050^1$	Neoplastic death vs variceal rupture $P = 0.050^1$	
Liver failure	8 (50.0)	8 (44.4)	581 (21.5)			
HCC rupture	3 (18.8)	4 (22.2)	172 (6.4)			
Variceal rupture	1 (6.2)	1 (5.6)	85 (3.1)	Cancer death vs GI bleeding $P = 0.013^1$		
GI bleeding	1 (6.2)	2 (11.1)	55 (2.0)			
Neoplastic death	0 (0)	0 (0)	1487 (55.1)			
Others	3 (18.8)	3 (16.7)	320 (11.9)			

^b $P < 0.01$, Statistically significant. ¹The calculated P -values did not reach statistical significance with Bonferroni correction; without the correction, however, P -values were below 0.05. ²The calculated P -values reached statistical significance with Bonferroni correction. HCC: Hepatocellular carcinoma; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; CL: Chemolipiodolization; TAE: Transcatheter arterial embolization; RFA: Radiofrequency ablation therapy; PEIT: Percutaneous ethanol injection therapy; MCT: Microwave coagulation therapy; GI: Gastrointestinal.

patients with AIH, 29/44 (65.9%) of HCC patients with PBC, and in only 2250/4941 (45.5%) of the general Japanese HCC population. We did not add cases with liver fibrosis (LF) to the incidence of liver cirrhosis (LC) in the general Japanese HCC population, which may be one of the reasons why the incidence of liver cirrhosis was surprisingly low. Additionally, we think that the HCC cases with PBC and AIH and with non-cirrhotic liver, in which sufficient examinations and successful treatments were performed because of their higher hepatic reserve, were likely to be reported and submitted for publication. The possibility of bias in the selection of the reported cases should be raised.

Another interesting finding was that the interval between HCC diagnosis and death was shorter for patients with autoimmune liver disease than for the general HCC population of Japan^[13]. Furthermore, although we found that serum AFP level did not vary widely, the maximum tumor size and number of HCC loci were considerably lower in patients with autoimmune liver disease than in general HCC patients (Table 4). One explanation for this finding may be a selection bias, as cases which were detected earlier and treated successfully were more likely to be submitted for publication. Despite a smaller tumor size and a lower number of HCC loci in patients with HCC arising in the setting of autoimmune liver disease at the time of HCC diagnosis, a shorter reported survival was not attributed to late detection of HCC and failure to survey patients with autoimmune liver disease for HCC, but was more likely to be due to advanced liver disease and cirrhosis. Future prospective studies will be needed to verify or

refute these findings.

Although CL and TAE were the most frequently selected treatment modalities across all patient groups (Table 5), many patients ultimately refused treatment due to advanced age or social circumstances. Medical treatments using CL or TAE may be common because HCC cases are often inoperable due to cirrhotic liver disease in these patients. While survival may be related to the choice of therapeutic options, inconsistencies in data reporting over multiple decades and across multiple medical centers made the calculation of survival data difficult.

Several mechanisms explaining the development of HCC from autoimmune liver diseases have been proposed: enhanced progression to cirrhosis through progressive autoimmune hepatitis, decreased antitumor immune responses caused by long-term administration of steroids and immunosuppressants, or virus-mediated hepatitis^[16,17]. In this study, we found significantly higher rates of HBV and HCV among PBC patients with HCC than among AIH patients with HCC. This finding may be attributable to the higher rates of blood transfusion in HCC patients with PBC ($P = 0.041$, Table 3). This result is supported by the findings of Shimizu *et al*^[18], who reported that 3/16 (19%) HCC patients with PBC tested positive for prior HBV and present HCV infections. Given the high rates of prior HBV infections among HCC patients with PBC, it is possible that prior HBV infection predisposes patients to HCC through HBV-DNA becoming integrated into hepatocyte DNA. It has been reported that even in patients who test negative for serum HCV-RNA and serum HBV-DNA (less

than the sensitivity of HBV-DNA), liver tissue samples frequently test positive for HCV-RNA or HBV-DNA. This suggests a possible role for positive HCV-RNA or HBV-DNA in hepatic tissue in the development of HCC^[19-23]. In the present study, however, only six HCC patients with AIH and two HCC patients with PBC were found to have detectable HBV-DNA and HCV-RNA in liver tissue samples. Aggressive liver biopsies should be taken to allow genetic analysis for HBV and HCV in liver tissue, in order to further study HCC cases with non-B, non-C hepatitis.

We reported high rates of HBV or HCV infection among HCC patients with PBC (Table 3); however, this is less surprising because international diagnostic criteria for AIH allocate negative points for positive HBV or HCV diagnostic tests^[14]. Furthermore there are no definitive histological features that allow a clinician or pathologist to distinguish AIH from chronic viral hepatitis. Thus, HBV or HCV infected patients are rarely classified as having AIH in the modern era. In contrast, the unique histological features of PBC and the relative specificity of AMA tests allow clinicians to diagnosis and report cases of concurrent PBC and chronic viral hepatitis with a greater degree of confidence.

It has been reported that HCC develops significantly more often in patients with concurrent PBC and HCV infection than in patients with AMA-positive PBC^[9]. The incidence of HCC associated with PBC has been suggested to have increased recently due to prolonged periods of liver cirrhosis resulting from longer survival on steroid therapy, concurrence of the hepatitis virus or alcohol intake with HCC, and the administration of immunosuppressants which may disturb immunoregulatory function^[24,25]. In a proportional hazards analysis of patients with PBC, Shibuya *et al.*^[11] found 3 factors to be independently associated with the development of HCC: age at time of diagnosis, male gender, and a history of blood transfusion. Our findings showed that HCC cases arising from PBC were more common in men and those with liver cirrhosis.

While the number of HCC cases arising from PBC is stated to be small, it has been reported that the calculated crude incidence of HCC was 492.4/100 000 person years, and that HCC has a relatively high prevalence in PBC^[7]. Furthermore, there is a dramatically increased risk for development of hepatobiliary malignancies in patients with PBC, with a relative risk of 46 ($P < 0.0001$) in women and 55 ($P < 0.0001$) in men^[26].

Finally, several questions remain for the clinician. Namely, should AIH and PBC patients be screened for HCC? Should screening be limited to cirrhosis? Does the clinical course after diagnosis differ from other HCC patients? Late-stage AIH and PBC patients should be screened for HCC just as in HCV-related cirrhosis, given the similar reported incidence of HCC development in late-stage PBC^[13]. Furthermore, Suzuki *et al.*^[27], reported very recently that patients of older age, male sex, history of blood transfusion, and any signs of portal hypertension or cirrhosis should be considered for HCC screening. A prospective study or a case control study

for AIH patients is needed similar to that conducted for PBC patients.

At present, HCC transformation in early-stage pre-cirrhotic AIH and PBC were thought to be very rare. However, a high incidence of HCC development was observed in AIH and PBC patients with overlapping HCV and HBV infection, including occult HBV infection^[9,19,20]. These patients should be closely followed using ultrasonography, CT-scanning and MRI of the abdomen, as well as tumor markers for HCC. Reports of HCC cases arising from "pure" AIH and PBC (with no history of blood transfusion, excessive alcohol intake, immunosuppressant administration, and with negative HBV and HCV serotyping) are rare^[2,27-31]. El-Serag *et al.*^[32], in a multivariate analysis reported that AIH itself is not significant; however, our study indicates that early-stage AIH and PBC patients also have the potential to develop HCC. We advocate that "pure" or "early-stage" AIH and PBC cases should also be regularly screened for HCC.

Our data also indicate that the clinical course after diagnosis of HCC with AIH and PBC differs from virus-associated HCC, although prospective studies are needed to confirm these results. Clinicians should note the common clinical features of HCC cases with AIH and PBC at diagnosis, treatment, and follow-up of these patients.

Lastly, our findings also beg the question of why HCC rupture is the second most common cause of death in both groups of patients examined. We have recently reported a pelioid-type HCC patient with PBC, who died from rupture of HCC^[33]. A pelioid change was observed more frequently in large poorly-differentiated and encapsulated HCC^[34], and the features of pelioid-type HCC were high blood flow into the HCC, high pressure in the tumor and fibrous capsular formation. It is unknown whether the ruptured HCCs in the present study had these features, as this study had severe limitations because it was retrospective. Tumors in such patients may grow rapidly, and pathophysiological factors shared by both patient groups may trigger the rupture of HCC. A prospective study on the cause of death and a pathologic study of ruptured HCC with AIH and PBC is awaited with great interest.

Further clinical and laboratory studies are needed to describe which pathological, biological and genetic features are common among HCC cases arising from AIH and PBC. How HCC in these patients relates to viral hepatitis also requires further clarification. The present study was retrospective; however, this is the first study to date that highlights the importance of these future research topics. Future prospective studies on these important subjects are required.

COMMENTS

Background

Hepatocellular carcinoma (HCC) development in autoimmune hepatitis (AIH) as well as in primary biliary cirrhosis (PBC) is a rare event. The common clinical features of HCC associated with AIH and PBC have not yet been extensively

described.

Research frontiers

In this study, we characterized these common features through a systematic review of the literature conducted using the Japana Centra Revuo Medicina database. We demonstrated common clinical features among cases of HCC arising from AIH and PBC in Japan.

Innovations and breakthroughs

We found common clinical features in HCC cases with AIH and PBC as follows, (1) HCC was more common in men than in women with AIH or PBC. (2) Many patients underwent chemolipiodolization (CL) or transcatheter arterial embolization (TAE). (3) Liver failure was the primary cause of death among patients in this study, followed by tumor rupture. (4) The survival interval between diagnosis and death was fairly short.

Applications

The present study was retrospective; however, this is the first study to date that highlights the common clinical features in HCC cases with AIH and PBC. Future prospective studies of these important subjects are required.

Peer review

This is a systematic literature review of HCC cases with AIH and PBC published throughout Japan. The review is clearly written and highlights a very interesting topic.

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Progression of Hypermethylation of the $p16^{INK4A}$ Gene from Normal Liver to Nontumorous Liver and Hepatocellular Carcinoma: An Evaluation Using Quantitative PCR Analysis

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Abstract The aim of this study was to determine to what extent hypermethylation of the $p16^{INK4A}$ ($p16$) gene promoter is increased in nontumorous liver tissues compared with in normal liver, using two quantitative methylation-specific polymerase chain reaction (MS-PCR) methods and a bisulfite sequencing method. Methylation of the $p16$ gene was detected more frequently in nontumorous liver than in normal liver using the TaqMan PCR method. Methylation indices also were significantly higher in nontumorous than in normal liver. However, the bisulfite sequencing method did not detect significantly more methylation of the $p16$ gene in nontumorous than normal liver, nor was there a significant difference in the level of $p16$ mRNA. There may be a greater proportion of cells which contain methylated $p16$ in nontumorous than in normal liver. However, the difference was so small that the functional relevance to hepatocarcinogenesis remains elusive.

Keywords $p16^{INK4A}$ · Methylation-specific PCR (MS-PCR) · Real-time quantitative PCR · Bisulfite sequencing · Nontumorous liver · Normal liver

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide and one of the leading causes of cancer death in Japan [1, 2]. Long-standing hepatitis B virus (HBV) or hepatitis C virus (HCV) infections are the most frequent cause of HCC and most HCCs arise in livers with chronic hepatitis and cirrhosis. Necroinflammation caused by these viruses or other toxic agents, such as alcohol and aflatoxins, induce a broad variety of genetic and epigenetic changes involving the inactivation of tumor suppressor genes and activation of oncogenes that contribute to a multistep transformation process, leading ultimately to the clinical manifestation of HCC [3, 4].

Epigenetic changes, including in the status of DNA methylation, are among the most common molecular alterations in human neoplasia [5, 6]. Cytosines are methylated in the human genome almost exclusively when located 5' to a guanosine. Regions with high G:C content (so-called CpG islands) are mostly unmethylated in normal tissue but may be methylated to varying degrees in human cancers, thus constituting tumor-specific changes [6, 7]. Recently, aberrant methylation in the promoters of a number of genes that are involved in cell growth, DNA repair, and signal transduction has been reported in early preneoplastic lesions in the lung, colon, esophagus, pancreas, and stomach [8–12]. These findings suggest that aberrant methylation and consequent downregulation of gene expression precedes tumor development and may constitute a preneoplastic stage. Because HCC arises in livers with

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chronic disease and its most characteristic feature is a high recurrence rate, several studies have focused on the status of DNA methylation in nontumorous liver tissue [13–15]. In addition, there have been several studies that sought to delineate methylation profiling of gene promoters applying quantitative PCR analysis [16–19].

The *p16^{INK4A}* (hereafter designated *p16*) gene, located on chromosome 9p21, encodes a critical negative regulator of cell cycle progression [20, 21] and is a major target for inactivation in HCC [22, 23]. There have been reports of a high rate of detection of the methylated *p16* gene and downregulation of *p16* protein in nontumorous liver tissues of HCC patients, suggesting a possible involvement of the methylation of that gene in the development of a preneoplastic stage [13, 24–27]. However, so far only a few quantitative analyses have addressed the association of the degree of *p16* gene methylation and the cancer-prone state of the liver by comparing nontumorous tissues with normal liver, as well as tissue from chronic liver disease [18]. In addition, few studies have analyzed the methylation status of 5'CpG islands in the *p16* gene using the bisulfite sequencing method for comparison of HCC, nontumorous, and normal liver tissues. In this study, we carried out two quantitative methylation-specific PCR (MS-PCR) analyses and used the bisulfite sequencing method to evaluate the methylation status of *p16*, especially focusing on nontumorous liver tissues that mostly had chronic inflammation and may constitute a preneoplastic stage.

Materials and Methods

Tissue Samples

HCC and surrounding liver tissues were obtained by surgical resection (20 men and 9 women). Twenty-eight HCC and 29 adjacent liver tissue specimens were available. Liver biopsy specimens obtained from 10 patients with chronic hepatitis (7 HCV; 3 HBV) and 12 normal liver tissue specimens obtained at surgical resection of metastatic liver tumors, cholangiocellular carcinomas, and gallbladder cancers, also were subjected to analysis. "Normal liver" means no inflammation or fibrosis, microscopically. Age, gender, viral markers, clinical staging, and size of HCCs, fibrosis levels [28] of nontumorous tissues, and some of these parameters in biopsy specimens and normal livers are shown in Table 1. Viral status HBV and HCV indicates HBsAg- and anti-HCV- positivity, respectively, and NBNC means negative for both markers.

Informed consent for the study was obtained from the patients and the study was conducted in accordance with the guidelines of the Ethics Committee of Niigata University.

Table 1 Background of patients

Operation (HCC and noncancerous liver tissue)	Total no.
Age (years)	
<50	4
≥50	25
Gender	
Male	20
Female	9
TNM stage	
I/II	14
III/IV	14
Tumor size	
≤30 mm	14
>30 mm	14
Virus infection	
HCV	15
HBV	9
NBNC	5
F stage	
F1-3	10
F4 (LC)	19
Biopsy (chronic hepatitis)	Total no.
Age (years)	
<50	5
≥50	5
Gender	
Male	7
Female	3
Virus infection	
HCV	7
HBV	3
F stage	
F1-3	8
F4 (LC)	2
Operation (normal liver)	Total no.
Age (years)	
<50	0
≥50	12
Gender	
Male	6
Female	6

Methylation-Specific PCR Analysis and Estimation of Methylation Index

DNA was extracted from liver tissues using a DNA isolation kit (Qiagen, Germany). DNA samples were modified by sodium bisulfite as reported previously [23]. Modified

DNA was subjected to MSP using primers specific for 5' CpG islands in the *p16* promoter, which were 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and 5'-CAACCCCAAACCACAACCATAA-3' for unmethylated DNA, 5'-TTATTAGAGGGTGGGGCGGATCGC-3' and 5'-GACCCCGAACCGCGACCGTAA-3' for methylated DNA (Fig. 1) [29].

We carried out quantitative real-time PCR using a Light Cycler (Roche Diagnostic, Mannheim, Germany). Detection and estimations of the ratio of methylated to unmethylated alleles of *p16* were carried out using the following methods.

Estimation of Methylation Index Using SYBR Green

For quantitative real-time PCR with SYBR green, 20 μ l PCR reactions contained 0.5 μ M of each primer, 2 μ l Light Cycler-DNA Master SYBR Green I (Roche), 2 mM MgCl₂, 1 μ l dimethyl sulfoxide (DMSO), and 2 μ l template DNA. PCR conditions were as follows: one cycle of denaturing at 95°C for 10 s, followed by 45 cycles of 95°C for 10 s, 65°C for 10 s, and 72°C for 6 s. Fluorescence was acquired at the end of each 72°C extension phase. The melting curves of the final PCR products were analyzed after 45 cycles of PCR amplification by cooling the samples to 70°C, then increasing the temperature to 99°C at a rate of 0.1°C/s. When detected by the PCR with primers specific for the methylated allele of *p16*, they were counted as an incidence (incidence of methylation, IM).

PCR products amplified by a Hot Start PCR (Takara, Japan), using primers to detect the methylated and unmethylated allele, were subcloned into pGEM[®]-T Easy vector (Promega, Madison, WI) according to the manufacturer's instructions. They were diluted serially and used to construct a standard correlation to quantify copy numbers of methylated and unmethylated *p16* alleles. The methylation index (MI) in each sample was calculated using the following equation [16, 30]:

```

. . . . . tccctccaga ggalltgagg gacagggtcg
gagggggctc ttccgccagc accggaggaa gaagaggag
gggctggctg ctcaccagag ggggggggg accgtgtccg
ctcggggctc gctgagaggg gtagagcagg cactgggccc
caggtagcag catggagccg ggggcggga gcagcatgga
gccttcgct gactggctgg ccacggcgc ggccggggg
gggtagagg aggtcgggc . . . . .

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Fig. 1 Locations of primer and probe sequences in the CpG island of the *p16^{INK-1A}* gene promoter used in the experiments. CpG sites possibly methylated are represented as bold and underlined. Primer sequences common to the SYBR green and the TaqMan probe methods are presented in shaded boxes. Probe sequence for methylated DNA-specific TaqMan PCR and primer sequences for the bisulfite sequencing method (no CpG site) are presented in hatched and in open boxes, respectively

Methylation index(MI) = $M/(M + U) \times 100$.

Estimation of Methylation Index Using TaqMan Probe

Quantitative real-time PCR with a TaqMan probe was used to detect each methylated and unmethylated allele, respectively (Fig. 1). DNA samples modified by sodium bisulfite were amplified using the primer set described above and the TaqMan probes 6FAM5'-AGTAGTATG GAGTCGGCGGGGG-3'TAMRA for the methylated allele [19] and 6FAM5'-AGGTAGTGGGTGGTGGGGA GTAGTATGGAGTTG-3'TAMRA for the unmethylated allele. PCR was carried out in a reaction volume of 20 μ l. Each PCR reaction contained 0.2 μ M of each primer, 0.1 μ M probe (Applied Biosystems, Foster City, CA), 4 μ l TaqMan Master (Roche), and 5 μ l template DNA. As in the case of PCR using SYBR green, cloned DNAs were used as standards to calculate the copy number of methylated and unmethylated DNA, and MIs were derived using the same equation.

Bisulfite Sequencing Method

The bisulfite sequencing method [31] was used to elucidate the complete methylation status of 5' CpG islands in the *p16* promoter. Briefly, primers which do not contain any CpG sites (5'-GAGGATTTGAGGGATAGGGT-3' for sense, 5'-CTACCTACTCTACCCCTCTC-3' for antisense; Fig. 1) were subjected to PCR analysis amplifying DNAs modified by sodium bisulfite. Each 50 μ l PCR reaction contained 0.2 μ M of each primer, 2 mM MgCl₂, 4 μ l 10 mM dNTP, 1 μ l DMSO, 0.75 units Hot Start PCR (Takara), and 2 μ l template DNA. PCR conditions were: one cycle of denaturing at 95°C for 5 min, followed by 38 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 60 s. Amplified DNAs were purified (Wizard PCR purification kit, Promega) from ultrapure agarose gels (Takara) and subcloned into pGEM[®]-T Easy vector (Promega). For this analysis, DNA samples derived from a HCC tissue that is highly methylated, from a nontumorous tissue that showed by TaqMan PCR the highest MI of the nontumorous tissues, and from a normal liver tissue that showed no methylation by the TaqMan PCR were selected. Ten (10) clones obtained from each of these samples were sequenced (Autosequencer, ABI).

Quantification of *p16* mRNA by Real-Time PCR

To correlate the MI with the expression level of *p16*, we analyzed *p16* mRNA levels quantitatively using the TaqMan PCR method. RNA was extracted from liver tissue using an RNA isolation kit (Qiagen). One microgram of RNA digested with DNase (Invitrogen) was converted to cDNA using a Transcriptor First Strand cDNA synthesis kit

(Roche) and subjected to PCR analysis. Primers and probe sequences of *p16* mRNA and β -actin as an internal control were obtained from the Universal probe library (Roche). PCR conditions were: one cycle of denaturing at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 1 s. Primer sequences were; 5'-GAC-CTGGCTGAGGAGCTG-3' (sense) and 5'-TTCAATCGG GGATGTCTGA-3' (antisense) for *p16* mRNA, 5'-ATTGG CAATGAGCGGTTC-3' (sense) and 5'-GGATGCCACA GGACTCCAT-3' (antisense) for β -actin mRNA. The *p16* mRNA level was quantified by the ratio to the level of β -actin mRNA.

Statistical Analysis

Chi-square test or Fisher's exact test were used for the comparison of the incidence of methylation. Unpaired Student's *t* test was used for the comparison of methylation indices among groups. The correlation coefficient was calculated as the Pearson product-moment correlation coefficient. $P < 0.05$ was considered to be statistically significant.

Results

Detection of Methylated and Unmethylated *p16* Alleles by Real-Time PCR and Construction of Standard Curves for Quantification

Figure 2 shows the detection (left) and quantification (right) of methylated and unmethylated *p16* alleles by real-time PCR with the SYBR green (a, b) and TaqMan probe (c, d) methods. Serially diluted control DNAs (cloned DNAs) were included in the assays and linear correlations between DNA concentration and cycle numbers were derived.

Comparison of the SYBR Green and TaqMan Probe Methods for Detection of the Incidence of Methylated *p16* Alleles

We applied the two methods of MS-PCR analysis to detect the methylated and unmethylated *p16* alleles quantitatively. The TaqMan probe method requires the methylation or nonmethylation of the three CG sites in the probe sequence, in addition to the methylation or nonmethylation of CG sites in the primer sequence, for amplification (Fig. 2), while only the methylation status within the primer sequence was involved in the SYBR green method. Thus, the detection rate of the methylated *p16* alleles should theoretically be higher with the SYBR green method than the more specific TaqMan probe method. In fact, 46 samples gave the same results with both methods

[25 (44%) both positive and 21 (37%) both negative]. Eleven (19%) samples were detectable only using the SYBR green method whilst no samples were detectable only using the TaqMan probe method. Figure 3 shows the correlation of MIs between the SYBR green and TaqMan methods using DNA derived from HCC. There was a significant correlation ($P < 0.01$) between the values obtained by the two methods.

IM in Normal Liver, Nontumorous Liver, and HCC as Clinicopathological Parameters

Table 2 shows the IM according to the SYBR green and TaqMan probe methods. The IM increased significantly in the order of normal liver, nontumorous liver, and HCC according to the TaqMan probe method. However, this tendency was not significant using the SYBR green method. The IM was higher in cirrhosis (F4) than chronic hepatitis (F3) by both methods, but this was not significant. The IM was significantly higher in the HCV-induced HCC than HBV and NBNC HCC. However, there was no significant difference in nontumorous tissues among chronic HCV, HBV, and NBNC livers (data not shown). We also compared IM between nontumorous liver tissues and biopsy specimens with chronic viral infections using the TaqMan probe method. These were 28% (8/29) and 30% (3/10), respectively (NS).

MI's Among Clinicopathological Parameters

MI's increased significantly in the order of normal liver, nontumorous liver, and HCC by both the SYBR green and TaqMan probe methods (Fig. 4). As was observed in the analysis of IM, MI's were higher in cirrhosis (F4) than chronic hepatitis (F1–3) by both methods, but this was not significant. In addition, HCV-induced HCC showed a significantly higher MI than other etiologies, but this tendency was not observed in the nontumorous liver tissues (data not shown). Differences in MI between nontumorous livers and biopsy specimens with chronic viral hepatitis were 1.962 ± 2.212 (mean \pm SD) and 2.242 ± 2.057 , respectively (NS).

Results of Bisulfite Sequencing and Cloning

PCR amplification with primers that did not contain any CpG site at the promoter of the *p16* gene was performed for bisulfite-treated DNA derived from one HCC, nontumorous liver (that with the highest MI), and normal liver. The PCR products were subcloned and ten clones from each of these samples were sequenced (Fig. 5). Almost all the CpG sites from HCC were methylated, except for clone 8 where only the first CpG site was methylated. On the other hand,

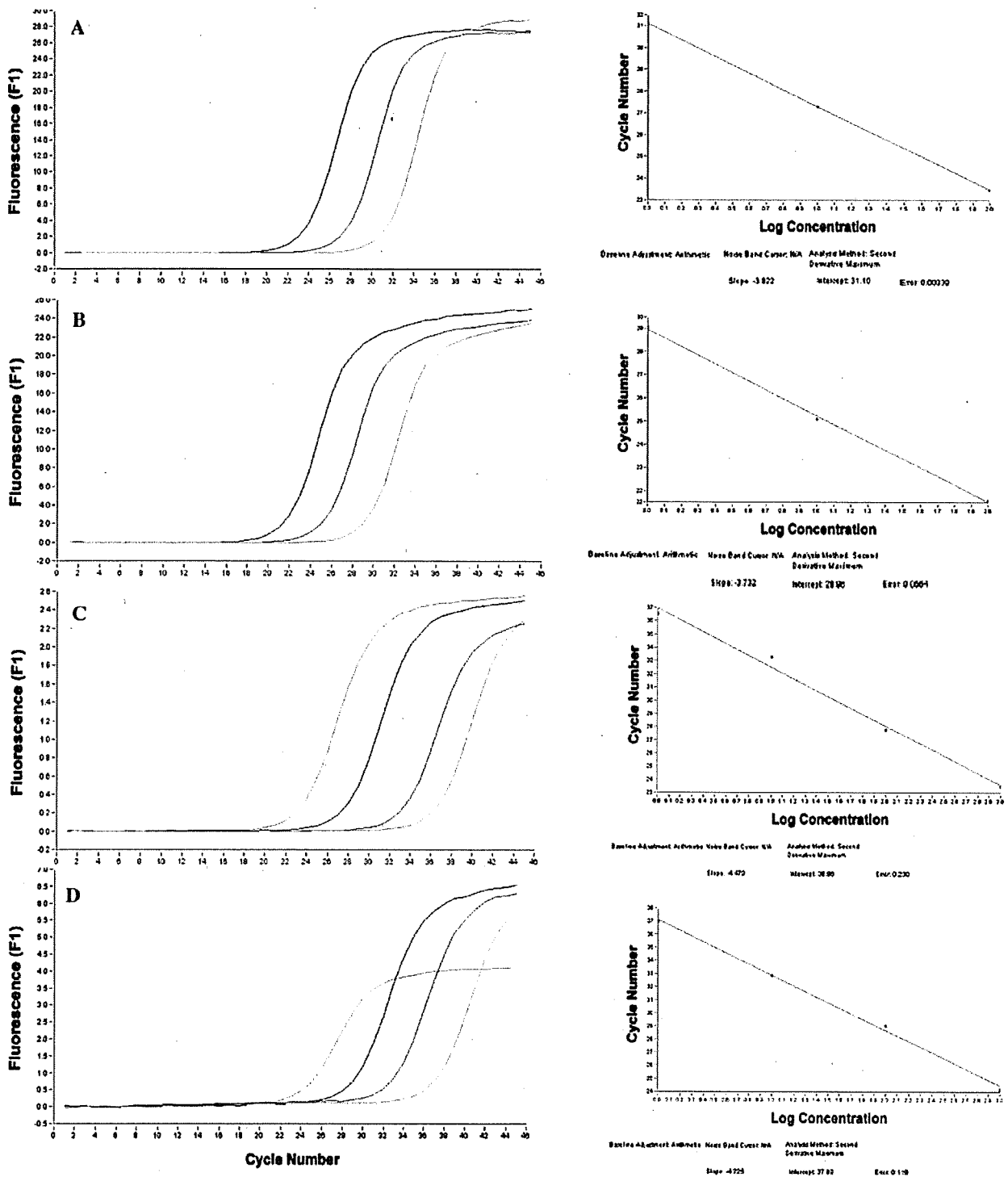


Fig. 2 Quantitative MS-PCR using serial dilutions of cloned DNA. The results of SYBR green method (a, b) and the TaqMan probe (c, d) for methylated (a, c) and unmethylated (b, d) targets are shown. PCR was performed using tenfold serial dilutions of cloned DNA

from 2 ng to 2 µg (left panels). Based on the amplification results, linear correlation between the concentration of DNA and cycle number was obtained using LightCycler software (right panels)

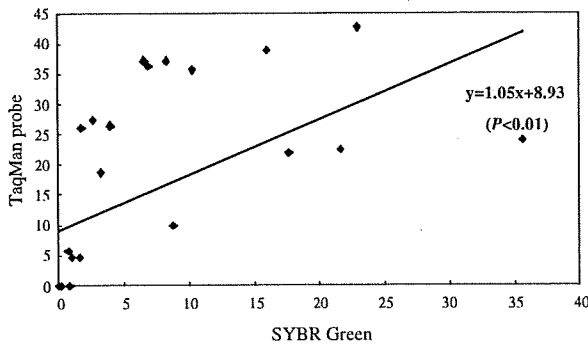


Fig. 3 Correlation of MIs between the SYBR green (horizontal axis) and TaqMan probe (vertical axis) methods using DNA from HCC tissues. A significant correlation ($P < 0.05$) was obtained

Table 2 Detection of methylated *p16* gene by SYBR green and TaqMan PCR methods

	SYBR	TaqMan
HCC	20/28 (71%)	17/28 (64%)*, **
NT	16/29 (55%)	8/29 (28%)*
NL	4/12 (33%)	0/12 (0%)**
NT; F0–3	4/10 (40%)	2/10 (20%)
NT; F4	12/19 (63%)	6/19 (32%)
HCC (stage I, II)	8/14 (57%)	6/14 (43%)
HCC (stage III, IV)	12/14 (86%)	11/14 (79%)
HCC (HCV)	14/14 (100%)*	12/14 (86%)*
HCC (HBV)	4/9 (44%)*	3/9 (33%)*
HCC (NBNC)	2/5 (40%)*	2/5 (40%)*

* $P < 0.05$, ** $P < 0.01$

almost all the CpG sites were unmethylated in nontumorous liver tissue and normal liver (except one site in the nontumorous tissue and two sites in the normal liver). Thus, unexpectedly we could not detect a significantly higher number of methylated CpG sites in the nontumorous tissue than normal liver, as was suggested by two quantitative MS-PCRs which showed a higher MI in the nontumorous tissue than in normal livers (Fig. 4a).

Results of Quantitative Real-Time PCR Analysis for mRNA of *p16* Allele

Fig. 6 shows a comparison of mRNA of *p16* levels in nontumorous tissue and normal livers. There was no significant difference.

Discussion

We used quantitative MSP with the SYBR green and TaqMan probe methods to obtain two independent

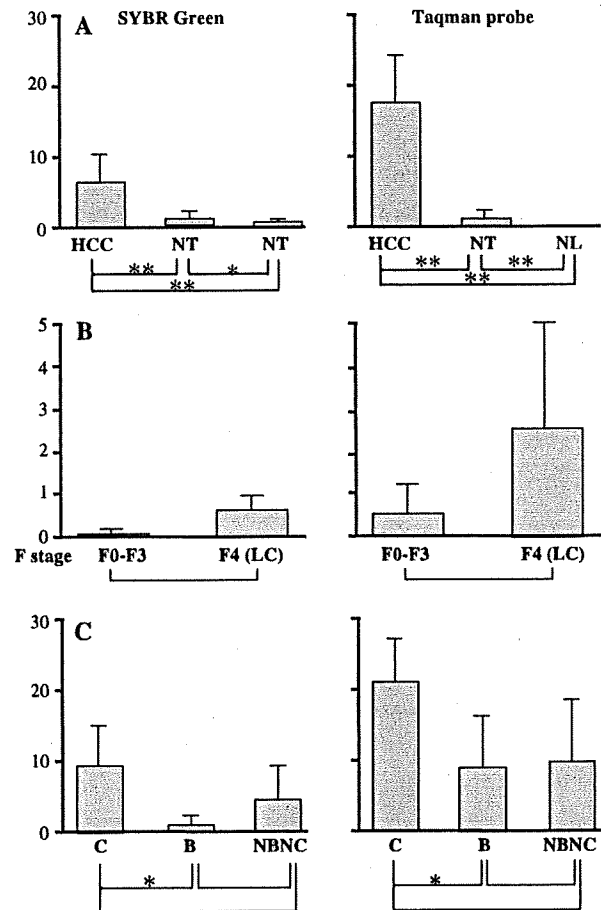


Fig. 4 Results of MIs (mean \pm SD) in the liver tissues by the SYBR green (left) and TaqMan probe (right) methods. **a** Comparison among HCC, nontumorous liver (NT), and normal liver (NL). There were significant differences between each set of comparisons ($*P < 0.05$, $**P < 0.01$). **b** Comparison between MIs between F1–3 and F4. **c** Comparison among HCV-, HBV-, and NBNC-HCC. The MI of HCV-HCC was significantly higher than other etiologies ($*P < 0.05$)

estimates of the degree of *p16* gene methylation. Methylated alleles were detected more frequently by the SYBR green method than by the TaqMan probe method that requires other methylated CpG sites in the probe sequence for amplification (Table 2). Theoretically, there should be no samples which were detected by the TaqMan probe method but not by the SYBR green method and this was the case. In addition, there was a significant correlation of MI using the SYBR green and TaqMan methods (Fig. 3). These results confirmed the accuracies of the assays.

Gene methylation increases with age [32]. Because the purpose of our experiments was to evaluate the degree of methylation in preneoplastic liver tissue by quantitative estimation of the methylated allele of *p16*, it was necessary to evaluate the methylation status in normal liver. There have been several studies that attempted to detect methylated *p16* in nonlesional liver by MS-PCR and the

Fig. 5 Results of bisulfite sequencing and cloning. Ten CpG sites (bold face letter) are present in the sequence amplified. Each circle corresponds to one site. The methylation status of CpG sites in one HCC (upper), nontumorous liver (middle), and normal liver (lower) was analyzed. The results of all ten clones are shown. Methylated CpG sites are shown by closed circles and unmethylated CpG sites are shown by open circles

GA GGATTTGAGG GACAGGGTCG GAGGGGGCTC TTCCGCCAGC ACCGGAGGAA
 GAAAGAGGAG GGGCTGGCTG GTCACCAGAG GGTGGGGCGG ACCGCGTGCG
CTCGGCGGCT GCGGAGAGGG GTAGAGCAGG CAG

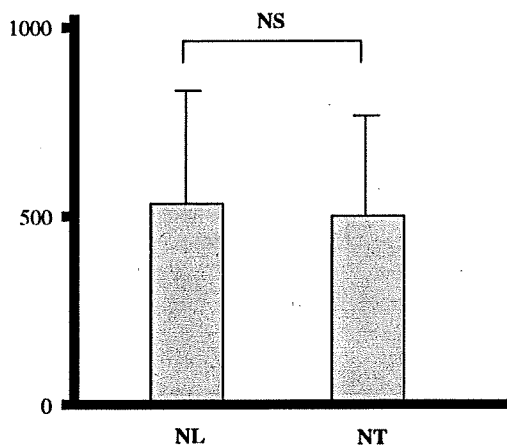
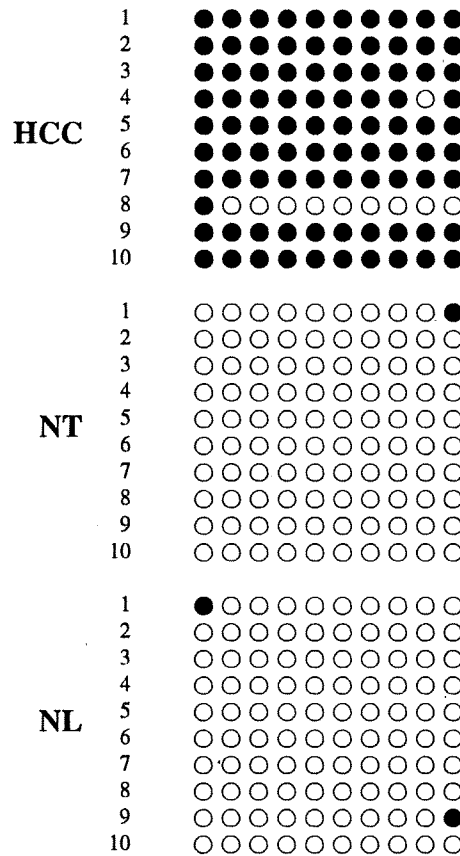


Fig. 6 Comparison of quantification of mRNA of the *p16* gene between nontumorous and normal liver tissues. The *p16* mRNA level was quantified by the ratio to the level of β -actin mRNA. The results are shown as mean \pm SD. There was no significant difference

detection rate was 0% to 7% [18, 33], much lower than in our report. Waki et al. [33] clarified the incidence of methylation of tumor suppressor and tumor-related genes, including the *p16* gene, in autopsy samples using a routine PCR method. The frequency of methylation was higher in the samples obtained from older individuals. However, they did not report the detection of *p16* gene methylation in liver. In contrast, we could detect *p16* gene methylation in normal liver tissues obtained during surgery carried out on livers without chronic disease. Sampling always was carried out carefully to avoid possible contamination with tumor tissues. In addition, methylation was detectable by the SYBR green method (40%) and not by the TaqMan probe method (0%), showing that the methylation was partial and located only in the binding site of the primers. These results lead us to conclude that detection of methylation of the *p16* gene in these tissues was an age-related phenomenon (all the patients were ≥ 50 years old) and not attributable to contamination with tumor tissues.

We found that methylation was detected more frequently in nontumorous (28%) than in normal (0%, $P < 0.05$) liver by the TaqMan probe method. The reason the SYBR green method did not yield a statistically significant result is a higher sensitivity and lower specificity than the TaqMan method, as described above.

There have been several reports of the detection of the methylated *p16* gene in livers with cirrhosis or chronic hepatitis [13, 24–27] using the MS-PCR method. Kaneto et al. [13] reported such methylation in 29.4% of cirrhosis and 23.5% of chronic viral hepatitis, and Li et al. [26] reported methylation in 16% of nontumorous liver tissues obtained at surgery for HCC. However, so far few quantitative analyses of methylated *p16* have been reported and there is little information on its involvement in the preneoplastic state of the liver. Interestingly, in our study, the mean MIs were significantly higher in the order of HCC, nontumorous liver, and normal liver by both the SYBR green and TaqMan methods (Fig. 4a). This result shows that the number of cells with methylated *p16* alleles may increase significantly as the pathological stages progress in the order of normal liver, nontumorous liver, and HCC in the samples where methylation was detected using the whole DNA.

Although not the main subject of our research, we made several additional observations. Firstly, both IM and MI were higher in HCV-associated HCC than in tumors associated with HBV and NBNC (Table 2 and Fig. 4c). This result was consistent with a previous report [26]. Interestingly, this tendency was not observed in the nontumorous livers (data not shown). Because there were no significant differences of clinical staging and tumor size between HCV-induced HCC and others (data not shown), stronger association of the methylation of *p16* with HCV-induced HCC than with other etiologies was suggested. A second observation is that we could detect *p16* gene methylation in nontumorous liver tissue from NBNC cases of HCC in contrast to previous studies that did not detect methylation in nontumorous livers with etiologies other than HBV and HCV [13, 34] (Fig. 4c). This discrepancy may come from differences in the sensitivities of the assays, because the methylated *p16* allele was detected even in normal livers in our study.

Although not significant, IM and MI were higher in livers with cirrhosis (F4) than in those F3 or less (Fig. 4b). Because cirrhosis has a higher risk for hepatocarcinogenesis than chronic hepatitis, this result supports the possible association of the increase in frequency of the *p16*-methylated allele with the development of a preneoplastic stage. However, IM and MI in the biopsy specimens from individuals with chronic viral infections were similar to those of nontumorous liver tissues. These results show that chronic inflammation may lead to the accumulation of the

cells with a methylated allele of the *p16* gene, irrespective of the coexistence of HCC.

We carried out bisulfite sequencing to confirm the result of MS real-time PCR. We analyzed ten clones from each to evaluate the methylation of each CpG site in the sequences amplified from a HCC, the nontumorous liver which showed the highest MI, and a normal liver (Fig. 5). Because the PCR primers do not contain any CpG sequence, this method is another means of clarifying the status of CpG methylation, not being so biased to overrepresentation of methylated alleles as PCR methods that detect and amplify only methylated sequences. Unexpectedly, we could not see any increase in methylation of CpG sites in the nontumorous liver compared with in the normal liver (one versus two sites), whereas most CpG sites in clones obtained from the HCC tissues were methylated. The interpretation may be that the number of cells with methylated alleles of the *p16* gene was increased in the nontumorous liver compared with in normal liver, but the degree was too small to be detected by the bisulfite sequencing method, which has a lower sensitivity than MS-PCR [35]. In addition, the levels of *p16* mRNA did not differ significantly between the nontumorous and normal livers. Thus, we could not correlate the increase in the numbers of cells that had methylated *p16* alleles with the significant downregulation of *p16* mRNA in nontumorous liver tissues.

In conclusion, although we suggest that one of the features of the preneoplastic stage of the liver is proliferation of cells with partially methylated *p16* alleles, the proportion of such cells may be low and the involvement of these cells in the initiation of hepatocarcinogenesis remains elusive. Recently, there was a report showing that the *RASSF1A* gene is increasingly methylated in nonlesional, regenerative, and neoplastic liver [15]. Analyzing the spread of hepatocytes with methylated alleles by quantitative evaluation of a number of gene promoters to elucidate a functional association with the development of HCC should be pursued in future studies.

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Serum Alpha-Fetoprotein Levels During and After Interferon Therapy and the Development of Hepatocellular Carcinoma in Patients with Chronic Hepatitis C

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Abstract The association between serum alpha-fetoprotein (AFP) levels during and after interferon (IFN) therapy and the development of hepatocellular carcinoma (HCC) was evaluated in patients with chronic hepatitis C (CHC). A total of 263 patients treated by IFN with or without ribavirin were enrolled in the study. Serum AFP levels during and after IFN therapy were investigated retrospectively, and statistical analysis was performed to identify the factors associated with HCC development. During IFN therapy, serum AFP levels significantly decreased, regardless of virologic response to treatment. Increased serum AFP levels (≥ 10 ng/ml) at the end of IFN therapy (EOT) was a close-to-significant variable affecting the development of HCC ($P = 0.057$), and a significantly higher cumulative incidence of HCC was seen in patients with increased serum AFP levels at EOT ($P = 0.021$). Serum AFP level at EOT is a possible predictor of HCC in CHC patients after IFN therapy.

Keywords Chronic hepatitis C · Interferon · Alpha-fetoprotein · Hepatocellular carcinoma

Introduction

Hepatitis C virus (HCV) has a worldwide prevalence. HCV infection frequently causes chronic liver disease leading to

liver cirrhosis, and increases the risk of hepatocellular carcinoma (HCC) [1–5]. Interferon (IFN)-based therapy has been used in patients with chronic hepatitis C (CHC), as it has been shown not only to eradicate HCV but also to reduce serum alanine aminotransferase (ALT) levels [5–9]. Moreover, several studies have indicated that IFN therapy reduces the rate of development of HCC and results in improved survival in patients with CHC [10, 11]. Nevertheless, there have been many reports of the detection of HCC in some patients with CHC even after successful eradication of HCV by IFN therapy [12, 13]. Although several factors, such as older age, male gender, and severe fibrosis, have been implicated [12, 13], the factors associated with the development of HCC after IFN therapy are still inconclusive.

Alpha-fetoprotein (AFP), a 70-kDa single-stranded glycoprotein, has been widely used as a diagnostic marker for HCC [14, 15]. Although elevated serum AFP level in patients with CHC has been shown to be a significant independent predictor of the development of HCC [4, 5], AFP levels are sometimes elevated in patients with chronic hepatitis and cirrhosis who have no evidence of HCC [16–18]. Especially among patients with advanced CHC, serum AFP values are frequently elevated (e.g., at a rate of 47% in patients with cirrhosis), even in the absence of HCC [20]. Therefore, the usefulness of AFP as a screening marker of HCC has been limited by its impaired specificity. Several studies have revealed that elevation of serum AFP levels in CHC is associated with female gender, elevated serum ALT level, prolonged prothrombin time, decreased platelet count, low serum albumin level, hepatic necroinflammation and fibrosis in biopsy specimens, and genotype 1b HCV infection [18–22]. In addition, IFN therapy decreases serum AFP levels in patients with CHC [20–24]. However, there is little knowledge about the relationship between the

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changes in serum AFP level associated with IFN therapy and the development of HCC. We therefore investigated the changes in serum AFP levels during and after IFN therapy, and we evaluated the clinical significance of changes in AFP value in the prediction of HCC development after IFN therapy.

Methods

Patients and Treatment

A total of 263 patients with CHC treated by IFN with or without ribavirin at hospitals participating in the Niigata Liver Disease Study Group from January 2000 to May 2007 were investigated retrospectively. All patients were confirmed to be seropositive for anti-HCV antibody and HCV-RNA. Patients with hepatitis B surface-antigen positivity, autoimmune hepatitis, or alcoholic liver disease, were excluded from the study. No patient had a previous history of treatment for HCC or had liver tumors detectable by ultrasound (US) or computed tomography (CT) before treatment. Histopathologic examinations before IFN therapy were performed in 174 patients. Hepatic inflammation and fibrosis of specimens were assessed according to the new Inuyama classification of chronic hepatitis in Japan [25]. There were four degrees of inflammation: A0 (no inflammation), A1 (mild), A2 (moderate), and A3 (severe). There were five degrees of fibrosis: F0 (no fibrosis), F1 (mild), F2 (moderate), F3 (severe), and F4 (cirrhosis). In accordance with approval of the study protocol by the institutional review board of Niigata University Medical and Dental Hospital, written informed consent was appropriately obtained from all of the individuals enrolled in the study. The study was carried out in accordance with the ethical guidelines of the 1975 Declaration of Helsinki.

The IFNs used in this study were pegylated-IFN (Peg-IFN) α -2b, Peg-IFN α -2a, recombinant IFN α -2b, consensus IFN α , and natural IFN α . Of the 263 patients, 114 (43.3%) were treated with Peg-IFN α -2b (1.5 μ g/kg weekly) and ribavirin (600–1,000 mg daily); 71 (27.0%) were treated with recombinant IFN α -2b (6–10 MIU three times weekly) and ribavirin (600–1,000 mg daily); 61 (23.2%) were treated with Peg-IFN α -2a (90–180 μ g weekly); ten (3.8%) were treated with consensus IFN α (9–18 MIU three times weekly); and seven (2.7%) were treated with natural IFN α (3–6 MIU two or three times weekly). Doses of IFNs and/or ribavirin were reduced when adverse effects, such as neutropenia or anemia, were observed. Most patients infected with genotype II HCV were treated for 24 weeks, and patients infected with genotype I HCV were usually treated for 48 weeks or more. All patients were followed for at least 24 more weeks after the end of treatment

(EOT). Qualitative polymerase chain reaction (PCR) assay 24 weeks after the end of treatment was used to evaluate efficacy of treatment according to elimination of HCV-RNA. Patients were classified by virologic response into three groups: sustained virologic response (SVR), relapse, and no response (NR). SVR was defined as negative HCV-RNA 24 weeks after EOT. Relapse was defined as negative HCV-RNA during IFN therapy and positive HCV-RNA 24 weeks after EOT regardless of the serum ALT level. NR was defined as positive HCV-RNA during IFN therapy and 24 weeks after EOT.

Laboratory Examinations and Follow-Up

The white blood cell count, red blood cell count, platelet count, serum ALT level, and serum albumin level were measured before treatment (at baseline) and at least once every 4 weeks after initiation of treatment. Serum AFP level was measured before treatment and once every 1 to 6 months during the follow-up period. HCV-RNA was tested before treatment and 24 weeks after treatment by qualitative PCR assay (Amplicor HCV version 2.0; Roche Diagnostics Co., Tokyo, Japan), with a detection limit of 50 IU/ml. Viral (HCV-RNA) load was measured before and during treatment by quantitative PCR assay (Amplicor HCV monitor version 2.0; Roche Diagnostics Co., Tokyo, Japan) with a detection limit of 500 IU/ml. HCV-RNA genotype was determined by an immunoserological typing method (Immucheck F-HCV Gr Kokusai; Sysmex, Kobe, Japan). Besides laboratory examinations, patients were screened for the presence of HCC by several imaging modalities (US, CT, and magnetic resonance imaging). The status of patients enrolled in this study was confirmed as of November 2007.

Statistical Analysis

In accordance with their pretreatment AFP values, the 263 subjects were divided into two groups: a low AFP group (AFP <10 ng/ml; $n = 191$) and a high AFP group (AFP ≥ 10 ng/ml; $n = 72$). Differences in the distributions of clinical features between the two groups were determined by Fisher's exact test and the Mann-Whitney test. Logistic regression analysis was performed with forward selection to identify the factors associated with serum AFP level at baseline. Wilcoxon signed-rank test was used to compare the changes in serum AFP level from baseline to EOT and from EOT to 24 weeks after EOT. A Cox's proportional hazards model was used to analyze the factors contributing to development of HCC; factors examined were age, gender, ALT, platelet count, albumin, AFP, histopathologic findings (inflammation and fibrosis), HCV genotype, HCV load, duration of treatment, and efficacy of IFN (SVR vs.

relapse and NR). Cumulative development of HCC based on serum AFP level (<10 ng/ml vs. \geq 10 ng/ml) at EOT was determined by the Kaplan–Meier method. Differences between the curves were evaluated by log-rank test. Statistical analyses were performed by SPSS 15.0 (SPSS Japan Inc., Tokyo, Japan). A *P*-value of <0.05 was considered statistically significant.

Results

Clinical Features of Patients Classified by Serum AFP Level at Baseline

A total of 263 patients were studied. The mean AFP level (mean \pm SD) at baseline was 15.0 ± 41.9 ng/ml, and the median was 5.1 ng/ml (range, 1.0–542.0). There were 71 patients with serum AFP \geq 10 ng/ml at baseline (27.0%). The serum AFP level usually remained elevated without large fluctuations, although the evident pattern of AFP elevation varied in each patient. Clinical features of patients classified by serum AFP level (<10 ng/ml vs. \geq 10 ng/ml) at baseline are shown in Table 1. Patients with elevated AFP (\geq 10 ng/ml) were characterized by older age ($P < 0.001$), high ALT level ($P < 0.001$), low platelet count ($P < 0.001$), low albumin level ($P < 0.001$), and progression of hepatic inflammation ($P < 0.001$) and fibrosis ($P < 0.001$). No significant difference was seen in gender, HCV genotype, or HCV-RNA viral load.

Multivariate logistic regression analysis revealed that the factors correlated with elevated serum AFP at baseline were low serum albumin level ($P = 0.001$) and severe hepatic inflammation ($P = 0.02$) (Table 2).

Changes in Serum AFP Level During and After IFN Therapy

Mean duration of IFN therapy was 39.6 ± 16.5 weeks. One hundred thirty-two (50.2%) patients were treated for 24 to 47 weeks, and 131 (49.8%) were treated for 48 or more weeks. Of the 263 patients treated with IFNs, 136 (51.7%) were defined as SVR, 51 (19.4%) as relapse, and 76 (28.9%) as NR. Serum AFP levels decreased gradually during IFN therapy without fluctuations, and a significant decrease was observed regardless of response to treatment. ($P < 0.001$, $P = 0.003$, and $P < 0.001$ for SVR, relapse, and NR, respectively). In the SVR group, a decrease in serum AFP level was also observed after treatment ($P = 0.037$) (Table 3). The significant decrease of serum AFP level was not associated with hepatic fibrosis, as seen in both patients with mild (F0-2) ($P < 0.001$) and severe hepatic fibrosis (F3 and F4) ($P = 0.012$) (Table 4).

Next, we evaluated the changes in serum AFP level according to the treatment regimens. Among patients treated with Peg-IFN α -2b, recombinant IFN α -2b and Peg-IFN α -2a, the decrease in the serum AFP level during IFN therapy was not associated with the treatment regimen ($P < 0.001$, $P < 0.001$ and $P = 0.001$, for Peg-IFN α -2b

Table 1 Clinical features of patients classified by serum AFP level at baseline

Characteristics	AFP <10 ng/ml (n = 191)	AFP \geq 10 ng/ml (n = 72)	<i>P</i> -value
Age (years)	54.3 \pm 11.8	60.0 \pm 8.6	<0.001
Gender (Female/Male)	84/107	41/31	0.072
ALT (IU/l)	85.8 \pm 88.1	104.5 \pm 66.3	<0.001
Platelet ($\times 10^4/\mu$ l)	16.8 \pm 5.6	13.1 \pm 4.8	<0.001
Albumin (g/dl)	4.4 \pm 0.3	4.0 \pm 0.5	<0.001
Histopathology			
Inflammation (A0-1/A2-3)	68/57	7/42	<0.001
Fibrosis (F0-2/F3-4)	116/9	30/19	<0.001
HCV-RNA			
Serology (Group 1/2)	131/57	54/18	0.446
Viral load (0-99/100-499/500 < KIU/ml)	18/39/99	8/16/30	0.588

ALT alanine aminotransferase, AFP alpha-fetoprotein, HCV hepatitis C virus. Values are expressed as mean \pm SD

Table 2 Multivariate logistic regression analysis for predicting elevated AFP (\geq 10 ng/ml) at baseline

Variables	Coefficient	SE	OR (95% CI)	<i>P</i> -value
Albumin (g/dl)	-2.502	0.724	0.082 (0.020–0.339)	0.001
Inflammation (A0-1/A2-3)	1.525	0.656	4.594 (1.270–16.617)	0.020

SE standard error, OR odds ratio, CI confidence interval

Table 3 Changes in serum AFP level during and after IFN therapy according to the virologic response

Virological response	Baseline	EOT	24 weeks after EOT
SVR			
Median AFP (ng/ml)	4.2	3.4*	3.3 [†]
(range)	(1.0–153.0)	(1.0–12.9)	(1.0–10.3)
Relapse			
Median AFP (ng/ml)	5.8	4.1**	3.9
(range)	(1.0–271.8)	(2.0–31.6)	(1.0–82.7)
NR			
Median AFP (ng/ml)	8.3	5.9*	6.0
(range)	(1.5–542.0)	(1.5–59.8)	(1.3–109.0)

Wilcoxon signed-ranks test was used. * $P < 0.001$, compared to baseline; ** $P = 0.003$, compared to baseline; [†] $P = 0.037$, compared to EOT

AFP alpha-fetoprotein, SVR sustained virologic response, NR non-response, EOT end of treatment

Table 4 Changes in serum AFP level during and after IFN therapy according to the hepatic fibrosis

Hepatic fibrosis	Baseline	EOT	24 weeks after EOT
Slight fibrosis, F0-2			
Median AFP (ng/ml)	4.4	3.5*	3.3
(range)	(1.0–153.0)	(1.0–59.8)	(1.0–37.4)
Advanced fibrosis, F3-4			
Median AFP (ng/ml)	14.1	6.5**	6.3
(range)	(2.6–542.0)	(1.5–54.4)	(1.8–82.7)

Wilcoxon signed-ranks test was used. * $P < 0.001$, compared to baseline; ** $P = 0.012$, compared to baseline

AFP alpha-fetoprotein, EOT end of treatment

and ribavirin, recombinant IFN α -2b and ribavirin, Peg-IFN α -2a, respectively) (Table 5). We were unable to evaluate the changes in serum AFP level in patients treated with consensus IFN α or natural IFN α owing to the small number of cases.

Risk Factors for Development of HCC

The mean observation period from initiation of IFN therapy was 162.4 ± 82.3 weeks (range, 89.7–367.7). HCC developed in seven patients within the study period. The final diagnosis of HCC was determined by imaging modalities. Two of the seven were patients defined as SVR and five as NR. The mean length of time from initiation of IFN therapy to diagnosis of HCC was 129.0 ± 77.3 weeks. Serum AFP levels at diagnosis of HCC were 2.2, 3.0, 4.0, 10.9, 11.9, 17.4, and 47.0 ng/ml. Of these seven patients, one showed F3 fibrosis, two were diagnosed clinically as

Table 5 Changes in serum AFP level during and after IFN therapy according to the treatment regimens

Treatment regimens	Baseline	EOT	24 weeks after EOT
Peg-IFN α-2b + RBV			
Median AFP (ng/ml)	5.0	3.9*	4.0
(range)	(1.4–77.8)	(1.5–22.2)	(1.4–26.3)
Rec-IFN α-2b + RBV			
Median AFP (ng/ml)	7.3	4.1*	4.0
(range)	(1.0–542.0)	(1.0–54.4)	(1.0–35.1)
Peg-IFN α-2a			
Median AFP (ng/ml)	4.0	3.8**	3.5
(range)	(1.0–271.8)	(1.5–31.6)	(1.0–82.7)

Wilcoxon signed-ranks test was used. * $P < 0.001$, compared to baseline; ** $P = 0.001$, compared to baseline

AFP alpha-fetoprotein, Peg-IFN pegylated-IFN, Rec-IFN recombinant IFN, RBV Ribavirin, EOT end of treatment

having cirrhosis, and the other four were diagnosed as having chronic hepatitis at the start of IFN treatment. In the follow-up period from EOT to diagnosis of HCC, a pattern of AFP increase, a stable AFP pattern and a pattern of AFP decrease were seen in three, three and one patients, respectively (data not shown). Table 6 represents the changes in serum AFP level during and after IFN therapy in the patients who developed HCC and in those who did not. Univariate Cox's regression analysis revealed that older age at the initiation of therapy ($P = 0.038$) was a significant prognostic indicator of the development of HCC after IFN therapy, and increased serum AFP levels at EOT ($P = 0.057$) was a close-to-significant variable (Table 7). We examined the cumulative incidence of HCC development on the basis of serum AFP level (<10 ng/ml vs. ≥ 10 ng/ml) at EOT (Fig. 1). Patients with AFP ≥ 10 ng/ml had a significantly higher incidence of HCC than did those with AFP <10 ng/ml by log-rank test ($P = 0.021$).

Table 6 Changes in serum AFP level during and after IFN therapy in those with patients who developed HCC and in those without HCC

Patients	Baseline	EOT	24 weeks after EOT
Patients developed HCC			
Median AFP (ng/ml)	8.5	17.9	7.6
(range)	(3.6–61.0)	(2.2–46.0)	(1.0–109.0)
Patients without HCC			
Median AFP (ng/ml)	5.1	3.9*	3.9
(range)	(1.0–542.0)	(1.5–59.8)	(1.0–82.7)

Wilcoxon signed-ranks test was used. * $P < 0.001$, compared to baseline

AFP alpha-fetoprotein, EOT end of treatment

Table 7 Factors associated with the development of HCC after IFN therapy by univariate Cox's regression analysis

Variables	HR (95% CI)	P-value
Pre-treatment baseline variables		
Age (years)	1.117 (1.006–1.239)	0.038
Gender (Female/Male)	1.888 (0.365–9.772)	0.449
ALT (IU/l)	0.996 (0.984–1.009)	0.570
AFP (ng/ml)	0.998 (0.980–1.017)	0.856
Platelet ($\times 10^4/\mu\text{l}$)	0.956 (0.830–1.102)	0.539
Albumin (g/dl)	1.569 (0.159–15.518)	0.700
Histopathology		
Inflammation (A0-1/A2-3)	0.624 (0.086–4.521)	0.641
Fibrosis (F0-2/F3-4)	1.362 (0.137–13.558)	0.792
HCV-RNA		
Serology (Group 1/2)	1.195 (0.231–6.187)	0.832
Viral load (0-99/100-499/500 < KIU/ml)	1.422 (0.346–5.849)	0.625
Duration of therapy (week)	0.997 (0.919–1.038)	0.453
Efficacy of IFN (SVR/Relapse/NR)	2.453 (0.476–12.652)	0.284
Post-treatment variables at EOT		
ALT (IU/l)	1.006 (0.998–1.014)	0.137
AFP (ng/ml)	1.048 (0.999–1.101)	0.057
Platelet ($\times 10^4/\mu\text{l}$)	1.020 (0.889–1.171)	0.777
Albumin (g/dl)	0.115 (0.005–2.776)	0.183

HR hazard ratio, CI confidence interval, ALT alanine aminotransferase, AFP alpha-fetoprotein, HCV hepatitis C virus, SVR sustained virologic response, NR non-response, EOT end of treatment

Discussion

Until now, there has been little information on the relationship between the changes in serum AFP level associated with IFN therapy and the development of HCC, although several reports have described the changes in serum AFP level during IFN therapy. Consistent with our results, Murashima et al. [21] have already reported that IFN reduces the serum

AFP level during treatment regardless of virologic response. Nomura et al. [23] also showed a significant decrease in serum AFP level during low-dose and long-term IFN monotherapy in 44 aged patients with CHC; SVR status was achieved in only two. In contrast, Chen et al. [22] demonstrated a significant decrease in serum AFP level during and after IFN therapy in an SVR group ($n = 26$), whereas no significant decrease in serum AFP level was observed in the non-SVR group ($n = 9$). The association of changes in serum AFP level during IFN therapy with the development of HCC was not examined in these studies.

We demonstrated that the serum AFP level in patients with CHC decreased during IFN therapy regardless of virologic response, and that, by univariate Cox's regression analysis, AFP level at EOT was a close-to-significant prognostic factor affecting the development of HCC. Moreover, a significantly higher cumulative incidence of HCC was seen in patients with increased serum AFP levels at EOT by log-rank test. Our results suggest that serum AFP level at EOT is a possible predictor of HCC development after IFN therapy and will be useful for considering additional treatments, such as long-term administration of low-dose IFN, to prevent HCC development.

AFP is a fetal glycoprotein produced by the yolk sac and fetal liver. Following birth, AFP is replaced by albumin as the major serum protein, and then the AFP level decreases rapidly. Consequently, AFP is not detectable in the sera of adults. However, production of AFP in adults occurs during liver regeneration and hepatocarcinogenesis. Therefore,

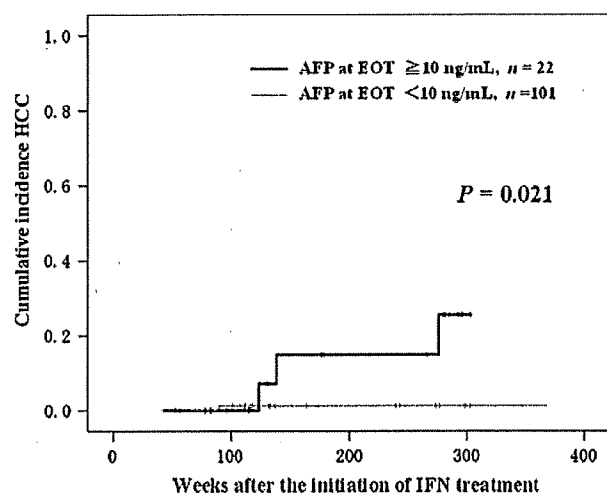


Fig. 1 Cumulative incidence HCC in two groups (serum AFP level $< 10\text{ ng/ml}$, $n = 101$; vs. $\geq 10\text{ ng/ml}$, $n = 22$) at the end of treatment. The high AFP group had a significantly higher incidence of HCC ($P = 0.021$). AFP alpha-fetoprotein, EOT end of treatment

AFP has been used as a diagnostic marker for HCC [14, 15], although AFP levels are sometimes elevated in patients with chronic hepatitis and cirrhosis who have no evidence of HCC [16–18]. The reported prevalence of elevated AFP in patients with CHC varies from 10 to 43% [18, 19, 26, 27]. This wide discrepancy among previous reports is probably due to differences in the definition of elevated AFP value, as well as in patient population, ethnicity, and sample size. Chu et al. [18] reported that 28.7% (33/115) of patients with CHC had an elevated serum AFP level (≥ 12 ng/ml). Chen et al. [22] also reported that an elevated AFP level (≥ 10 ng/ml) was seen in 33% (41/123) of patients with CHC. Our results revealed that 27.0% (72/263) of patients with CHC had an elevated serum AFP level (≥ 10 ng/ml). This result is consistent with those of previous reports from Asian countries [18, 22].

Our multivariate logistic regression analysis revealed that hepatic inflammation and serum albumin level were significant independent factors associated with serum AFP level at baseline. Consistent with our results, several reports have shown that serum AFP level is correlated with the degree of hepatic inflammation in liver biopsy specimens [20]. Moreover, a number of studies have shown a relationship between elevated serum AFP level and decreased serum albumin level in patients with CHC [18, 21]. One of the reasons for these associations is simply the fact that advanced liver damage results in decreased production of albumin. In addition, reciprocal changes in albumin and AFP gene transcription during liver regeneration may account for the relationship between elevated serum AFP level and decreased serum albumin level [28, 29]. Although advanced hepatic fibrosis has been reported as the most common cause of serum AFP elevation [18, 22], our multivariate analysis was unable to demonstrate any association between serum AFP level and advanced hepatic fibrosis. In the present study, however, stage 3–4 fibrosis was significantly more frequent in the group showing elevated serum AFP levels. Moreover, the association of an elevated AFP level with a low albumin level may be secondary to a decrease of liver synthetic function due to cirrhosis. We speculated that one of the reasons for the discrepancy might be the fact that only 49 patients with an elevated serum AFP level underwent liver biopsy.

Many reports have described elevated serum AFP level, together with age, gender, and liver histologic stage, as independent risk factors for HCC in patients with CHC [4, 5]. Our results revealed that the hepatic inflammation was a significant independent factor associated with serum AFP level at baseline. These results indicate that active inflammation in the liver is one of the most important factors influencing serum AFP level, and suggest that this persistent inflammation results in hepatocarcinogenesis. In contrast, a number of reports have revealed that AFP itself

can enhance the proliferation of mammalian tumor cells. Wang and Xu et al. [30] have reported that human AFP increases the proliferation of mouse H-22 hepatoma cells, and that the growth stimulatory activity of AFP could be abolished by anti-AFP antibodies. Wang and Xie et al. [31] showed that AFP enhances the proliferation of human HCC cells, and they suggested that this effect was mediated by an AFP/receptor autocrine pathway. In addition, AFP stimulates the expression of some oncogenes in human HCC cells and has immunosuppressive activity [32, 33]. These results suggest that not only hepatic inflammation accompanied by elevation of serum AFP level, but AFP itself, is associated with hepatocarcinogenesis.

Our univariate Cox's regression analysis revealed that serum AFP level at EOT was a close-to-significant prognostic variable affecting the development of HCC, although serum AFP level before treatment was not. Consistent with these results, Arase et al. [24] reported that in patients with CHC who were treated with IFN or herbal medicine, elevated serum AFP level (>10 ng/ml) after treatment was a statistically significant independent prognostic factor in the development of HCC. There are some factors other than AFP that could have a potential association with the development of HCC. Several studies have implicated obesity, diabetes, intake of alcohol, and smoking, as risk factors for HCC [34–37]. However, we did not investigate these factors in the present retrospective study. In addition, we were unable to exclude the possibility of occult HBV infection in patients with HCC, although all patients were negative for HBsAg.

In conclusion, our results revealed that serum AFP level decreased during IFN therapy regardless of the virologic response. In addition, serum AFP level at EOT was a close-to-significant prognostic factor for HCC development. These results suggest that serum AFP level at EOT should be utilized in making decisions on additional therapeutic options, such as long-term administration of low-dose IFN, to reduce the risk of HCC development in patients in whom HCV elimination by routine IFN therapy has failed. We realize our study has some limitations as follows: first, it was a retrospective analysis; second, various kinds of IFN were used; and third, the duration of IFN therapy varied from 24 to 90 weeks. Further prospective studies in which patients are treated by the current standard protocol-Peg-IFN with ribavirin-for a set period of time will confirm the relationship between changes in serum AFP level associated with IFN therapy and the development of HCC.

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