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.

REFERENCES

1 **Cowling DC**, Mackay IR, Taft LI. Lupoid hepatitis. *Lancet* 1956; **271**: 1323-1326

2 **Park SZ**, Nagorney DM, Czaja AJ. Hepatocellular carcinoma in autoimmune hepatitis. *Dig Dis Sci* 2000; **45**: 1944-1948

3 **Floreani A**, Niro G, Rosa Rizzotto E, Antoniazzi S, Ferrara F, Carderi I, Baldo V, Premoli A, Olivero F, Morello E, Durazzo M. Type I autoimmune hepatitis: clinical course and outcome in an Italian multicentre study. *Aliment Pharmacol Ther* 2006; **24**: 1051-1057

4 **Kaplan MM**. Primary biliary cirrhosis. *N Engl J Med* 1996; **335**: 1570-1580

5 **Loof L**, Adami HO, Sparen P, Danielsson A, Eriksson LS, Hultcrantz R, Lindgren S, Olsson R, Prytz H, Ryden BO. Cancer risk in primary biliary cirrhosis: a population-based study from Sweden. *Hepatology* 1994; **20**: 101-104

6 **Turissini SB**, Kaplan MM. Hepatocellular carcinoma in primary biliary cirrhosis. *Am J Gastroenterol* 1997; **92**: 676-678

7 **Floreani A**, Biagini MR, Chiaramonte M, Milani S, Surrenti C, Naccarato R. Incidence of hepatic and extra-hepatic malignancies in primary biliary cirrhosis (PBC). *Ital J Gastroenterol* 1993; **25**: 473-476

8 **Miyake Y**, Iwasaki Y, Terada R, Okamaoto R, Ikeda H, Makino Y, Kobashi H, Takaguchi K, Sakaguchi K, Shiratori Y. Persistent elevation of serum alanine aminotransferase levels leads to poor survival and hepatocellular carcinoma development in type 1 autoimmune hepatitis. *Aliment Pharmacol Ther* 2006; **24**: 1197-1205

9 **Floreani A**, Baragiotta A, Leone MG, Baldo V, Naccarato R. Primary biliary cirrhosis and hepatitis C virus infection. *Am J Gastroenterol* 2003; **98**: 2757-2762

10 **Maruyama H**, Iwamura S, Watanabe S. Three autopsy cases of hepatocellular carcinoma arising from primary biliary cirrhosis (PBC). *Okayama Igakkai zasshi* 1990; **102**: 261-263

11 **Shibuya A**, Tanaka K, Miyakawa H, Shibata M, Takatori M, Sekiyama K, Hashimoto N, Amaki S, Komatsu T, Morizane T. Hepatocellular carcinoma and survival in patients with primary biliary cirrhosis. *Hepatology* 2002; **35**: 1172-1178

12 **Krasner N**, Johnson PJ, Portmann B, Watkinson G, Macsween RN, Williams R. Hepatocellular carcinoma in primary biliary cirrhosis: report of four cases. *Gut* 1979; **20**: 255-258

13 **Caballeria L**, Pares A, Castells A, Gines A, Bru C, Rodes J. Hepatocellular carcinoma in primary biliary cirrhosis: similar incidence to that in hepatitis C virus-related cirrhosis. *Am J Gastroenterol* 2001; **96**: 1160-1163

14 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938

15 **Ikai I**, Arii S, Okazaki M, Okita K, Omata M, Kojiro M, Takayasu K, Nakanuma Y, Makuuchi M, Matsuyama Y, Monden M, Kudo M. Report of the 17th Nationwide Follow-up Survey of Primary Liver Cancer in Japan. *Hepatol Res* 2007; **37**: 676-691

16 **Wang KK**, Czaja AJ. Hepatocellular carcinoma in corticosteroid-treated severe autoimmune chronic active hepatitis. *Hepatology* 1988; **8**: 1679-1683

17 **Miyake Y**, Sakaguchi K, Tanaka H, Nakamura S, Kobayashi Y, Fujioka S, Iwasaki Y, Shiratori Y. Development of hepatocellular carcinoma in a woman with HBV- and HCV-negative autoimmune hepatitis with unsatisfactory response to Corticosteroid. *Intern Med* 2005; **44**: 949-953

18 **Shimizu A**, Koyama M, Okuda Y, Takase K, Nakano T, Tameda Y. Hepatocellular carcinoma in primary biliary cirrhosis: a case report and review of the Japanese literature. *Hepatogastroenterology* 1998; **45**: 2352-2355

19 **Pollicino T**, Squadrito G, Cerenzia G, Cacciola I, Raffa G, Craxi A, Farinati F, Missale G, Smedile A, Tiribelli C, Villa E, Raimondo G. Hepatitis B virus maintains its pro-oncogenic properties in the case of occult HBV infection. *Gastroenterology* 2004; **126**: 102-110

20 **Pollicino T**, Raffa G, Costantino L, Lisa A, Campello C, Squadrito G, Levrero M, Raimondo G. Molecular and functional analysis of occult hepatitis B virus isolates from patients with hepatocellular carcinoma. *Hepatology* 2007; **45**: 277-285

21 **Ryder SD**, Koskinas J, Rizzi PM, McFarlane IG, Portmann BC, Naoumov NV, Williams R. Hepatocellular carcinoma complicating autoimmune hepatitis: role of hepatitis C virus. *Hepatology* 1995; **22**: 718-722

22 **Nakai T**, Shiroishi O, Kawabe T. Prognosis and positive rate of HBV-DNA in liver tissue of the operative cases of hepatocellular carcinoma with non-B, non-C hepatitis. 40th Annual Meeting of Liver Cancer Study Group of Japan, 2004: 150

23 **Kubo S**, Oba K, Hirohashi K, Tanaka H, Shuto T, Takemura S, Yamamoto T, Tamori A, Enomoto M, Nishiguchi S. Alcohol abuse as an etiologic factor for hepatocellular carcinoma in Japan. *Hepatol Res* 2005; **31**: 73-78

24 **Yamada N**, Nomoto M, Ichida T, Aoyagi Y. Primary biliary cirrhosis -diagnosis of condition, therapy, prognosis -2. Malignancy with primary biliary cirrhosis. Tokyo: Chugai igakusha, 2003: 68-75

25 **Miura Y**, Kaneda Y, Toyama J, Katayama S, Takigawa Y, Nakadate I, Saito Y, Yamazaki K, Hanme T, Yoshida T, Kashiwabara N, Suzuki K, Sato S, Masuda T. Icteric type hepatoma in a patient with primary biliary cirrhosis. *Kan Tan Sui* 1994; **28**: 129-134

26 **Nijhawan PK**, Therneau TM, Dickson ER, Boynton J, Lindor KD. Incidence of cancer in primary biliary cirrhosis: the Mayo experience. *Hepatology* 1999; **29**: 1396-1398

27 **Suzuki A**, Lymp J, Donlinger J, Mendes F, Angulo P, Lindor K. Clinical predictors for hepatocellular carcinoma in patients with primary biliary cirrhosis. *Clin Gastroenterol Hepatol* 2007; **5**: 259-264

28 **Watanabe M**, Moritani M, Hamamoto S, Uchida Y, Ishihara S, Adachi K, Kinoshita Y. Hepatocellular carcinoma complicating HCV-negative autoimmune hepatitis without corticosteroid therapy. *J Clin Gastroenterol* 2000; **30**: 445-446

29 **Ogata S**, Maeyama S, Tobe N, Koike J, Uchikoshi T, Takahashi Y, Okuse C, Osada T, Tomoe M, Hayashi T, Suzuki M, Iino S. An autopsy case of hepatocellular carcinoma arising from autoimmune Hepatitis. *Kanzo* 2000;

41: 48-52

30 **Takenawa H**, Sakuma I, Yamaoka K, Yamane M, Shaura K, Sakai H, Ikeda T, Marumo F, Sato C. Hepatocellular carcinoma complicating primary biliary cirrhosis--a case report and a review of the literature. *Nippon Shokakibyo Gakkai Zasshi* 1994; **91**: 2127-2132

31 **Watanabe H**, Danjo N, Shijo H. A case report of viral marker negative hepatocellular carcinoma (HCC) associated with primary biliary cirrhosis (PBC) stage-II. *Kanzo* 2000; **41**: 413-418

32 **El-Serag HB**, Richardson PA, Everhart JE. The role of diabetes in hepatocellular carcinoma: a case-control study among United States Veterans. *Am J Gastroenterol* 2001; **96**: 2462-2467

33 **Watanabe T**, Aikawa K, Kanefuji T, Yamazaki K, Hirono H, Hasegawa K, Soga K, Shibasaki K, Umezu H, Nomoto M. Pelioid-type hepatocellular carcinoma with numerous eosinophilic infiltrations in a patient with primary biliary cirrhosis. *Hepatol Res* 2008; **38**: 421-427

34 **Adachi E**, Maeda T, Kajiyama K, Kinukawa N, Matsumata T, Sugimachi K, Tsuneyoshi M. Factors correlated with portal venous invasion by hepatocellular carcinoma: univariate and multivariate analyses of 232 resected cases without preoperative treatments. *Cancer* 1996; **77**: 2022-2031

S- Editor Cheng JX **L- Editor** Webster JR **E- Editor** Yin DH

Digestive Diseases and Sciences

10.1007/s10620-008-0611-5

Original Article

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

So Kurita[1], Shogo Ohkoshi[1] ✉, Masahiko Yano[1], Kazuhide Yamazaki[1], Kenta Suzuki[1], Yohei Aoki[1], Yasunobu Matsuda[1], Toshifumi Wakai[2], Yoshio Shirai[2], Takafumi Ichida[3] and Yutaka Aoyagi[1]

(1) Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences of Niigata University, 1-754, Asahimachi-Dori, Niigata-city 951-8122, Japan

(2) First Department of Surgery, Graduate School of Medical and Dental Sciences of Niigata University, 1-754, Asahimachi-Dori, Niigata-city 951-8122, Japan

(3) Division of Gastroenterology and Hepatology, Juntendo University School of Medicine and Juntendo University Hospital of Shizuoka, Shizuoka, Japan

✉ **Shogo Ohkoshi**
Email: okoshi@med.niigata-u.ac.jp

Received: 23 March 2007 **Accepted:** 20 June 2007 **Published online:** 19 November 2008

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 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.

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

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.

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].

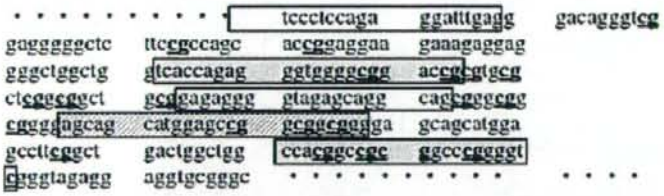

Fig. 1 Locations of primer and probe sequences in the CpG island of the *p16* ^{INK4A} gene promoter used in the experiments. CpG sites possibly methylated are represented as bold and underlined. Primer sequences common to the SYBR green and the TaqMan probe methods are presented in shaded boxes. Probe sequence for methylated DNA-specific TaqMan PCR and primer sequences for the bisulfite sequence method (no CpG site) are presented in hatched and in open boxes, respectively

---

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 μl PCR reactions contained 0.5 μM of each primer, 2 μl Light Cycler-DNA Master SYBR Green I (Roche), 2 mM $MgCl_2$, 1 μl dimethyl sulfoxide (DMSO), and 2 μl 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]:

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

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′-AGTAGTATGGAGTCGGCGGCGGG-3′ TAMRA for the methylated allele [19] and 6FAM5′-AGGTAGTGGGTGGTGGGGAGTAGTATGGAGTTG-3′TAMRA for the unmethylated allele. PCR was carried out in a reaction volume of 20 μl. Each PCR reaction contained 0.2 μM of each primer, 0.1 μM probe (Applied Biosystems, Foster City, CA), 4 μl TaqMan Master (Roche), and 5 μl 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 μl PCR reaction contained 0.2 μM of each primer, 2 mM $MgCl_2$, 4 μl 10 mM dNTP, 1 μl DMSO, 0.75 units Hot Start PCR (Takara), and 2 μl 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′-GACCTGGCTGAGGAGCTG-3′ (sense) and 5′-TTCAATCGGGGATGTCTGA-3′ (antisense) for *p16* mRNA, 5′-ATTGGCAATGAGCGGTTC-3′ (sense) and 5′-GGATGCCACAGGACTCCAT-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.

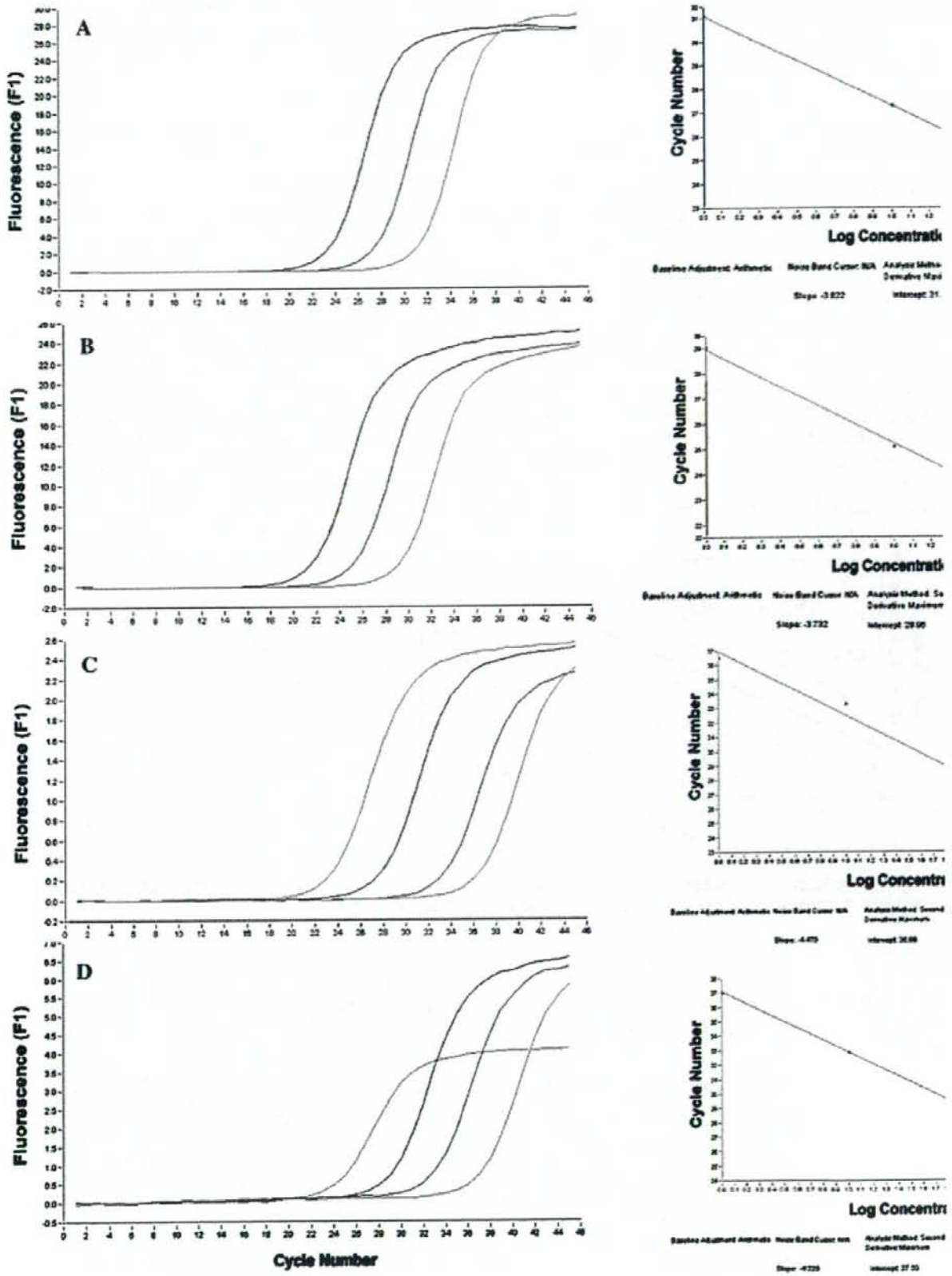
A
B
C
D
Fluorescence (F1)
Cycle Number
Log Concentrati

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)

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.

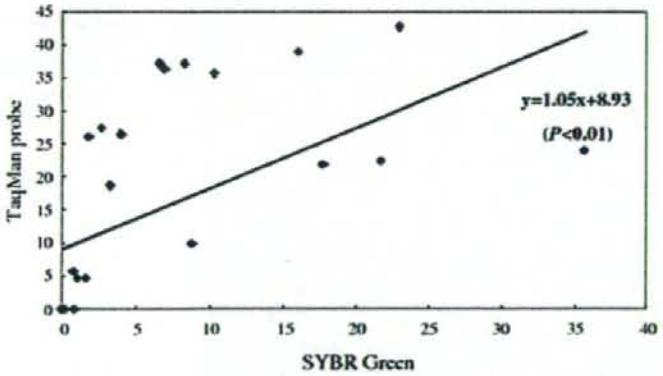

Fig. 3 Correlation of MIs between the SYBR green (horizontal axis) and TaqMan probe (vertical axis) methods using DNA from HCC tissues. A significant correlation ($P < 0.05$) was obtained

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).

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%)*

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

MIs Among Clinicopathological Parameters

MIs 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, MIs 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 ± SD) and 2.242 ± 2.057, respectively (NS).

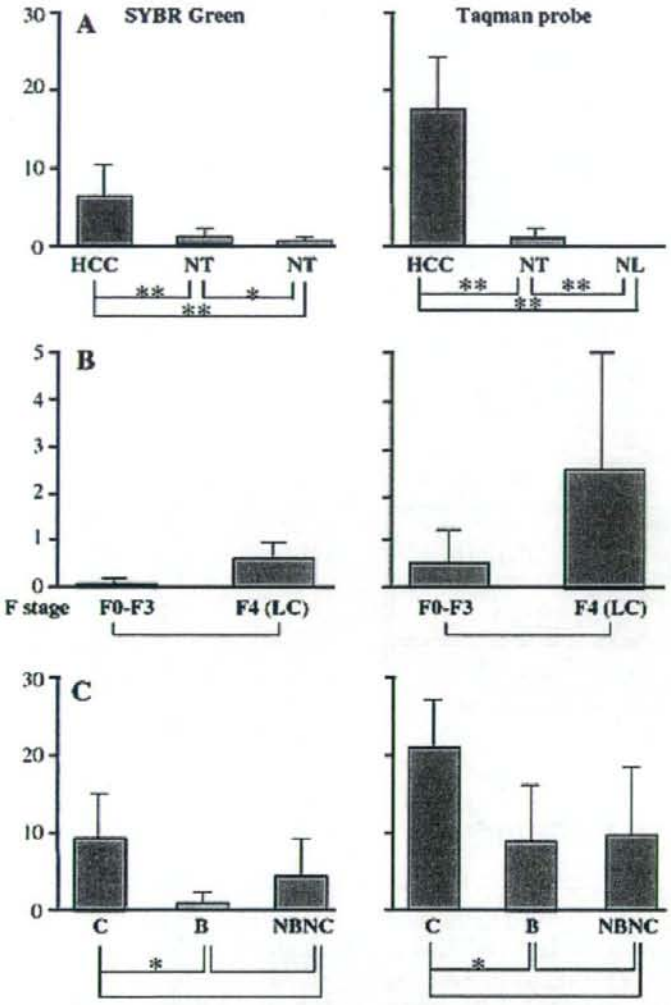

Fig. 4 Results of MIs (mean ± SD) in the liver tissues by the SYBR green (left) and TaqMan probe (right) methods. **a** Comparison among HCC, nontumorous liver (NT), and normal liver (NL). There were significant differences between each set of comparisons (*$P < 0.05$, **$P < 0.01$). **b** Comparison 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 (*$P < 0.05$)

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, 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).

GA GGATTTGAGG GACAGGGTCG GAGGGGGCTC TTCCGCCAGC ACCGGAGGAA

GAAAGAGGAG GGGCTGGCTG GTCACCAGAG GGTGGGGCGG ACCGCGTGCG

CTCGGCGGCT GCGGAGAGGG GTAGAGCAGG CAG

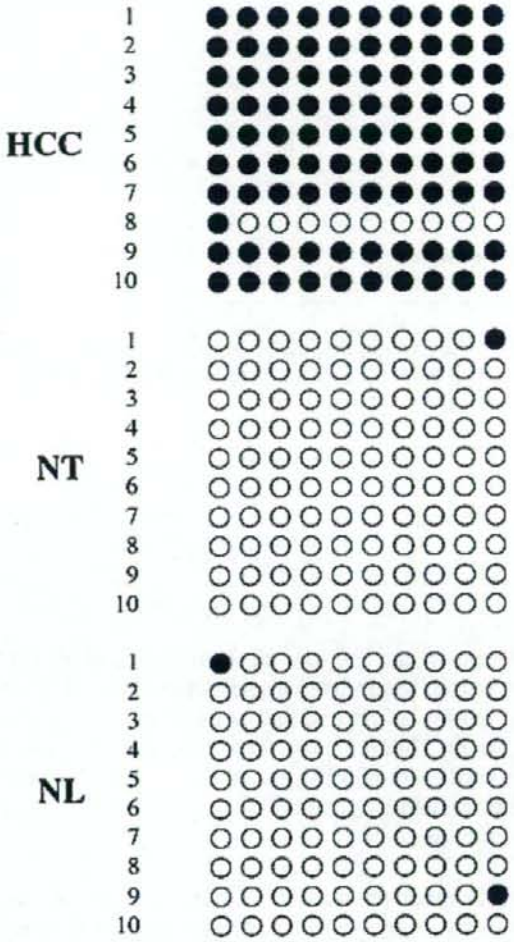

Fig. 5 Results of bisulfite sequencing and cloning. Ten CpG sites (bold face letter) are present in the sequence amplified. Each circle corresponds to one site. The methylation status of CpG sites in one HCC (upper), nontumorous liver (middle), and normal liver (lower) was analyzed. The results of all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

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.

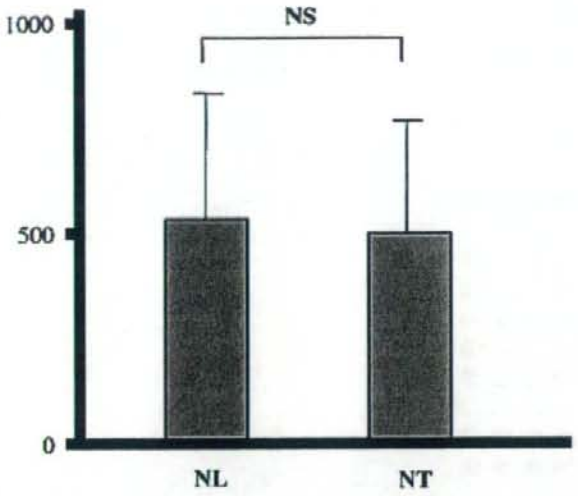

Fig. 6 Comparison of quantification of mRNA of the *p16* gene between nontumorous and normal liver tissues. The *p16* mRNA level was quantified by the ratio to the level of β-actin mRNA. The results are shown as mean ± SD. There was no significant difference

Discussion

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

References

1. Okuda K. Hepatocellular carcinoma: recent progress. *Hepatology*. 1992;15:948–963.
PubMed | cross ref | ChemPort

2. Shiratori Y, Yoshida H, Omata M. Different clinicopathological features of hepatocellular carcinoma in relation to causative agents. *J Gastroenterol*. 2001;36:73–78.
PubMed | [SpringerLink] | ChemPort

3. Feitelson MA, Sun B, Satiroglu Tufan NL, Liu J, Pan J, Lian Z. Genetic mechanisms of hepatocarcinogenesis. *Oncogene*. 2002;21:2593–2604.
PubMed | cross ref | ChemPort

4. Farazi PA, DePinho RA. Hepatocellular carcinoma pathogenesis: from genes to environment. *Nat Rev Cancer*. 2006;6:674–687.
PubMed | cross ref | ChemPort

5. Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res*. 1998;72:141–196.
PubMed | cross ref | ChemPort

6. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415–428.
PubMed | cross ref | ChemPort

7. Bird A. The essentials of DNA methylation. *Cell*. 1992;70:5–8.
PubMed | cross ref | ChemPort

8. Belinsky SA, Nikula KJ, Palmisano WA, et al. Aberrant methylation of *p16* ^{INK4a} is an early event in lung cancer and a potential biomarker for early diagnosis. *Proc Natl Acad Sci USA*. 1998;95:11891–11896.
PubMed | cross ref | ChemPort

9. Hsieh CJ, Klump B, Holzmann K, Brochard F, Gregor M, Porschen R. Hypermethylation of the p16INK4a promoter in colectomy specimens of patients with long-standing and extensive ulcerative colitis. *Cancer Res*. 1998;58:3942–3945.
PubMed | ChemPort

10. Klump B, Hsieh CJ, Holzmann K, Gregor M, Porschen R. Hypermethylation of the CDKN2/p16 promoter during neoplastic progression in Barrett's esophagus. *Gastroenterology*. 1998;115:1381–1386.
PubMed | cross ref | ChemPort

11. Wilentz RE, Geradts J, Maynard R, et al. Inactivation of the p16 (INK4A) tumor-suppressor gene in pancreatic duct lesions: loss of intranuclear expression. *Cancer Res*. 1998;58:4740–4744.
PubMed | ChemPort

12. Lee JH, Park SJ, Abraham SC, et al. Frequent CpG island methylation in precursor lesions and early gastric adenocarcinomas. *Oncogene*. 2004;23:4646–4654.
PubMed | cross ref | ChemPort

13. Kaneto H, Sasaki S, Yamamoto H, et al. Detection of hypermethylation of the *p16* ^{INK4A} gene promoter in

chronic hepatitis and cirrhosis associated with hepatitis B or C virus. *Gut*. 2001;48:372–377.
PubMed CrossRef ChemPort

14. Lee S, Lee HJ, Kim JH, Lee HS, Jang JJ, Kang GH. Aberrant CpG island hypermethylation along multistep hepatocarcinogenesis. *Am J Pathol*. 2003;163:1371–1378.
PubMed ChemPort

15. Di Gioia S, Bianchi P, Destro A, et al. Quantitative evaluation of *RASSF1A* methylation in the non-lesional, regenerative and neoplastic liver. *BMC Cancer*. 2006;6:89–100.
PubMed CrossRef

16. Lo YM, Wong IH, Zhang J, Tein MS, Ng MH, Hjelm NM. Quantitative analysis of aberrant *p16* methylation using real-time quantitative methylation-specific polymerase chain reaction. *Cancer Res*. 1999;59:3899–3903.
PubMed ChemPort

17. Hoque MO, Begum S, Topaloglu O, et al. Quantitative detection of promoter hypermethylation of multiple genes in the tumor, urine, and serum DNA of patients with renal cancer. *Cancer Res*. 2004;64:5511–5517.
PubMed CrossRef ChemPort

18. Lehmann U, Berg-Ribbe I, Wingen LU, et al. Distinct methylation patterns of benign and malignant liver tumors revealed by quantitative methylation profiling. *Clin Cancer Res*. 2005;11:3654–3660.
PubMed CrossRef ChemPort

19. Harden SV, Tokumaru Y, Westra WH, et al. Gene promoter hypermethylation in tumors and lymph nodes of stage I lung cancer patients. *Clin Cancer Res*. 2003;9:1370–1375.
PubMed ChemPort

20. Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature*. 1994;368:753–756.
PubMed CrossRef ChemPort

21. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature*. 1993;366:704–707.
PubMed CrossRef ChemPort

22. Baek MJ, Piao Z, Kim NG, et al. p16 is a major inactivation target in hepatocellular carcinoma. *Cancer*. 2000;89:60–68.
CrossRef ChemPort

23. Matsuda Y, Ichida T, Matsuzawa J, Sugimura K, Asakura H. *p16 INK4A* is inactivated by extensive CpG methylation in human hepatocellular carcinoma. *Gastroenterology*. 1999;116:394–400.
PubMed CrossRef ChemPort

24. Kondo Y, Kanai Y, Sakamoto M, Mizokami M, Ueda R, Hirohashi S. Genetic instability and aberrant DNA methylation in chronic hepatitis and cirrhosis—a comprehensive study of loss of heterozygosity and microsatellite instability at 39 loci and DNA hypermethylation on 8 CpG islands in microdissected specimens from patients with hepatocellular carcinoma. *Hepatology*. 2000;32:970–979.
PubMed CrossRef ChemPort

25. Roncalli M, Bianchi P, Bruni B, et al. Methylation framework of cell cycle gene inhibitors in cirrhosis and associated hepatocellular carcinoma. *Hepatology*. 2002;36:427–432.

PubMed CrossRef ChemPort

26. Li X, Hui AM, Sun L, et al. p16^{INK4A} hypermethylation is associated with hepatitis virus infection, age, and gender in hepatocellular carcinoma. *Clin Cancer Res*. 2004;10:7484–7489.
PubMed CrossRef ChemPort

27. Qin Y, Liu JY, Li B, Sun ZL, Sun ZF. Association of low p16INK4a and p15INK4b mRNAs expression with their CpG islands methylation with human hepatocellular carcinogenesis. *World J Gastroenterol*. 2004;10:1276–1280.
PubMed ChemPort

28. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Sheuer PJ. Classification of chronic hepatitis: diagnosis, grading, and staging. *Hepatology*. 1994;19:1513–1520.
PubMed ChemPort

29. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA*. 1996;93:9821–9826.
PubMed CrossRef ChemPort

30. Kim BN, Yamamoto H, Ikeda K, et al. Methylation and expression of p16^{INK4A} tumor suppressor gene in primary colorectal cancer tissues. *Int J Oncol*. 2005;26:1217–1226.
PubMed ChemPort

31. Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res*. 1994;22:2990–2997.
PubMed CrossRef ChemPort

32. Issa JP. CpG-island methylation in aging and cancer. *Curr Top Microbiol Immunol*. 2000;249:101–118.
PubMed ChemPort

33. Waki T, Tamura G, Sato M, Motoyama T. Age-related methylation of tumor suppressor and tumor-related genes: an analysis of autopsy samples. *Oncogene*. 2003;22:4128–4133.
PubMed CrossRef ChemPort

34. Narimatsu T, Tamori A, Koh N, et al. *p16* promoter hypermethylation in human hepatocellular carcinoma with or without hepatitis virus infection. *Intervirology*. 2004;47:26–31.
PubMed CrossRef ChemPort

35. Muhlisch J, Bajanowski T, Rickert CH, et al. Frequent but borderline methylation of *p16* ^{INK4A} and *TIMP3* in medulloblastoma and sPNET revealed by quantitative analyses. *J Neurooncol*. 2007;83:17–29. (online first).
PubMed SpringerLink ChemPort

Dig Dis Sci
DOI 10.1007/s10620-008-0642-y

ORIGINAL ARTICLE

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

Yasushi Tamura · Satoshi Yamagiwa · Yohei Aoki · So Kurita · Takeshi Suda · Shogo Ohkoshi · Minoru Nomoto · Yutaka Aoyagi · The Niigata Liver Disease Study Group

Received: 22 September 2008 / Accepted: 12 November 2008

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

Y. Tamura (✉) · S. Yamagiwa · Y. Aoki · S. Kurita · T. Suda · S. Ohkoshi · M. Nomoto · Y. Aoyagi
Division of Gastroenterology and Hepatology, Graduate School of Medical and Dental Sciences, Niigata University, 757 Asahimachi-dori 1, Chuo-ku, Niigata 951-8510, Japan
e-mail: ytamura@med.niigata-u.ac.jp

The Niigata Liver Disease Study Group
The Niigata Liver Disease Study Group, Niigata, Japan

Published online: 18 December 2008

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-IFNα-2a, recombinant IFNα-2b, consensus IFNα, and natural IFNα. Of the 263 patients, 114 (43.3%) were treated with Peg-IFNα-2b (1.5 μg/kg weekly) and ribavirin (600–1,000 mg daily); 71 (27.0%) were treated with recombinant IFNα-2b (6–10 MIU three times weekly) and ribavirin (600–1,000 mg daily); 61 (23.2%) were treated with Peg-IFNα-2a (90–180 μg weekly); ten (3.8%) were treated with consensus IFNα (9–18 MIU three times weekly); and seven (2.7%) were treated with natural IFNα (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.