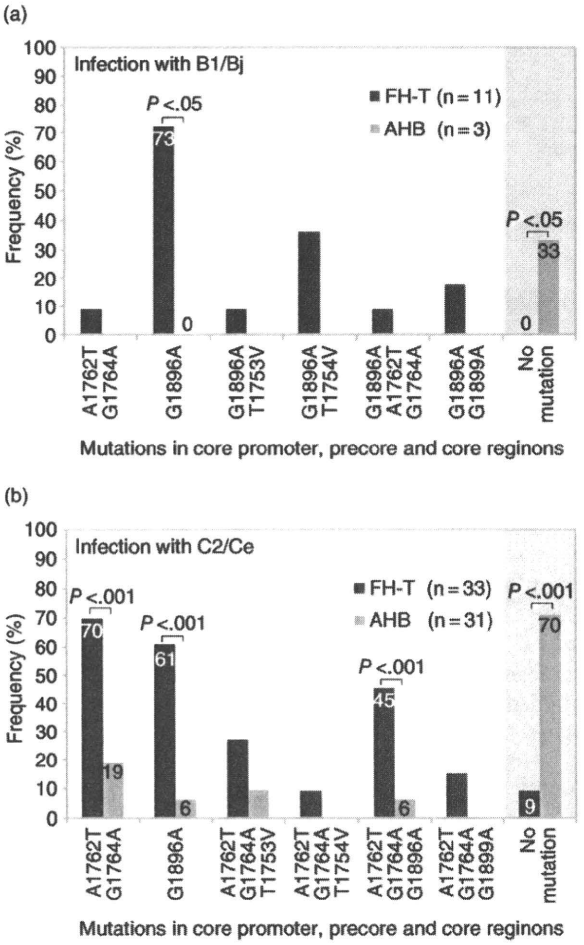

Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs. 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	*P*-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C ($n = 12$)	AE-C ($n = 12$)	Differences *P*-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.[31] Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.[13–16] Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.[19–21] It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.[22]

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,[32] and downregulated by the A1762T/G1764A double mutation at the transcription level.[33,34] Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.[35] Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,[35] the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tollerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.[36,37] In the absence or decrease of HBeAg, therefore, hosts would mount vigor cytotoxic T-cell responses to core epitopes excessively presented on hepatocytes, and develop severe liver injuries culminating in FHB.[38]

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case–control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.[8] These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;[13–16] these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.[18] Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient

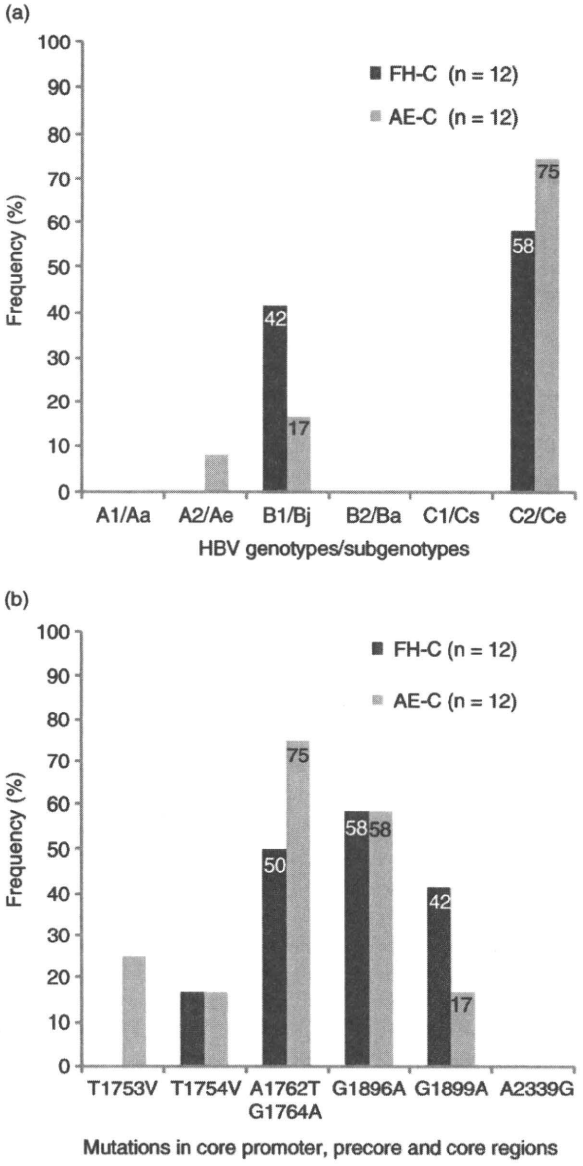

Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

HBV infection (Table 2). In particular, G1896A mutation was the most important factor associated with the development of FHB. Host responses, represented by T.bil, contributed to the development of FHB as well.

As for HBV genotypes, B1/Bj alone was significantly more frequent in the FH-T patients in univariate analysis. In the patients infected with B1/Bj, G1896A was more frequent in those with FH-T than AHB. In *in vitro* replication analysis, Ozasa *et al.*[15] observed extremely high expressions of intra- and extracellular HBV DNA in culture transfected with an HBV clone of B1/Bj genotype having the G1896A mutation; a high replication would be induced by this pre-core mutation for the induction of FHB. Our clinical results stand in support of this *in vitro* analysis. Taken altogether, chances for developing severe acute or FH would be high in the patients with acute hepatitis who are infected with HBV/B1 having the pre-core mutation. By contrast, in patients infected with C2/Ce, G1896A or A1762T/G1764A, or both was much more frequent in the FH-T patients than AHB. Of note, the co-occurrence of G1896A and A1762T/G1764A mutations was invariably accompanied by either FHB or acute severe hepatitis B in this study. Hence, these pre-core and core-promoter mutations might have addictive or synergetic effects for exacerbating hepatitis, when they emerge in the patients infected with C2/Ce. Such high-risk patients deserve special care and surveillance for signs and symptoms of fulminant or severe acute hepatitis B.

In the present study, serum levels of HBV DNA were significantly higher in the patients with FH-T than AHB. High serum levels of HBV DNA have been reported in patients with FHB;[39] they are followed by rapid decrease as the sequel of virus elimination operated by vigorous immune responses. Because of rapid and extensive elimination of HBV by the host immune system, HBV DNA in serum, in general, has decreased to low levels in patients with FHB at the presentation.[40] HBV DNA levels may be subject to the time that has elapsed from the onset of hepatitis to its measurement.[39] Also, serum levels of core protein (the product of the C gene) closely correlate with serum HBV DNA levels in patients with hepatitis B,[27] and they were compared between the FH-T patients and AHB. The core protein was determined by the newly developed CLEIA method; it is much easier and less expensive than the determination of HBV DNA. The level of core protein has turned out to be marginally higher in the FH-T patients than AHB (Table 1), and therefore might not contribute to an early diagnosis of FHB by transient infection.

Fulminant hepatitis B by AE of ASC is assumed as a different clinical condition from FHB by transient HBV infection. In this study, as there was no case–control study on virological factors associated with FHB for the patients with AE of ASC, we also attempted to identify virological factors associated with the development of FHB in the 12 FH-C and the 12 AE-C patients who were

matched for age as well as sex. Disappointingly, no differences of virological factors such as HBV genotypes and pre-core mutations, which were strongly associated with the development of FHB by transient infection, were found between the FH-C and AE-C patients (Fig. 3a,b). Furthermore, there were also no significant differences about HBeAg-positive rate and the levels of serum HBV DNA or core protein (Table 3), suggesting that several host factors may play a more important role in the development of FHB in ASC instead of virological factors. In this case–control study, however, there seems to be some problems: a small number of patients, different duration of HBV infection, different clinical stage (ASC or CHB) at the onset of AE, and HBV quasispecies complexity. Further investigations are needed to identify factors associated with FHB precipitating in asymptomatic HBV carriers.

In conclusion, virological factors associated with enhancement of viral replication seemed to be important for the development of FHB in the patients by transient HBV infection. But no virological factors were identified for differentiation of the FH-C patients from the AE-C patients. Hence, the pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

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Interleukin-18 promoter polymorphisms and the disease progression of Hepatitis B virus-related liver disease

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In this study, we aimed to explore whether interleukin-18 (IL-18) gene-promoter polymorphisms are associated with the outcome of hepatitis B virus (HBV) infection. In all, 204 chronically HBV-infected patients were recruited in this study. Of the 204 HBV-infected patients, 43 were considered to be inactive HBV carriers based on the sustained normalization of serum alanine aminotransferase (ALT) together with seropositivity for the antibody to hepatitis B e-antigen (anti-HBe). A total of 161 patients were found to have chronic progressive liver disease, which included cirrhosis. In these HBV-infected patients, the frequencies of AA genotype of IL-18 gene-promoter polymorphisms at position -607 and C allele at position -137 were significantly higher in inactive HBV carriers compared with those in patients with chronic progressive liver disease. These polymorphisms of the IL-18 promoter regions (-607 and -137) could be associated with different outcomes of HBV infection. (Translational Research 2009;153:91–96)

Abbreviation: ALT = alanine aminotransferase; CI = confidence interval; CPLD = chronic progressive liver disease; anti-HBe = hepatitis B e-antigen; HBsAg = hepatitis B surface antigen; HBV = hepatitis B virus; HCC = hepatocellular carcinoma; IFN-γ = interferon-γ; IL-18 = interleukin-18; OR = odds ratio; PCR = polymerase chain reaction; SNP = single nucleotide polymorphism; Th_1 = T-helper type 1; TMA = transcription-mediated amplification

Hepatitis B virus (HBV) infection is one of the most prevalent chronic viral diseases in the world.[1] Most individuals with persistent HBV infection develop chronic hepatitis, which can progress to cirrhosis or hepatocellular carcinoma (HCC).[2] Increasing evidence indicates that genetic factors influence the natural history of chronic liver diseases.[3] Furthermore, several recent advances concerning the polymorphism of cytokines that control the host response to the virus could play an important role in determining the outcome of HBV infection.[4] Previous studies have shown that the capacity of cytokine production varies among individuals and correlates with single nucleotide polymorphisms (SNPs) in the promoter region of various cytokine genes.[5] Also, cytokine gene polymorphisms were associated with the severity of the liver disease in patient with HBV infection,[6] which may provide clues to clarify the mechanism for the progression of viral hepatitis.

Interleukin-18 (IL-18) was first described as an interferon-γ (IFN-γ)–producing factor, and it has multiple functions including the activation of cytotoxic T lymphocytes or natural killer cells and the promotion

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AT A GLANCE COMMENTARY

Background

The mechanisms underlying pathogenesis and the progression of chronic hepatitis B virus (HBV) infection have not been properly elucidated. Accordingly, no satisfactory therapeutic approaches are available to treat the millions of chronic HBV carriers. Although chronic HBV infection is induced by HBV, the immune system plays an important, yet poorly understood, role in its pathogenesis.

Translational Significance

In this study, we investigated a relationship between interleukin (IL)-18 promoter polymorphisms and the disease progression of chronic HBV-related liver diseases. Our data suggest that polymorphisms at the IL-18 gene-promoter region could be associated with different outcomes of HBV infection.

of T-helper type 1 (Th_1)-type immune responses.[7] Considering these multiple functions, IL-18 may activate effector cells that are involved in the cytotoxicity against pathogens or malignant cells. Because it is involved in the inflammatory cytokine network, IL-18 could have an important role in the development of chronic inflammatory diseases.[8] Recent findings show that the IL-18 gene-promoter region regulates the gene expression of this cytokine.[9] Interestingly, 2 SNPs at position -607 A/C and -137 G/C within the IL-18 promoter region were suggested to alter the IL-18 promoter activity.[9] Taking this evidence into consideration, we investigated the possible role of the SNPs of IL-18 gene-promoter region in the progression of chronic hepatitis B.

PATIENTS AND METHODS

Patients. A total of 204 patients, who were positive for the hepatitis B surface antigen (HBsAg) and who visited the clinics for treatment of liver disease at Nagasaki University Hospital or at National Nagasaki Medical Center between August 1999, and December 2003, were enrolled in this study. Sixty three healthy volunteers (33 males and 30 females) served as a control group. The patients were followed regularly with measurements of serum alanine aminotransferase (ALT) and HBV markers, such as HBsAg, hepatitis B e-antigen (HBeAg), and anti-HBe, using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan) every 3–6 months. Patients were also examined with ultrasonography or computed tomography of the liver every 3–6 months. Serum HBV-DNA was detected by the transcription-mediated amplification (TMA) method[10] as described previously. The results were expressed as the logarithm of the genome equivalent per milliliter (LGE/mL). The detection limit of this method is 3.7 LGE/mL. The patients receiving IFN therapy or antinucleoside analog reverse transcriptase inhibitor therapy were excluded in this study. The study protocol was approved by the Ethics Committees of both the Nagasaki University Hospital and the National Nagasaki Medical Center, and informed consent was obtained from each individual. Of 204 patients, 43 (20 male, 23 female; mean age ± standard deviation [SD], 55 ± 18 years) were considered to be inactive HBV carriers based on sustained normal serum ALT levels together with seropositivity for anti-HBe throughout the study. Of 43 inactive HBV carriers, all 31 patients tested for HBV-DNA had serum HBV-DNA levels less than 10^5 copies/mL (Table I). In addition, 161 of the 204 HBV carriers were considered to have chronic progressive liver disease (CPLD) such as chronic hepatitis or cirrhosis, which is manifested by elevated serum ALT levels and by clinical or histologic findings on liver tissue study during the follow-up period. This group comprised 114 males and 47 females (mean age ± SD, 50 ± 14 years). The ratio of males to females was significantly higher in CPLD patients compared with those in inactive HBV carrier ($P = 0.005$) (Table I). In accordance with the previous report, this ratio demonstrated that the patient gender affects the disease progression in HBV infection.[11] The group of patients with CLPD was classified into the following 3 subgroups: (1) 62

Table I. Clinical characteristics of 204 HBV carrier

Variable	Patients with inactive HBV carrier (n = 43)	Patients with CPLD (n = 161)
Sex (male/female)	20/23	114/47
Age (years)	55 ± 18	50 ± 14
HBeAg/anti-HBe status	0/43	53/109
Chronic hepatitis/cirrhosis		68/93
Serum ALT (IU/L)	21 ± 8	74 ± 101
HBV-DNA	(n = 31)	(n = 148)
$< 10^5$ copies/mL	31	60
$\geq 10^5$ copies/mL	0	88
Liver biopsy specimen available during study period	(n = 6)	(n = 51)
Stage of fibrosis		
F0–F2	6	15
F3–F4	0	36

NOTES: Age and serum ALT are expressed as mean ± SD.

Table II. Primers for the SNP analysis

PCR	SNP	Primer	Sequence
1st	-607 (A/C)+	-607ControlF	5'-CTTTGCTATCATTCCAGGAA-3'
	-137 (G/C)	-137R	5'-AGGAGGGCAAAATGCACTGG-3'
2nd	-607	-607BtF	5'-biotin-CTTTGCTATCATTCCADGGAATAGAAAGTTT-3'
		-607BseR	5'-TGCTGTATCAGATGCAAGCCAGACGGATACCATGAGGAGAATTTTAT-3'
2nd	-137	-137BtR	5'-biotin-ACTGCTGTCGGCACTCCTTGGGCCCGC-3'
		-137BseF	5'-GAGGTACAGGTTTTGGAAGGCACAGAGCCCAACTGAGGAGGAAGAAA-3'

patients with chronic hepatitis (CH), (2) 52 patients with cirrhosis, and (3) 47 patients with HCC. Of 47 HCC patients, 41 patients (87%) had cirrhosis.

Of 204 HBV carriers, 57 had undergone liver biopsy during the study period, and their degree of liver fibrosis were assessed using the METAVIR system.[12] To ensure a sufficient number of patients in each category, the severity of fibrosis was classified into 2 categories: F0–F2 and F3–F4. Among 57 patients, 36 patients in the CPLD group were classified as category F3–F4. (Table I).

DNA extraction. Genomic DNA was isolated from whole blood using the QIAamp DNA blood protocol (Qiagen Ltd., West Sussex, United Kingdom) according to the manufacturer's instructions.

IL-18 (-607/-137) genotyping. Two SNPs at position -607 A/C (rs1946518) and -137 C/G (rs187238) in the IL-18 gene promoter were determined by the ligation-mediated genotyping method[13] with a slight modification. A 849-bp fragment that contained the 2 SNPs was amplified by polymerase chain reaction (PCR) using primers -607ControlF and -137R (Table II), LA Taq polymerase (Takara Bio Inc., Shiga, Japan), and approximately 20 ng genomic DNA. The products were subjected to the 2nd PCR to amplify 178-bp and 174-bp fragments for genotyping at -607 and -137 by the primer pairs of -607BtF and -607BseR and of -137BtR and -137BseF, respectively. The second PCR products for the 2 SNPs were mixed and digested by BseRI to generate a 2-base overhang at the SNP sites. The 2 adapters complementary to the 2 possible 2-base overhang generated from a single SNP were prepared by annealing oligonucleotides, one of which was labeled with 2 different fluorescent dyes (FITC/TexasRed for -607C/A or Cy3/Cy5 for -137G/C) and mixed. The BseRI digest was ligated with the mixed adapters by Ligation Convenience Kit (Nippon Gene Co., Ltd., Toyama, Japan) at room temperature (Table III). The biotinylated DNA fragments were bound to Dynabeads M-280 Streptavidin (Dynal Biotech [Invitrogen], Tokyo, Japan) were subjected to the fluorescence measurement after extensive washing. The reactions after the 2nd PCR were automated using MagSNiPer FD (PSS Co., Ltd., Chiba, Japan) equipped with a 12-channel paramagnetic beads handling unit.[14,15]

Table III. Oligonucleotides for adapter preparation

SNP (genotype)	Sequence†
-607 (C)	5'-FITC-TACAAGATTCTGAAGACACCACCCAT CCTTGT-3'
-607 (A)	5'-TexasRed-TACAAGATTCTGAAGACACCACCCA TCCTTTT-3'
-137(C)	5'-Cy3-TACAAGATTCTGAAGACACCACCCATCCT TGA-3'
-137(G)	5'-Cy5-TACAAGATTCTGAAGACACCACCCATCCT TCA-3'
Common*	5'-AAGGATGGGTGGTGTCTTCAGAATCTTGTA -3'

*The oligonucleotide was annealed to each oligonucleotide above.
†The nucleotides at the position of the SNPs are underlined.

Statistical analysis. Results are expressed as mean ± SD. Comparison of the allele and genotype frequencies of different groups were performed using the chi-square test or the Fisher exact test. IL-18 allele frequencies were tested for the Hardy-Weinberg equilibrium for both patients and controls using the Cochrane-Armitage test. The significance level was set at a P value of 0.05.

RESULTS

We investigated the distribution of IL-18 promoter -137 (C/C) and -607 (A/C) polymorphisms in 204 Japanese HBV-infected patients (case) and 63 healthy volunteers (control). The genotypes at the positions of IL-18 promoter -137 (C/C) and -607 (A/C) polymorphisms were in Hardy-Weinberg equilibrium in both the case subjects and the control subjects (Tables IV and V).

The genotype frequencies of IL-18 promoter polymorphisms (-607 and -137) in each subgroup of HBV-infected patients are summarized in Table VI. These 204 HBV-infected patients were divided into the 2 groups; 43 patients were considered to be the inactive HBV carrier, and 161 were found to have chronic progressive liver diseases (CPLD). The genotype frequencies of IL-18 promoter polymorphisms (-607 and -137) in these 2 groups of HBV carriers are summarized in Table VII. With regard to the -607 genotypes, 6 (14.0%) inactive HBV carriers had the CC genotypes,

Table IV. Frequencies of IL-18 gene-promoter genotypes (-607 and -137) in case subjects

Locus	Genotype	Observed number (%)	Expected number*	P value†
IL-18 -607	A/A	55 (27.0)	64.2	
	A/C	119 (58.3)	105.5	0.46
	C/C	30 (14.7)	39.3	
IL-18 -137	G/G	167 (81.9)	164.1	
	G/G	32 (15.7)	37.9	0.37
	C/C	5 (2.5)	2.2	

*Expected phenotype frequencies based on observed allele frequencies and assuming Hardy-Weinberg equilibrium.
†*P* values were calculated using the Cochran-Armitage test for Hardy-Weinberg equilibrium at individual loci.

Table V. Frequencies of IL-18 gene promoter genotypes (-607 and -137) in control subjects

Locus	Genotype	Observed number (%)	Expected number*	P value†
IL-18 -607	A/A	20 (31.7)	19.48	0.50
	A/C	30 (47.6)	31.10	
	C/C	13 (20.6)	12.42	
IL-18 -137	G/G	52 (82.5)	51.57	0.48
	G/C	10 (15.9)	10.68	
	C/C	1 (1.6)	0.57	

*Expected phenotype frequencies based on observed allele frequencies and assuming Hardy-Weinberg equilibrium.
†*P* values were calculated using the Cochran-Armitage test for Hardy-Weinberg equilibrium at individual loci.

19 (44.2%) had the AC genotype, and 18 (41.9%) had the AA genotype. Of the CPLD group, 24 patients (14.9%) had the CC genotype, 100 (62.1%) had the AC genotype, and 37 (23.0%) had the AA genotype (Table VII). The frequency of the AA genotype was significantly lower in CPLD compared with that in the inactive HBV carriers (odds ratio [OR], 0.41; 95% confidence interval [CI], 0.20–0.84).

Although the frequency of the A allele at position -607 in inactive HBV carriers seems to be higher compared with those of CPLD patients (inactive HBV carrier; 64.0% vs CPLD; 54.0%), no significant difference was found between the inactive HBV carriers and the CPLD groups (Table VIII). However, the frequency of the C allele at position -137 was found to be significantly higher in the inactive HBV-carrier group compared with that in the CPLD group (Table VIII, inactive HBV carrier, 17.4% vs CPLD, 8.4%, $P = 0.024$).

To elucidate the relationship between these polymorphisms and the fibrosis staging, we divided the patients who received liver biopsy into 2 groups by the degree of fibrosis staging (the F0–F2 group, n = 21, and the F3–F4 group, n = 36). No significant difference was

Table VI. Genotype frequencies in patients with HBV

	Patients with HBV			
Genotype	Inactive HBV carrier (n = 43) (%)	CH (n = 62) (%)	Liver cirrhosis (n = 52) (%)	HCC (n = 47) (%)
Locus -607				
CC	6 (14.0)	6 (9.7)	10 (19.2)	8 (17.0)
CA	19 (44.2)	42 (67.7)	32 (61.5)	26 (55.3)
AA	18 (41.9)	14 (22.6)	10 (19.2)	13 (27.7)
Locus -137				
GG	31 (72.1)	53 (85.5)	40 (76.9)	43(91.5)
GC	9 (20.9)	9 (14.5)	11 (21.2)	3 (6.4)
CC	3 (7.0)	0 (0)	1 (19)	1 (2.1)

Table VII. The distribution of IL-18 genotype in inactive HBV carriers and CPLD patients

	Inactive HBV Carrier (n = 43)		CPLD (n = 161)			
	n	%	n	%	OR (95% CI)	P value
Genotype -607						
A/A	18	41.9	37	23.0	0.41 (.020–0.84)	0.022
A/C	19	44.2	100	62.1	2.07 (1.05–4.09)	0.051
C/C	6	14.0	24	14.9	1.08 (0.41–2.84)	>0.999
Genotype -137						
G/G	31	72.1	136	84.5	2.11 (0.95–4.65)	0.099
G/C	9	20.9	23	14.3	0.63 (0.27–1.48)	0.407
C/C	3	7.0	2	1.2	0.17 (0.03–1.04)	0.108

NOTES: P-value: The Fisher exact test.

discovered in IL-18 gene-promoter polymorphisms (-607 or -137) between these 2 groups (Table IX).

DISCUSSION

The susceptibility to persistent HBV infection is governed by several factors, which include the age at infection. When infection is acquired during the early neonatal period from an HBV-infected mother, only 10% of children will eliminate the virus. In contrast, when infection is acquired during childhood or later, up to 90% will eliminate the virus spontaneously.[16] Twin studies indicate that in addition to the age at infection, the host genetic background influences the outcome of HBV infection.[3] Elimination of HBV infection requires an innate and adaptive humoral and cell-mediated immune response.[17]

Previous studies have shown that the capacity of cytokine production varies among individuals and correlates with the polymorphisms in the promoter region of

Table VIII. Frequency alleles of IL-18 gene-promoter polymorphism in inactive HBV carriers and CPLD patients

Loci	Inactive HBV carrier (n = 86)	CPLD (n = 322)	P value
Locus -607			
C	31 (36.0)	148 (46.0)	0.128
A	55 (64.0)	174 (54.0)	
Locus -137			
G	71 (82.6)	295 (91.6)	0.024
C	15 (17.4)	27 (8.4)	

Table IX. The distribution of IL-18 genotype and fibrosis

	F0–F2 (n = 21)		F3–F2 (n = 36)		OR (95% CI)	P value
	n	%	n	%		
Genotype -607						
A/A	6	28.6	8	22.2	0.71 (.021–2.44)	0.591
A/C	13	61.9	21	58.3	0.86 (0.29–2.59)	0.791
C/C	2	9.5	7	19.4	2.29 (0.43–12.23)	0.461
Genotype -137						
G/G	17	81.0	30	83.3	1.10 (0.29–4.76)	>0.999
G/C	4	19.0	5	14.9	0.69 (0.16–2.90)	0.712
C/C	0	0.0	1	2.8	NA	>0.999

NOTES: P-value: The Fisher exact test.
ABBREVIATIONS: NA, not available.

cytokine genes.[5] Furthermore, cytokine gene polymorphisms have been shown to be associated with the disease progression of HBV infection.[4] It is crucial to identify genetic factors that determine the outcome of HBV infection, because these factors may reveal new therapeutic opportunities for patients with chronic HBV infection.

IL-18, which is a proinflammatory cytokine that belongs to the IL-1 family, induces IFN-γ production in T cells and natural killer cells, playing an important role in the Th_1 response.[7] However, the role of IL-18 in regulation of HBV infection has yet to be fully defined. IL-18 exerts a synergistic effect on IFN- γ production and induces antiviral activities.[18,19] It was also reported that IL-18 inhibited HBV replication in livers of HBV transgenic mice.[20] In addition, IL-18 is also known to induce the production of Th_2 cytokine, such as IL-4 and IL-13.[21] The dual role of IL-18 in Th_1 and Th_2 cytokine production could be implicated in the immune response against HBV infection.

In the current study, we compared the distributions of IL-18 gene promoter polymorphisms among Japanese HBV-infected patients with different clinical outcomes. Our results demonstrated that the frequencies of the -607 AA genotype and the -137 C allele were significantly higher in the inactive HBV carriers compared with those in patients with CPLD. The results of the current study suggest that the -607 AA genotype and the -137 C allele have a protective effect on the disease progression of HBV-related liver disease. Although the -607 A/A genotype was associated with a reduced risk of the progression of HBV infection (OR, 0.41), the -607 A/C genotype was associated with an increased risk of the progression of HBV infection (OR, 2.07). The mechanisms for the differential effects of these 2 genotypes on the development of HBV infection are not clear in this study. It was reported that these 2 polymorphisms -137 G/C and -607 C/A were in strong linkage disequilibrium.[22] In our study, there was a large difference in -137 C allele frequencies between the -607 AA genotype and the -607 AC or CC genotypes (-607 AA:21.7% vs -607 AC/CC:7.1%). It is possible that the protective effect of the -607 AA genotype on the development of HBV infection are attributable to the other loci, which are in linkage with -607 AA genotype, such as -137 C allele.

The presence of the C allele at position -607 (C/C+C/A) has been shown to be associated with a higher risk of cirrhosis and HCC in HBV-infected patients.[23] However, we did not find a significant association of the -607 genotype and severe fibrosis and HCC occurrence in our HBV-infected patients.

The current findings lead to address the question as to how IL-18 polymorphisms are related to the progression of HBV-related liver disease. Giedraitis et al[9] demonstrated that the allele C at -137 has been shown experimentally to disrupt the confirmed H4TF-binding site, whereas nucleotide substitution at -607 (C→A) may disrupt a the potential cyclic-adenosine-monophosphate–responsive element-binding site.[9] Furthermore, in an IL-18 promoter transcription activity assay, it was demonstrated that the presence of both A and C alleles at positions -607 and -137 in the same haplotype is associated with low promoter activity.[9] Our results may not be in good accordance with these findings of Giedratis et al.[9] Mechanisms underlying the relationship between the IL-18 gene promoter polymorphisms and the outcome of HBV infection are not clear in our study. Zhang et al reported that the carriage of the allele C at position -137 in the promoter of IL-18 gene may play a protective role in the development of HBV infection, and the AA genotype at position -607 may be associated with HBV-DNA replication.[24] Our results are concordant with these previous findings that IL-18 promoter polymorphisms could influence the outcome of HBV infection. More recently, Hirankarn et al[25] demonstrated an association between -607 A/A polymorphism and the susceptibility of chronic HBV infection. However, the state of HBV infection and the degree of liver damage in their studied population were not described. To establish firmly the relationship between IL-18 gene

promoter polymorphisms and the risk of the progression of HBV infection, more large-scale studies are required that include individuals of other ethnicities.

In conclusion, we have attempted to elucidate the role of genetic polymorphisms of IL-18 gene in the outcome of HBV infection. Our data suggest that the polymorphisms at the IL-18 gene promoter region (-607 and -137) may affect the development and progression of HBV-related liver disease.

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Long-term trends of the incidence of hepatocellular carcinoma in the Nagasaki prefecture, Japan

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Abstract. The incidence of hepatocellular carcinoma (HCC) in Japan is still increasing. The aim of the present study was to analyze the epidemiological trend of HCC in the Western area of Japan, Nagasaki. A total of 1,807 patients with HCC diagnosed at our two hospitals between 1981 and 2005 were consecutively recruited for this study. Cohorts of patients with HCC were categorized into five-year intervals. The etiology of HCC was categorized into four groups: HCC-B: HBsAg positive, HCVAb negative, HCC-C: HCVAb positive, HBsAg negative, HCC-BC: both of HBsAg and HCVAb positive and HCC-nonBC: both of HBsAg and HCVAb negative. The number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak from 1995 to 2000 and thereafter decreased. On the other hand, the number and ratio of the HCC-nonBC cases continued to increase in the whole period. The male/female ratio of HCC-C patients decreased from 6.4 in the period 1981-1985 to 1.9 in 2001-2005, indicating clearly the increase of female patients. On the other hand, the male/female ratio of other types of HCC patients did not change during the period. HCC patients rapidly increased from 1981 to 2000 and this increase was originated from that of HCC-C. The increase of the median age and the number of female patients with HCC-C was also demonstrated. The increase in the number and the proportion of the HCC-nonBC patients was also significant.

Introduction

Primary liver cancer is the most common primary cancer of the liver accounting for ~6% of all human cancers. It is estimated that half a million cases occur worldwide annually, making primary liver cancer the fifth most common malignancy in men and the ninth in women (1-6). Hepatocellular carcinoma (HCC) accounts for 85 to 90% of primary liver cancers (7) and the age-adjusted HCC mortality rate has increased in recent decades in Japan (8). Similarly, a trend of increasing rates of HCC has been reported from several developed countries in North America, Europe and Asia (9,10). HCC often develops in patients with liver cirrhosis caused by hepatitis B virus (HBV), hepatitis C virus (HCV), excessive alcohol consumption, or nonalcoholic fatty liver disease. Of the hepatitis viruses which cause HCC, HCV is predominant in Japan (11-14).

Although the age-adjusted incidence of HCC has increased in Japan, sequential changes in background features of HCC patients are not fully understood (15). Yoshizawa reports that deaths due to HCC in Japan have continued to increase in males, particularly in those older than 60 years of age in the past 3 decades, although the reasons for this are unclear (16). To clarify factors affecting epidemiological changes in Japanese HCC patients, especially the change in age distribution and gender, we analyzed the underlying features of HCC patients in a two major liver center-based study.

Patients and methods

Patients. A total of 1,807 patients with HCC diagnosed between January 1981 and December 2005 in the Liver Disease Center, National Nagasaki Medical Center and in the outpatient clinic of The First Department of Internal Medicine, Nagasaki University Hospital, were consecutively recruited for this study. The diagnosis of HCC was based on AFP levels and imaging techniques including ultrasonography (USG), computerized tomography (CT), magnetic resonance imaging (MRI), hepatic angiography (HAG) and/or tumor biopsy. The diagnostic criteria for HCC were either a confirmative tumor biopsy or elevated AFP (≥20 ng/ml) and neovascularization in HAG and/or CT. Cohorts of patients with HCC were categorized into five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005).

Etiology of HCC. Sera were stored at -80°C until use. A diagnosis of chronic HCV infection was based on the presence of HCVAb (microparticle enzyme immunoassay; Abbott

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Key words: hepatitis C virus, hepatocellular carcinoma, aging, Japan

Table I. The characteristics of HCC patients, 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Gender						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
Age (y.o) (IQR)	57 (6.5)	61 (5.1)	63 (5.4)	66 (5.1)	68 (6.3)	64 (6.5)
Hepatitis virus						
HCC-B	95	70	80	67	100	412
HCC-C	111	213	240	292	278	1134
HCC-B+C	8	8	9	11	10	46
HCC-nonBC	26	25	40	49	75	215

Gender: 2000-2005 vs. 1981-1985 p=0.0003; 2000-2005 vs. 1986-1990 p≤0.0001; 2000-2005 vs. 1991-1995 p=0.1330; 2000-2005 vs. 1996-2000 p=0.0197. Age: 2000-2005 vs. 1981-1985 p≤0.0001; 2000-2005 vs. 1986-1990 p≤0.0001; 2000-2005 vs. 1991-1995 p≤0.0001 and 2000-2005 vs. 1996-2000 p=0.0292. IQR, interquartile range.

Laboratories) and HCV-RNA detected by polymerase chain reaction (PCR), whereas diagnosis of chronic HBV infection was based on the presence of hepatitis B surface antigen (HBsAg) (enzyme-linked immunosorbent assay; Abbott Laboratories).

Statistical analysis. The data were analyzed by the Mann-Whitney test for the continuous ordinal data between two qualitative variables. The standard deviation was calculated based on the binomial model for the response proportion. $P<0.05$ was considered statistically significant.

Results

Clinical features of the studied patients. A total of 1,807 patients with HCC were diagnosed at our hospital from 1981 to 2005. There were 1,347 male (75%) and 460 female (25%) patients, with a median age of 64 years. The proportion of patients diagnosed as HCC-B (HBV-associated: HBsAg positive, HCVAb negative) was 23% (412 of 1,807), whereas 63% (1,134 of 1,807) had HCC-C (HCV-associated: HCVAb positive, HBsAg negative) and an additional 3% (46 of 1,807) had HCC associated with both viruses. The remaining 215 patients (12%) showed both of the virus markers negative.

As shown in Table I and Fig. 1, the number and proportion of HCC-B cases decreased from 1986 to 1990 and thereafter stabilized, whereas those of HCC-C reached the peak in the period 1996-2000 and thereafter decreased. The number and proportion of the HCC-nonBC (HBsAg and HCVAb negative) cases continued to increase in the whole period.

Background features for patients with HCC. Fig. 2 shows the median age at diagnosis of HCC-B, HCC-C and HCC-nonBC in five-year intervals (1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005). The median age of patients at diagnosis of HCC-C showed a steadily significant increase from 58 to 69 years of age during the period. The median age of patients with HCC-B and HCC-nonBC did not significantly change during the period.

Fig. 3 shows the age distribution of patients with HCC-B and HCC-C with the five 5-year intervals. There was no difference in the age distribution of patients with HCC-B during these periods. In contrast, HCC-C obviously had a trend to increase in the number of patients aged >65 years.

Table I shows that the male/female ratio of HCC patients decreased from 4.2 in the period 1981-1985 to 2.1 in 2001-2005, indicating clearly the increase of female patients. In analysis of patients in HCC-C, the male/female ratio in the periods 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 were 6.4, 4.8, 2.5, 2.7 and 1.9, respectively (1981-1985 vs. 2001-2005, p≤0.0001) (Table II). The ratio became clearly smaller, indicates an increase in female patients with HCC-C. On the other hand, the male/female ratio of other types of HCC patients did not significantly change during the period.

Discussion

This was a two major liver center-based study designed to examine the sequential change in the background of HCC patients during the past 25 years, 1981-2005. More than 80% of our patients had chronic HBV or HCV infections. During the observation period, the number and proportion of HCC-B cases decreased in the period 1986-1990 and thereafter reached a plateau, whereas HCC-C reached a peak in the period 1995-2000 and thereafter slightly decreased. On the other hand, the number and the proportion of HCC-nonBC gradually increased in the periods of 1981-1985, 1986-1990, 1991-1995, 1996-2000 and 2001-2005 being 26 (11%), 25 (8%), 40 (11%), 49 (12%) and 75 (16%), respectively. Previous studies from Japan reported that the proportion of HCC-C had been increased and reached a plateau in the

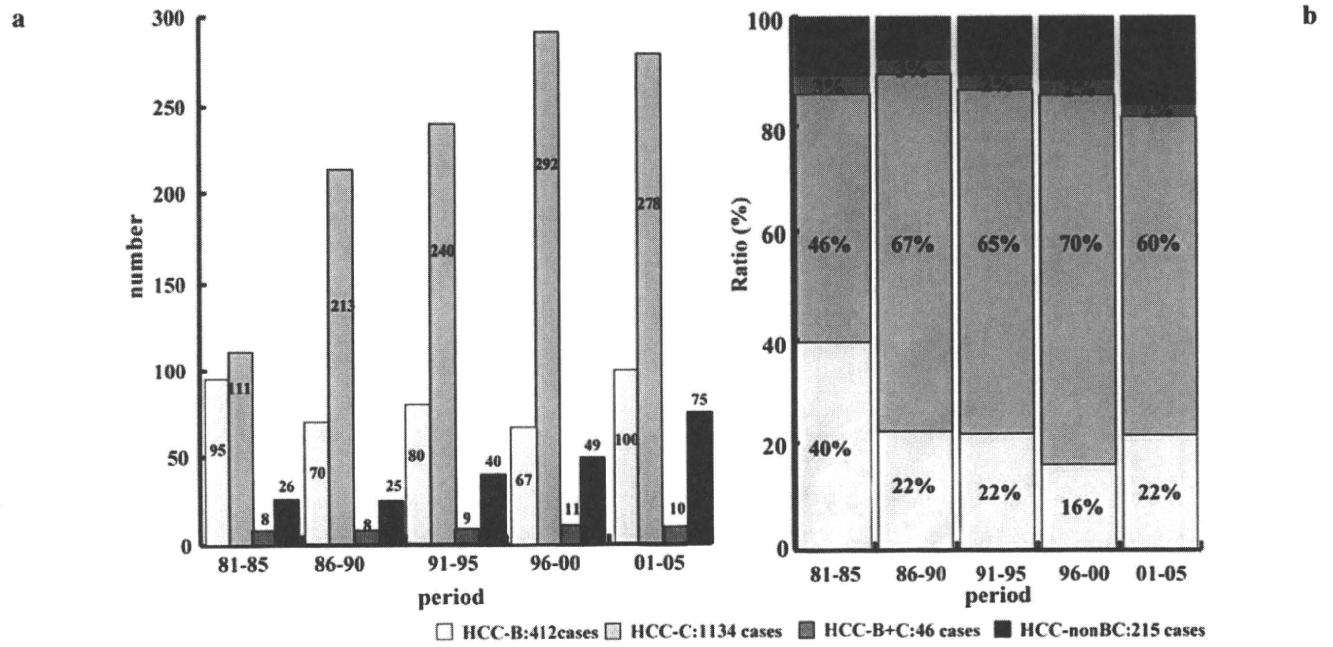

Figure 1. Sequential changes in the number (a) and ratio (b) of HCC patients categorized by etiology during the period 1981-2005 with 5-year intervals.

Table II. The number and male/female ratio of HCC patients during the period of 1981-2005.

Period	1981-1985	1986-1990	1991-1995	1996-2000	2001-2005	Total
Number	240	316	369	419	463	1807
Total						
Male	194	257	268	314	314	1347
Female	46	59	101	105	149	460
Ratio (male/female)	4.2	4.4	2.7	3.0	2.1	2.9
HCC-B						
Male	69	54	61	55	74	313
Female	26	16	19	12	26	99
Ratio (male/female)	2.7	3.4	3.2	4.6	2.9	3.2
HCC-C						
Male	96	176	172	212	182	838
Female	15	37	68	80	96	296
Ratio (male/female)	6.4	4.8	2.5	2.7	1.9	2.8
HCC-nonBC						
Male	21	20	29	40	51	1347
Female	5	5	11	9	24	460
Ratio (male/female)	4.2	4.0	2.6	4.4	2.1	2.9

HBV and nBnC: NS. HCV: 2000-2005 vs. 1981-1985 p≤0.0001; 2000-2005 vs. 1986-1990 p≤0.0001; 1996-2000 vs. 1981-1985 p=0.0033; 1996-2000 vs. 1986-1990 p=0.0084; 1991-1995 vs. 1981-1985 p=0.0024 and 1991-1995 vs. 1986-1990 p=0.0058.

period of 1981-2001 (8,15,17-19). However, in our study, the number and proportion of HCC-C cases decreased in the period 2001-2005. This may be due to interferon therapy associated with a decreased incidence of HCC (20-24). Iron depletion for chronic hepatitis C patients is a promising modality for lowering the risk of progression to HCC (25,26). Oral supplementation with oral branched-chain amino acids has been useful in the prevention HCC (27). Finally, the chronically HCV-infected population is aging in Japan. Yoshizawa reported that age-specific prevalence for the presence of HCVAb among ~300,000 voluntary blood donors from Hiroshima in 1999 clearly increased with the

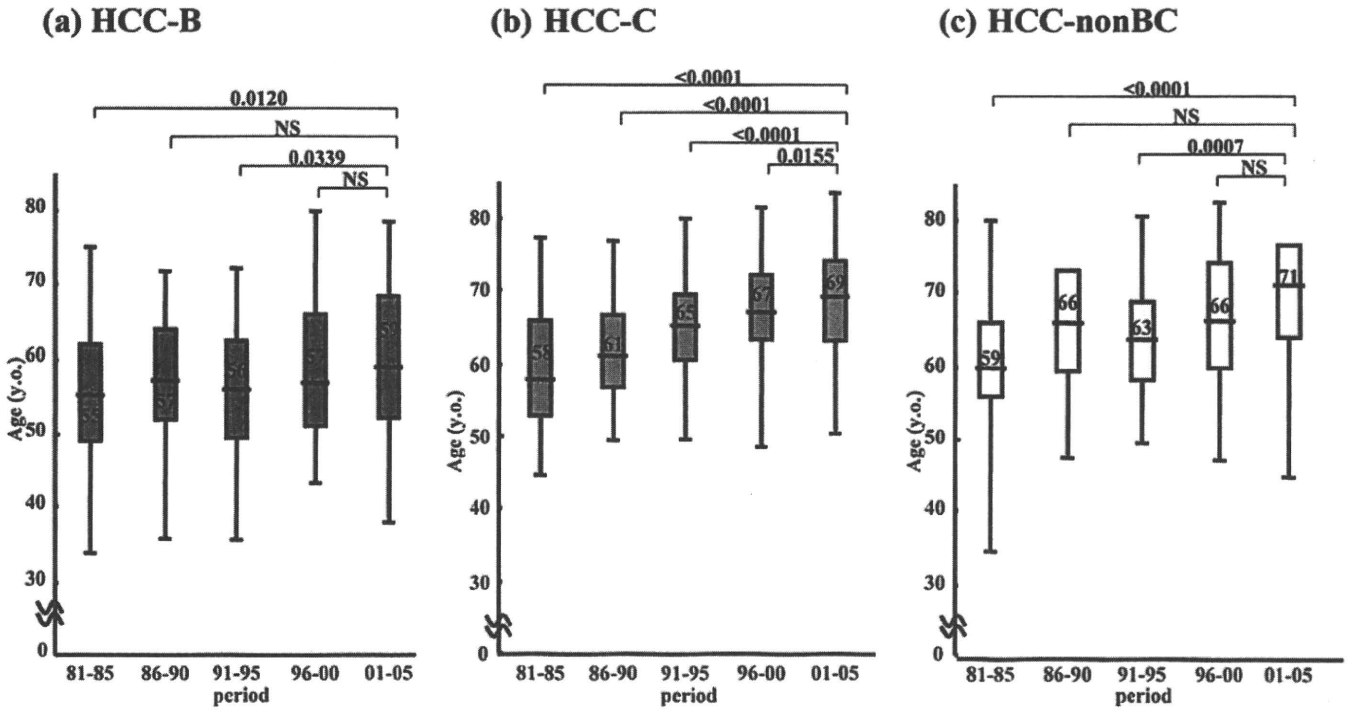

Figure 2. Sequential changes in the median age of HCC patients categorized by etiology during the period, 1981-2005 with 5-year intervals. (a) HCC-B, (b) HCC-C and (c) HCC-nonBC type $p<0.05$.

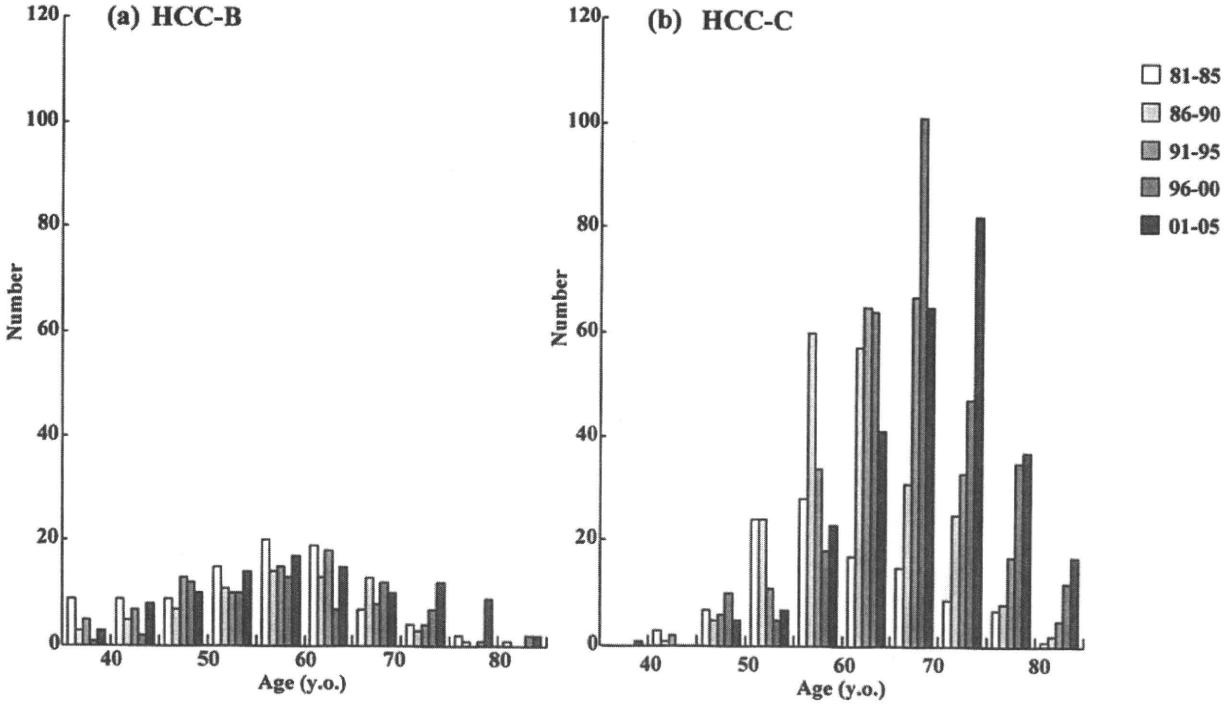

Figure 3. Changes in the age distribution of patients with HCC-B and HCC-C during the period, 1981-2005 with 5-year intervals.

age, reaching the highest proportion of 7% in individuals who were >70 years old (15,16). In this study, the median age of patients with HCC-C steadily increased from 58 to 69 years of age during the studied period. *i.e.* HCV infected people become older and they were regarded as a high risk for HCC.

In almost all populations, males have higher liver cancer proportions than females, with the male/female ratios usually averaging between 2:1 and 4:1 (7). However, the male/female ratio of HCC in Japan was 4.5 in the period 1983-1985 and 2.57 in 2000-2001 (17). In analysis of background features among HCC patients, HCC-B and HCC-nonBC cases revealed no significant change, whereas the male/female ratio of patients with HCC-C steadily decreased from 6.4 to 1.9 during the period. We suggest that the increase of female

patient with HCC-C was caused by the aging of HCV infected people. The increase of females among HCC patients was considered to increase because of HCC-C.

It is known that 2 to 4 decades of chronic HCV infection are required to develop cirrhosis and subsequent HCC (28-31). The number of HCC cases has increased in Japan, because individuals infected with HCV in the past have grown old and have reached the cancer-bearing age. The prevalence of HCV infection in young Japanese individuals is low and the incidence of HCVAb is very low because of preventative actions against HCV infection such as the screening of blood products for HCV and the use of sterile medical equipment (32). Additionally, we showed that the number and proportion of patients with HCC-C cases decreased together with an increase in the median age, whereas the number and ratio of HCC-nonBC steadily increased during the studied period. Based on these findings it may be expected that the incidence of HCC-nonBC in Japan may continue to increase even after the consequence of the HCV epidemic level off in the near future, although Japan is far advanced with regard to HCC-C.

In summary, HCC patients rapidly increased from 1981 to 2000 and this increase originated from HCC-C and the increase of the median age and the number of female patients with HCC-C. Increase in the number and proportion of the HCC-nonBC patients are also significant.

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HEPATOLOGY

Detection of HBV core promoter and precore mutations helps distinguish flares of chronic hepatitis from acute hepatitis B

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Abstract

Background and Aim: Acute exacerbation of chronic hepatitis B has to be distinguished from acute hepatitis, because treatment strategies differ between them.

Methods: Mutations in the core promoter and precore region of hepatitis B virus (HBV) were determined in 36 patients with acute exacerbation of chronic hepatitis B, in whom alanine aminotransferase (ALT) increased above 500 IU/L, as well as the 36 patients with acute hepatitis.

Results: Mutations in the core promoter (A1762T/G1764A) and precore region (G1896A) were more frequent in patients with acute exacerbation of chronic hepatitis than acute hepatitis (81% *vs* 19%; $P < 0.0001$ and 58% *vs* 6%; $P < 0.0001$, respectively). Of the 19 patients with mutations in both the core promoter and precore region, 17 (89%) had acute exacerbation of chronic hepatitis. In contrast, among the 32 patients with the wild-type for both the core promoter and precore region, 29 (89%) developed acute hepatitis. By multivariate analysis, the double mutation in the core promoter was predictive of acute exacerbation in chronic hepatitis with the highest odds ratio at 26.4.

Conclusions: In patients with hepatitis B having ALT levels >500 IU/L, mutations in the core promoter and precore region are useful in distinguishing acute exacerbation of chronic from acute HBV infection. Detection of these mutations would be useful for commencing prompt antiviral treatments on patients with acute exacerbation of chronic hepatitis for a better prognosis.

Introduction

There are two clinical entities of acute liver disease induced by hepatitis B virus (HBV).[1] Acute hepatitis is induced by immune responses of hosts for eliminating HBV. Most cases of acute hepatitis clear hepatitis B surface antigen (HBsAg) from serum and resolve infection within 6 months after the onset. Acute exacerbation of hepatitis, by contrast, occurs in individuals chronically infected with HBV. They have been infected perinatally or in an early infancy and are tolerant to HBV. Later in their lives, however, the tolerance to HBV is terminated, and immune responses are elicited in them. As a result, severe hepatitis can develop along with subjective symptoms and abnormalities in liver function tests. It is therefore difficult to distinguish acute hepatitis from acute-on-chronic hepatitis. The antibody to hepatitis B core antigen (anti-HBc) of the IgM class is used to distinguish acute from chronic HBV infection. However, IgM anti-HBc develops in some patients with chronic hepatitis during acute exacerbation, in titers overlapping with those of acute hepatitis.[2,3] Hence, high-titred anti-HBc can not always differentiate between acute and acute-on-chronic hepatitis B.

HBV is a small, partially double-stranded DNA virus made of approximately 3200 nucleotides (nt). Since its replication involves the reverse transcription of pregenome RNA,[4] mutations occur more frequently in HBV than in other DNA viruses.[5] Individuals persistently infected with HBV have hepatitis B e antigen (HBeAg) in serum initially. Later in their lives, they lose HBeAg and develop antibodies to HBeAg (anti-HBe). The seroconversion is induced by mutations in two different domains of HBV-DNA. The double mutations in core promoter (A1762T/G1764A) interfere with the transcription of precore RNA and reduce the expression of HBeAg precursor.[6] G-to-A mutation at nt 1896 in the precore region converts codon 28 for tryptophan (TGG) to a stop codon (TAG), and terminates the translation of HBeAg precursor.[7,8] These mutations in the core promoter and precore region are reported in patients with fulminant hepatitis,[9–11] as well as in those with chronic active hepatitis.[12]

In the present study, core promoter and precore mutations were determined in patients with acute exacerbation of chronic hepatitis and those with acute hepatitis. The results obtained indicate that these mutations would be useful for distinguishing acute-on-chronic from acute hepatitis B.

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Methods

Patients

During a 5-year period from 2000 through 2004, 36 patients with acute hepatitis B were admitted to National Hospital Organization Nagasaki Medical Center. The diagnosis of acute hepatitis B was made for patients who presented with signs and symptoms suggestive of acute hepatitis (nausea, jaundice, fever, abdominal pain, and enlarged liver) and who were positive for HBsAg and/or IgM anti-HBc, negative for anti-HCV as well as IgM anti-HAV, and had alanine aminotransferase (ALT) values exceeding five-times the upper limit of normal (40 U/L). The loss of HBsAg from serum within 6 months after onset was confirmed in all patients. Infectious sources of acute hepatitis B were sexual contacts in 25 patients, illicit intravenous drugs in four, and unknown in the remaining seven patients.

Among 261 patients with chronic hepatitis B who had been followed up during the same period, acute exacerbation developed in 36 (14%), and 30 of them (83%) reported a family history of HBV infection. HBsAg had persisted for 1 year or longer and ALT increased to >500 IU/L in them all. All the 36 patients with acute-on-chronic hepatitis B underwent a liver biopsy. Fibrosis stages were F0 in 1, F1 in 4, F2 in 16, F3 in 8, and F4 in 7, and activity grades were A1 in 3, A2 in 9, and A3 in 24. The five patients in mild fibrosis stages (F0 or F1) had been infected with HBV for longer than 6 months before they suffered from acute exacerbation, thereby excluding the possibility of acute HBV infection.

Fulminant hepatitis was diagnosed by prothrombin time <40% and hepatic encepharopathy of grade II or higher, and acute severe hepatitis by prothrombin time ≥40% and encephalopathy of grade I or less. Among the 36 patients with acute hepatitis, one developed fulminant hepatitis and five came down with severe hepatitis. Among the 36 patients with acute exacerbation of chronic hepatitis B, one developed fulminant hepatitis and one had severe hepatitis. The two patients with fulminant hepatitis died of advanced hepatic failure; their family members did not agree with liver transplantation.

Mutations in the core promoter and precore region were determined in sera from patients obtained when they presented with acute hepatitis or acute exacerbation of chronic hepatitis.

Informed consent was obtained from each patient, and the study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the institution's human research committee.

Determination of mutations in precore region and core promoter

HBV-DNA was recovered from serum (50 μL) with use of the SMITEX-R&D extraction kit (Medical Biological Laboratories [MBL], Nagoya, Japan). The stop-codon mutation in precore region (G1896A) was determined by enzyme-linked mini-sequence assay (ELMA) with a commercial kit (MBL).[13,14] HBV-DNA solution (50 μL) was mixed with ELMA solution (50 μL), and subjected to polymerase chain reaction (PCR). Amplification products were delivered to wells in a microtiter plate that had been coated with probes for the wild-type or mutant; they had G or A at the position 1896. Reaction was determined by colorimetry, and an optical density >0.100 was judged positive, while that of ≤1.00 was regarded negative. Positive reading on the well for wild-type only was recorded as the wild-type; that on mutant well alone as the mutant type; and positive readings both on wild-type and mutant wells were classified as the mixed type.

Mutations in core promoter were determined by enzyme-linked specific probe assay (ELSPA) with commercial kits (MBL).[13,14] HBV-DNA solution (50 μL) was mixed with ELMA solution (50 μL), and subjected to PCR. Amplicons were transferred to three wells in a microtiter plate which had been coated with different probes. One of wells was coated with probe for the wild-type with A1962/G1764 and another with that for the mutant type with T1762/A1764, and the third with a highly preserved HBV-DNA sequence for guaranteeing successful amplification by PCR. Determination was possible when optical density of the control well exceeded 0.800 and that of the well for the wild-type or mutant was higher than 0.400, in accordance with the decision table in package inserts of the kit.

The sensitivity and specificity of the ELSPA and MBL kits were examined on cloned wild-type and mutant-type HBV-DNA. Reproducible results were obtained with a sensitivity of 100 copies/100 μL of HBV-DNA.[13,14]

Statistical analysis

Categorical variables were compared between groups by the χ^2-test and Fisher's exact test, and continuous variables by the Student's *t*-test. Influence of various factors on the manifestation of disease was evaluated by logistic regression in univariate and multivariate analyses. Analyses were performed with SAS software (SAS Institute Japan, Tokyo, Japan), and differences were considered significant when the *P*-value exceeded 0.05.

Results

Comparison of patients with acute exacerbation of chronic hepatitis and acute hepatitis

Table 1 compares clinical and virological characteristics between patients with acute exacerbation of chronic hepatitis and acute hepatitis. Men predominated (86% *vs* 58%; $P < 0.01$) and platelets counts were lower (177 ± 56 *vs* $238 \pm 60 \times 10^3/mm^3$; $P < 0.0001$), while IgM anti-HBc was less frequent (58% *vs* 97%; $P < 0.0001$) in patients with acute-on-chronic than acute hepatitis. Distribution of HBV genotypes was no different between patients with acute-on-chronic and acute hepatitis, and genotype C accounted for ~90% and genotype B for only 8% in them both.

Mutations in the core promoter and precore region

The double mutation in core promoter (A1762T/G1764A) and precore mutation (G1896A) were more frequent in patients with acute-on-chronic than acute hepatitis (81% *vs* 19% and 58% *vs* 6%, respectively; $P < 0.0001$ for each). Of the five patients with fulminant or severe acute hepatitis, four (80%) possessed mutations in the core promoter and/or precore region. Table 2 summarizes mutations in the core promoter and precore region in

Table 1 Clinical and virological characteristics of patients with acute exacerbation of chronic hepatitis and those with acute hepatitis

Features	Acute exacerbation of chronic hepatitis ($n = 36$)	Acute hepatitis ($n = 36$)	Differences
Men	31 (86%)	21 (58%)	$P = 0.009$
Age (years)	36 ± 13 (16–62)	38 ± 19 (16–87)	NS
Albumin (g/dL)	4.1 ± 0.4 (3.1–5.0)	4.0 ± 0.5 (2.4–5.1)	NS
ALT (IU/L)	1499 ± 577 (808–2740)	1792 ± 785 (209–2990)	NS
Total bilirubin (mg/dL)	4.0 ± 4.2 (0.4–17.2)	5.6 ± 5.3 (0.8–21)	NS
Platelets (x 10^3/mm^2)	177 ± 56 (72–313)	238 ± 60 (75–356)	$P < 0.0001$
Prothrombin time (%)	71 ± 21 (36–114)	77 ± 27 (5–120)	NS
IgM anti-HBc	21 (58%)	35 (97%)	$P < 0.0001$
HBV genotypes			
B	3 (8%)	3 (8%)	NS
C	33 (92%)	32 (89%)	NS

ALT, alanine aminotransferase; anti-HBc, antibody to hepatitis B core antigen; HBV, hepatitis B virus; NS, not significant.

Table 2 Clinical manifestation of hepatitis B virus of the wild-type or with mutations in the core promoter and/or precore region

Core promoter (nt 1762/1764)	Precore region (nt 1896)	Acute exacerbation of chronic hepatitis	Acute hepatitis
Wild	Wild ($n = 32$)	3 (9%)	29 (91%)
Wild	Mutant ($n = 17$)	12 (71%)	5 (29%)
Mutant	Wild ($n = 7$)	4 (100%)	0
Mutant	Mutant ($n = 19$)	17 (89%)	2 (11%)

the patients with acute exacerbation of chronic hepatitis and those with acute hepatitis. Of the 19 patients with the mutant type both for core promoter and precore region, 17 (89%) were those with chronic hepatitis who had developed acute exacerbation. Of the 32 patients infected with the wild-type both for core promoter and precore region, in contrast, 29 (91%) had been diagnosed with acute hepatitis.

Factors contributing to the differentiation of acute exacerbation of chronic hepatitis from acute hepatitis

Univariate and multivariate analyses were performed for sorting out factors predictive of acute exacerbation in patients with chronic hepatitis B (Table 3). In univariate analysis, male gender, low platelet counts, negative IgM anti-HBc, and mutations in the core promoter, as well as the precore region, predicted the acute exacerbation of chronic hepatitis. In multivariate analysis, only male gender, negative IgM anti-HBc, and the double mutation in the core promoter were predictive of acute exacerbation of chronic hepatitis. Among these three parameters, the core promoter mutation had the highest odds ratio at 26.4.

Discussion

Acute HBV infection in adulthood is mostly self-limited, and rarely becomes chronic.[15] Acute exacerbation can emerge in chronic hepatitis, however, making it difficult to differentiate from acute self-limited hepatitis. The prognosis is more severe for acute-on-chronic than acute hepatitis B; it can transit swiftly to decompensation and cirrhosis.[16] Recently, many antiviral drugs have been introduced, including lamivudine, adefovir-dipivoxyl, and entecavir, and they can prevent the development of decompensation and cirrhosis in patients with chronic hepatitis B.[17,18] Hence it is necessary to diagnose the acute exacerbation in patients with chronic hepatitis B in order to start treatment with antiviral drugs immediately.[19–24]

In persistent HBV infection, mutations in the core promoter and/or precore region accumulate with time, as hosts seroconvert from HBeAg to anti-HBe. In the present series of 36 patients with the exacerbation of chronic hepatitis, core promoter and precore mutations were found more frequently than in the 36 patients with acute hepatitis (81% *vs* 19%; $P < 0.0001$ and 58% *vs* 6%; $P < 0.0001$, respectively). Of the 19 patients with mutations both in the core promoter and precore region, in particular, 17 (89%) had developed acute exacerbation of chronic hepatitis. In remarkable contrast, of the 32 patients with the wild-type both for the core promoter and precore region, 29 (91%) had acute HBV infection. By multivariate analysis, core promoter mutations were predictive of chronic HBV infection with the highest odds ratio at 26.4.

For acute hepatitis B, the wild-types both for the core promoter and precore region had positive and negative predictive values of 90% (29/32) and 81% (29/36), respectively. For acute exacerbation of chronic hepatitis B, mutation in either the core promoter or precore region had positive and negative predictive values of 83% (33/40) and 92% (33/36), respectively. Taken altogether, determination of mutations in the core promoter and precore region would be helpful in distinguishing between chronic and acute HBV infections in the patients who present themselves with serum HBsAg and ALT levels exceeding 500 IU/L. HBV genotypes can influence the development of core promoter and precore mutations.[25] They would have made little difference in patients in this study; the majority of them were infected with HBV genotype C.

Patients with acute HBV infection possess IgM anti-HBc in high titers, which can differentiate them from those with acute-on-chronic heaptitis.[26,27] IgM anti-HBc appears in considerably high titers in sera of some patients with chronic hepatitis undergoing acute exacerbation.[2,3] It is therefore difficult to differentially diagnose acute from chronic infection by IgM-anti-HBc alone. Based on the results obtained in this study, mutations in the core promoter and precore region would improve the diagnosis of acute exacerbation in chronic hepatitis.

Table 3 Odds ratio for the acute exacerbation of chronic hepatitis

Factors	Univariate analysis (95% confidence interval)	Multivariate analysis (95% confidence interval)
Male gender	4.4 (1.4–14.0); $P = 0.0115$	9.0 (1.0–76.6); $P = 0.0455$
Platelets < 100 × 10^3/mm^3	6.7 (2.4–19.0); $P = 0.0003$	4.0 (0.7–23.1); $P = 0.1275$
Negative IgM anti-HBc	25.0 (3.1–203.2); $P = 0.0026$	21.6 (1.7–267.5); $P = 0.0167$
Core promoter mutations	17.2 (5.3–55.2); $P < 0.0001$	26.4 (3.6–192.6); $P = 0.0013$
Precore mutation	23.8 (5.0–114.7); $P < 0.0001$	5.0 (0.7–37.2); $P = 0.1138$

anti-HBc, antibody to hepatitis B core antigen.

As we have reported previously, however, these mutations are frequent in patients with fulminant or severe acute hepatitis.[13] In this study, also, four of the five (80%) patients with fulminant or severe acute hepatitis possessed mutations in the core promoter and/or precore region. This would have to be taken into consideration when using these mutations to differentiate between acute-on-chronic and acute hepatitis.

It is hoped that our findings indicating the usefulness of the core promoter and precore mutations, obtained in limited numbers of patients with acute and chronic HBV infection, would be extended in further studies for prompting antiviral treatment in patients with chronic hepatitis who develop acute exacerbation.

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