Correlation Between Level of Hepatic IFNAR2 Levels and Response to IFN Therapy

We also examined the correlation between the outcome of IFN treatment and the level of hepatic IFNAR2 in 55 patients with chronic HCV infection [7]. Fifteen of 55 (27%) patients ,whose post-treatment level of serum HCV-RNA diminished to undetectable levels on RT-PCR, were judged as sustained viral responders (SVR). In contrast, serum HCV-RNA was still detectable after treatment in the remaining 40 (73%) non-sustained viral responders (Non-SVR) (Fig. 2).

The mean level of hepatic IFNAR2 in SVR (2.7±1.1U, n=15) was higher than that of Non-SVR (1.6±1.1 U, n=40) (P<0.001). Univariable and multivariable logistic regression analysis were also performed to evaluate the various factors influencing the efficacy of IFN therapy, such as the hepatic IFNAR2 level (IFN-R level), serum HCV-RNA level, HCV serotype, ALT level, age, gender, degree of fibrosis and inflamatory activity of the liver. Univariable logistic regression analysis showed that IFN-R level, HCV-RNA level, HCV serotype and degree of inflamatory activity of the liver achieved statistical significance and they were subjected to multiple logistic regression. Multivariate analysis was performed using these four factors. Among these factors, both IFN-R level (P=0.0056) and HCV-RNA level (P=0.0097) were important factors influencing the efficacy of IFN therapy in this study (Table 1).

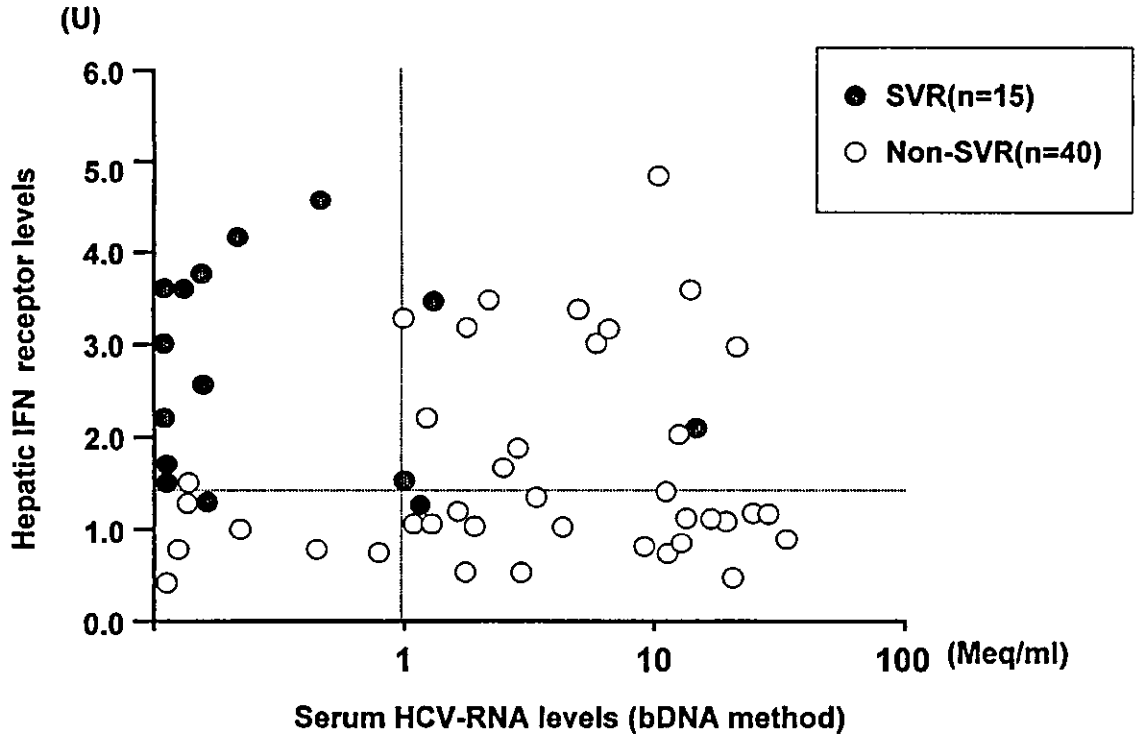

Fig. 2. Relationship between response to IFN therapy, level of expression of hepatic IFNAR2 and serum HCV-RNA levels in 55 patients with chronic hepatitis C. SVR: Sustained Responders (n=15), Non-SVR (n=40).

Table1. Predictive factors of the effectiveness of IFN monotherapy in patients (n=55) with chronic hepatitis C by multivariable logistic regression analysis

Predictor	Estimate	S.E.	P-value
IFN-AR2 level	−1.4578	0.5266	0.0056
HCV-RNA level	0.3403	0.1316	0.0097
Activity (liver biopsy)	−1.3815	0.7018	0.0490
HCV-genotype	−1.2536	1.0601	0.2370

We observed a clear relationship between response to IFN therapy and the level of expression of hepatic IFNAR2. Notwithstanding the wide experience in the use of IFN treatment in CHC, little is known about the host mechanisms that influence the response, or the lack of response, to this agent in individual cases. Because the receptor is a key element in the IFN response, we examined the usefulness of a monoclonal antibody that recognizes IFNAR2 in monitoring the expression of type I IFN receptor in patients with liver disease. In our study, pretreatment levels of hepatic IFNAR2 expression were significantly higher in patients with SVR than Non-SVR, suggesting that a poor response to IFN might be due to a deficient expression of hepatic IFNAR2 in CHC.

In confirmation of the results of previous studies, SR in the current study had low pretreatment serum HCV RNA levels than NR, and the frequency of a sustained response in patients infected with HCV genotype lb (serotype 1) was less than in those infected with other HCV genotypes (serotype 2). Both serum HCV RNA levels and HCV genotypes are considered as strong predictors of sustained response. Interestingly, our results showed that among patients with low level viremia or HCV serotype 2 who were expected to have SVR, hepatic IFNAR2 in these patients were significantly higher than in Non-SVR. To explain the reason of IFN resistance in patients with favorable virus-related factors on the response to IFN therapy, the level of hepatic IFN receptor should be considered as an important patient-related factor influencing the response to IFN therapy (Fig. 2). Logistic analysis in this study also demonstrated that the level of hepatic IFN receptor was an important predictor of IFN therapy. Furthermore, the monitoring of expression of type I IFN receptor in patients before IFN therapy may help to predict treatment outcome. Indeed, this approach would provide a better management of IFN therapy by deciding the time of IFN treatment.

In conclusion, we demonstrated that the expression level of hepatic IFNAR2 correlated with the response to IFN therapy in patients with CHC and that measurement of hepatic IFNAR2 levels might be useful in predicting the response to IFN therapy. Resistance to IFN treatment in patients with CHC might be due to low levels of hepatic IFNAR2. Further studies are necessary to understand the role of IFNAR2 and to allow the discovery of new therapies for patients resistance to treatment with IFN.

IFN Signals

As both the type I and type II IFN receptors lack intrinsic kinase domains, they require the association with a family of non-receptor tyrosine kinases known as Janus-Kinases (JAKs) to facilitate the tyrosine phosphorylation of STAT proteins. IFN exert their signals through receptor-associated Janus-Kinases (JAKs) and signal transducers by phosphorylation of STAT1 and STAT2. After binding to receptors, IFNs inititate gene activation through phosphorylation of intermediary proteins. The receptor-associated kinase Tyk2, JAK1 and JAK2, phosphorylate STAT1 and STAT2 (Fig. 3) [12].

Type I IFN (IFN-α and IFN-β) induces the formation of a heterotrimetric transcription factor complex, interferon-stimulated gene factor 3 (ISGF3), which consists of signal transducers and activators of transcription 1 and 2 (STAT1 and STAT2) and p48 (ISGF3γ), after binding to its receptor. ISGF3 translocates into the nucleus and binds to to a specific gene sequence termed the IFN-stimulated response element (ISRE), characterized by a conserved 15 base pair element. The ISRE has a consensus sequence GAAN(N)GAAA (N=any nucleotide) to a specific gene sequence termed the IFN-stimulated response element (ISRE), characterized by a conserved 15 base pair element. The ISRE has a consensus sequence GAAN(N)GAAA (N=any nucleotide). The ISRE is a necessary and sufficient component of the induction of many genes by IFN [13].

IFN-γ binds a different receptor and activates STAT1, which, as a homodimer, activates a related but distinct nucleotide sequence resulting in transcription of

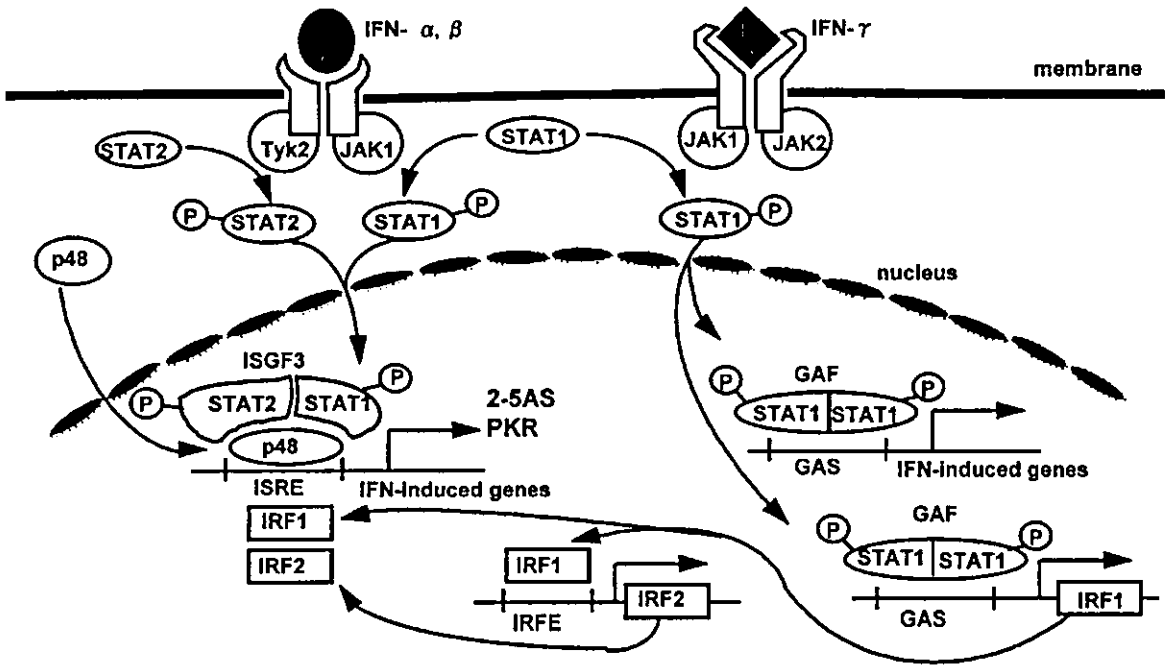

Fig. 3. The signal transduction pathway for IFNs

different genes and synthesis of a different set of proteins. An IFN-γ activation site (GAS) is composed of the consensus nucleotide sequence AANNNNNTT (N=any nucleotide) and confers responsiveness to IFN-γ after binding of a.STAT-1a homodimer [13].

Interferon regulatory factor-1 (IRF-1) is another important factor induced by type I and II IFNs. Since the sequence of IRF-1 binding site termed IRF-E overlaps with ISRE, IRF-1 also binds to ISRE sequence and activates the interferon-inducible gene transcription. p48 and IRF-1 show a homology within their amino-terminal regions and are members of the IRF family together with other IRFs [14].

IFN induces some interferon-inducible genes such as 2,5 oligo-adenylate synthetase (2-5AS) and double-stranded RNA-dependent protein kinase (PKR), inhibit the post-transcriptional stages of viral replication. The 2-5AS are a family of enzymes that convert ATP into unusual 2,5-oligo-adenylates (2-5A), which in turn activate a dominant ribonuclease, RnaseL, to degrade viral RNAs. The other antiviral enzyme, PKR, inhibits viral protein synthesis. It is activated by autophosphorylation of serine/threonine residues in the presence of dsRNA. Activated PKR phosphorylates eukaryotic translation initiation factor 2α (eIF-2α). Phosphorylated eIF-2α cannot participate in protein synthesis. Therefore, inhibition of PKR is essential for viral replication [15].

Recently it has been reported that JAB (JAK-binding protein), as a new molecule, strongly inhibits IFN-signaling by blocking JAK activity through directly binding to its kinase domain, and the loss of responsiveness to the antiviral effect of IFN-β in NIH-3T3 fibroblasts ectopically expressed JAB. It has been suggested that JAB might provide the influence on unresponsiveness and resistance to the IFN-therapy in some patients with viral disease [16].

Correlation Between Level of Hepatic IFN-Related Molecules and Response to IFN Therapy in Patients with Chronic Hepatitis C

Recently A.Katsume and M.Kohara, in the Department of Microbiol & Cell Biology in Tokyo Metro Institute Medical Science, developed quantitative method of IFN signals using real time detection polymerase chain reaction (RTD-PCR) based on Taq-Man chemistry. We examined IFN signals in liver tissue obtained from 22 patients with chronic hepatitis C later treated with IFN. According to viremia status after treatment, we divided into two groups: Sustained viral responders (SVR:n=9) and Non-sustained viral responders (Non-SVR:n=13). We examined mRNA level (expressed as Unit:U) of IFN receptor (IFN-R), double strand RNA-activated protein kinase (PKR), 2, 5 oligo-adenylate synthetase (2-5AS), interferon regulatory factor1(IRF1), IRF2, JAK-binding protein (JAB) and cytokine-inducible SH2 protein3 (CIS3) in liver tissue. It was shown that the mean levels of both IFN-R (1.195±0.311 U) and JAB (3.911±3.284U) in SVR were

significantly (P<0.01) higher than in NR (IFN-R:0.822±0.173U, JAB:1.471± 0.619). There was no difference of hepatic mRNA levels of PKR, 2-5AS, IRF1, IRF2 and CIS3 between SVR and Non-SVR (Table 2). This study also confirm that the levels of IFN-R are closely correlated with response to IFN therapy. But the expression levels of hepatic JAB mRNA, whose function was a strong inhibition of IFN-signaling by blocking JAK activity, were unexpectedly decreased in patients with Non-SVR group compared with SVR group. This paradoxical phenomenon may reflect the failure in IFN signal transduction in IFN resistant patients, because the expression of JAB is induced by IFN stimulation.

The action of IFN network such as IFN-R and JAB can contribute on the efficacy of IFN response. The resistance to IFN therapy might be due to low levels of hepatic IFN-R. A thorough understanding of intracellular IFN network that govern the IFN response will lay the foundation to identify the mechanisms that alter these effects in treatment of chronic hepatitis C.

Table 2. mRNA levels of several IFN related signals and IFN response in patients (n=22) with chronic hepatitis C

	SVR (n=9)	Non-SVR(n=13)	*P*-value
HCV-RNA	0.296±0.610	6.204±8.101	0.0424
IFN-R	1.195±0.311	0.822±0.173	0.0017
2-5AS	3.654±1.553	4.271±2.773	0.5539
PKR	1.507±0.637	1.272±0.621	0.3934
JAB	3.911±3.284	1.471±0.619	0.0157
CIS3	0.763±0.474	0.450±0.258	0.058
IRF1	4.648±1.343	3.595±1.530	0.1115
IRF2	3.633±0.953	3.062±0.674	0.1140

References

1. Uze G, Lutfalla G, Gresser. Genetic transfer of a functional human interferon -α receptor into mouse cells: cloning and expression of its cDNA. Cell 1990;60:225-234.
2. Novick D, Cohen B, Rubinstein M. The human interferon-α/β receptor: characterization and molecular cloning. Cell 1994;77:391-400.
3. Domanski P, Witte M, Kellum M, Rubinstein M, Hackett R, Pitha P, Colamonici OR. Cloning and expression of a long form of the beta subunit of the interferon alpha beta receptor that is required for signaling. J Biol Chem 1995;270:21606-11.
4. Pfeffer LM, Basu L, Pfeffer SR, Yang CH, Murti A, Russell-Harde D, Croze E. The short form of the interferon alpha/beta receptor chain 2 acts as a dominant negative for type I interferon action. J Biol Chem 1997;272:11002-5.
5. Mizukoshi E, Kaneko S, Kaji K, Terasaki S, Matsushita E, Muraguchi M, Ohmoto Y,

Kobayashi K. Serum levels of soluble interferon Alfa/Beta receptor as an inhibitory factor of interferon in the patients with chronic hepatitis C. Hepatology 1999;30:1325-31.

6. Han CS, Chen Y, Ezashi T, Roberts RM. Antiviral activities of the soluble extracellular domains of type I interferon receptors. Proc Natl Acad Sci USA 2001;98:6138-43.
7. Yatsuhashi H, Fujino T, Matsumoto T, Inoue O, Koga M, Yano M. Immunohistochemical analysis of hepatic interferon alpha-beta receptor level: Relationship between receptor expression and response to interferon therapy in patients with chronic hepatitis C. J Hepatol 1999;30:995-1003.
8. Navarro S, Colamonici OR, Llombart-Bosch A. Immunohistochemical detection of the type I interferon receptor in human fetal, adult, and neoplastic tissues. Mod Pathol 1996;9:150-156.
9. Volpes R, van den Oord JJ, De Vos R, Depla E, De Ley M, Desmet VJ. Expression of interferon-gamma receptor in normal and pathological human liver tissue. J Hepatol 1991;12:195-202.
10. Ishimura N, Fukuda R, Fukumoto S. Relationship between intrahepatic expression of interferon-a receptor mRNA and the histological progress of hepatitis C virus-associated chronic liver diseases. J Gastroenterol Hepatol 1996;11:712-717.
11. Miyajima I, Sata M, Gondo K, Suzuki H, Tanigawa K. Interferon accumulation in cirrhotic rat liver. J Vir Hepat 1996;3:23-27.
12. David M. Signal transduction by type I interferons. Biotechniques 2002;Suppl:58-65.
13. Borden EC. Gene regulation and clinical roles for interferons in neoplastic diseases. Oncologist 1998;3:198-203.
14. Harada H, Taniguchi T, Tanaka N. The role of interferon regulatory factors in the interferon system and cell growth control. Biochimie 1998;80:641-50.
15. Kalvakolanu DV. Virus interception of cytokine-regulated pathways. Trends Microbiol 1999;7:166-71.
16. Yasukawa H, Misawa H, Sakamoto H, Masuhara M, Sasaki A, Wakioka T, Ohtsuka S, Imaizumi T, Matsuda T, Ihle JN, Yoshimura A. The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. EMBO J 1999 ;18:1309-20.

3000 CA Rotterdam, The Netherlands
[2]Department of Pathology, Erasmus MC, University Medical Center Rotterdam, Room CA 326, P.O Box 2040, 3000 CA Rotterdam, The Netherlands

Acknowledgements

The authors thank Dr A.W. Langerak from the department of Immunology of the Erasmus MC Rotterdam, for performing the clonal rearrangement studies.

References

[1] Przybylski GK, Wu H, Macon WR, Finan J, Leonard DGB, Felgar RE, et al. Hepatosplenic and subcutaneous panniculitis-like γ/δ T cell lymphomas are derived from different Vδ subsets of γ/δ T lymphocytes. J Mol Diagn 2000;2:11–19.
[2] Wlodarska I, Martin-Garcia N, Achten R, De Wolf-Peeters C, Pauwels P, Tulliez M, et al. Fluorescence in situ hybridisation study of chromosome 7 aberrations in hepatosplenic T-cell lymphoma: isochromosome 7q as a common abnormality accumulating in forms with features of cytologic progression. Genes Chromosomes Cancer 2002;33:243–251.
[3] Khan WA, Yu L, Eisenbrey AB, Crisan D, Al Saadi A, Davis BH, et al. Hepatosplenic gamma/delta T-cell lymphoma in immunocompromised patients. Report of two cases and review of literature. Am J Clin Pathol 2001;116:41–50.
[4] Iannitto E, Barbera V, Quintini G, Cirrinsione S, Leone M. Hepatosplenic gamma/delta T-cell lymphoma: complete response induced by treatment with pentostatin. Br J Haematol Jun; 2002;117: 995–996.

doi:10.1016/S0168-8278(03)00272-1

Interleukin-10 promoter polymorphisms and liver fibrosis progression in patients with chronic hepatitis C in Japan

To the Editor:

Long-term morbidity and mortality in patients with chronic hepatitis C differ with race [1]. The development of hepatocellular carcinoma is more frequently observed in Japanese patients than in Western patients [1], and the same trend is observed for liver-related death. The reasons for this have not been cleared. We hypothesized that polymorphisms in the interleukin (IL)-10 gene promoter in Japanese patients may be a host factor contributing to the persistence of viremia and causing the unique time course of liver fibrosis that is strongly associated with the mortality of the hepatitis C virus (HCV) infection. We examined the inheritance of three biallelic polymorphisms in the IL-10 gene promoter, at positions −1082, −819, and −592 from the transcription start site, which produce three different haplotypes: GCC, ACC, and ATA.

Fifty-two Japanese healthy subjects (31 men and 21 women, aged 21–61 years [mean ± SD, 31 ± 7]) and 114 posttransfusion hepatitis C patients who had been infected for more than 30 years were analyzed. All the liver tissue specimens were obtained by needle biopsies. Samples were evaluated as described by Desmet et al. [2]. Stages F0 or F1, F2 or F3 and F4 were defined as 'slow or no-progression' (34 patients), 'intermediate progression' (33 patients) and 'rapid progression' (47 patients), respectively. In the three groups, there were no significant differences in the following eight factors: sex, age at infection, virus genomic type, HCV-RNA titer, excessive alcohol consumption, interferon treatment, duration from infection to entry, and age at entry. We compared haplotype frequency in the group using the χ^2 test with Yates' correction. Multiple logistic regression analysis was used to reveal any independent factor that contributed to the fibrotic stage. The Ethics Committees of the Nagasaki University Hospital and the National Nagasaki Medical Center approved the study protocol, and informed consent was obtained from each individual.

The distribution of haplotypes in patients with posttransfusion chronic hepatitis C and in control patients did not differ. Interestingly, the slow-progression group had the putative high IL-10 producing GCC haplotype to more often than the intermediate and rapid progression groups ($P < 0.01$). Individuals with the GCC haplotype were more likely to have less hepatic fibrosis, compared with individuals with the ATA or ACC haplotypes. This relationship remained significant ($P = 0.01$, OR = 0.1, 95% CI 0.01–0.9) after adjustment for potential confounding factors (Table 1).

As liver fibrosis may progress with time, it is difficult to estimate the potential degree of progression of a given patients [1,3]. In our study, however, the interval between HCV infection and the time at which liver tissue specimens were obtained was more than 30 years. It is unlikely that patients in the slow-progression stage in our study will progress to a severe fibrosis stage because rapid progression usually takes less than 20 years from infection to cirrhosis [1,3].

The IL-10 haplotype has a role in determining disease severity, but does not seem to be important in susceptibility [4]. We show that the distribution of haplotypes of the IL-10 promoter is very different in different races. The GCC haplotype is more frequent in Western populations than in

Table 1
IL-10 promoter haplotypes in chronic hepatitis C and healthy subjects

Haplotype (−1082/−819/−592)	Healthy subjects (controls)				Japanese chronic HCV patients		
	Spanish ($2n = 710$)	White ($2n = 482$)	Caucasoid ($2n = 238$)	Japanese ($2n = 104$)	Non-progressive F0/1 ($2n = 68$)	Intermediate F2/3 ($2n = 66$)	Progressive F4 ($2n = 94$)
ATA	0.25	0.22	0.21	0.69	0.67	0.6	0.68
				$n = 72$	$n = 46$	$n = 40$	$n = 64$
ACC	0.33	0.29	0.28	0.27	0.24	0.35	0.3
				$n = 28$	$n = 16$	$n = 23$	$n = 29$
GCC	0.42	0.49	0.51	0.04	0.09	0.05	0.01
				$n = 4$	$n = 6$	$n = 3$	$n = 1$

Note: Data on the Spanish control subjects are from Jose et al., Liver 2002;22:245–251. Data on the white control subjects are reprinted with from Sam Lim et al., Lancet 1998;352:113. Data on the Caucasoid control subjects are from Hajeer et al., Scand J Rheumatol 1998;27:142–145.

the Japanese population (Table 1) and, therefore, the association between disease severity and haplotype may differ with race.

Our results lead to the conclusion that, in chronic hepatitis C, the putative high IL-10 production haplotype GCC is more likely to be associated with inhibition of the progression of liver fibrosis. In fact, endogenous IL-10 has been shown to reduce the intrahepatic inflammatory response and to limit hepatotoxicity in several models of liver injury. Moreover, recombinant IL-10 has been reported to decrease hepatic inflammation and reduce liver fibrosis in patients with chronic hepatitis C [5]. Our findings suggest that IL-10 promoter polymorphisms have an important role in chronic inflammation and fibrogenesis in this disease. Because the frequency of the high IL-10 producing GCC haplotype in the Japanese population is less than in Western populations, this may be one of the reasons why Japanese chronic hepatitis C patients have a worse prognosis than Western patients do. However, the mechanisms of genetic action are complex. Further studies are needed to address the effects of these polymorphisms on IL-10 expression and to confirm these observations in Western populations.

Hisayuki Hamada[1,2], Hiroshi Yatsuhashi[1], Koji Yano[1], Kokichi Arisawa[2], Kazuhiko Nakao[2], Michitami Yano[1]
[1]*Clinical Research Center, WHO Collaborating Center for Reference and Research on Viral Hepatitis, National Nagasaki Medical Center, Kubara 2-1001-1, Omura 852-8562, Nagasaki, Japan*
[2]*First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki, Japan*
[3]*Department of Preventive Medicine and Health Promotion, Nagasaki University School of Medicine, Nagasaki, Japan*

References

[1] Pagliaro L, Peri V, Linea C, Camma C, Giunta M, Magrin S. Natural history of chronic hepatitis C. Ital J Gastroenterol Hepatol 1999;31:28–44.
[2] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology 1994;19:1513–1520.
[3] Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet 1997;349:825–832.
[4] Lim S, Crawley E, Woo P, Barnes PJ. Haplotype associated with low interleukin-10 production in patients with severe asthma. Lancet 1998;352:113.
[5] Nelson DR, Lauwers GY, Lau JY, Davis GL. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: a pilot trial of interferon non-responders. Gastroenterology 2000;118:655–660.

doi:10.1016/S0168-8278(03)00246-0

Cutae morphea associated with chronic hepatitis C

To the Editor:

Chronic hepatitis C (HCV) infection has been associated with numerous dermatologic conditions including lichen planus [1], porphyria cutanea tarda [2], cutaneous necrotizing vasculitis [3], erythema nodosum and multiform [4], urticaria [5] and, more recently systemic sclerosis [6]. To our knowledge, there has been no previous report of cutae morphea (localized scleroderma) among patients with HCV. In this communication, we report two patients with HCV-RNA-positive HCV infection and cutae morphea.

Case # 1 (WB): A 51-year-old African American (AA) male was referred to our service for further evaluation and management of his long-term HCV seropositivity. Two years earlier he noticed skin pigmentation and 'tightness' as well as articular and bone pains in all four extremities that got progressively worse. He was seen by a rheumatologist

THE AMERICAN JOURNAL OF GASTROENTEROLOGY
© 2002 by Am. Coll. of Gastroenterology
Published by Elsevier Science Inc.

Vol. 97, No. 8, 2002
ISSN 0002-9270/02/$22.00
PII S0002-9270(02)04283-1

Influence of Interleukin-10 Gene Promoter Polymorphisms on Disease Progression in Patients Chronically Infected With Hepatitis B Virus

Seiji Miyazoe, M.D., Keisuke Hamasaki, M.D., Keisuke Nakata, M.D., Yuji Kajiya, M.D., Kayo Kitajima, M.Sc., Kazuhiko Nakao, M.D., Manabu Daikoku, M.D., Hiroshi Yatsuhashi, M.D., Michiaki Koga, M.D., Michitami Yano, M.D., and Katsumi Eguchi, M.D.

First Department of Internal Medicine, Nagasaki University School of Medicine, Nagasaki; Health Research Center, Nagasaki University, Nagasaki; and National Nagasaki Medical Center, Nagasaki, Japan

OBJECTIVES: The role of host genetic factors in chronic hepatitis B virus (HBV) infection is not fully understood. We studied the influence of tumor necrosis factor-α (TNF-α) and interleukin-10 (IL-10) gene promoter polymorphisms on disease progression in HBV carriers.

METHODS: The sample population included 213 Japanese HBV carriers and 52 healthy volunteers. Of 213 HBV carriers, 66 were considered to be asymptomatic carriers based on the sustained normalization of serum ALT together with seropositivity for the antibody to hepatitis B e antigen (anti-HBe), and 147 were found to have chronic progressive liver disease including cirrhosis. Five biallelic polymorphisms in the TNF-α gene promoter and three biallelic polymorphisms in the IL-10 gene promoter were analyzed by polymerase chain reaction in combination with direct sequencing or restriction fragment length polymorphism assay.

RESULTS: Allelic distributions of both gene promoters were not significantly different between HBV carriers and healthy volunteers. In HBV carriers, the TNF-α gene promoter polymorphisms were not linked to disease progression. In contrast, allelic frequencies of T and A at positions -819 and -592, respectively, in the IL-10 gene promoter, as well as the frequencies of ATA haplotype at positions $-1082/-819/-592$ (which is characterized with low capacity for IL-10 production), were significantly higher in asymptomatic carriers than in patients with chronic progressive liver disease. Even after adjusting for individuals positive for anti-HBe, such a relationship could be found between the two groups.

CONCLUSION: In chronic HBV infection, inheritance of the IL-10 gene promoter polymorphisms is involved in a host genetic factor that is relevant to disease progression. (Am J Gastroenterol 2002;97:2086–2092. © 2002 by Am. Coll. of Gastroenterology)

INTRODUCTION

Hepatitis B virus (HBV) is the most common cause of acute and chronic liver disease worldwide, especially in several areas of Asia and Africa. It is estimated that HBV is present in a reservoir of more than 350 million chronic carriers, representing more than 5% of the world population (1, 2). Perinatal transmission of HBV, particularly mother-to-child transmission of the virus during the perinatal period (3–5), is a common source of chronic infection, resulting in 25% lifetime risk of death from cirrhosis or primary liver cancer (6).

The immunopathogenic mechanisms of HBV, by which a portion of chronic cases evolve into progressive liver disease, are under investigation (7). The long-term follow-up studies indicate that HBV carriers who are positive for antibody to hepatitis B e antigen (HBeAg), with sustained normalization of the serum levels of ALT, have a favorable outcome with a low risk of progression of liver damage including cirrhosis (8, 9). Recent studies have shown that certain mutations of the infected HBV confer survival advantage over the wild-type HBV by evading the host immune response or by enhanced viral replication, and that the emergence of such mutant viruses can modify the clinical course of the disease (10–12). Taken together, family studies in China provide some evidence that the host genetic factors influence viral persistence, as higher concordance rates were found for HBeAg persistence in identical compared with nonidentical twins (13). The majority of host genetic studies with HBV have focused on human leukocyte antigen associations (14–16), but one particular allele has not been clearly identified in association with HBV persistence or disease severity.

The role of cytokines in the viral clearance and the host immune response to the virus has been investigated. Several proinflammatory cytokines such as Th1 cytokines (including IL-2 and IFN-γ) and TNF-α are believed to participate in elimination of HBV (7, 17–20). In contrast, IL-10, a Th2

Table 1. Clinical Characteristics of 213 HBV Carriers

Variable	Asymptomatic Carriers (ASC) (N = 66)	Patients With Chronic Progressive Liver Disease (N = 147)
Sex (male/female)	33/33	105/42
Age (yr)*	51 ± 18	49 ± 15
HBeAg/anti-HBe status	/66	55/92
Definite ASC/chronic hepatitis-ASC	46/20	
Chronic hepatitis/cirrhosis		58/89
Serum ALT (IU/L)†	21 ± 8	92 ± 122
History of IFN-α therapy	6	25
Liver biopsy specimen available during study period	6	47
Stage of fibrosis		
F0–F2	5	16
F3–F4	1	31

* Age is expressed as mean ± SD.
† Serum ALT represents mean ± SD at baseline in each group, and difference is significant between the two groups ($p < 0.0001$).

cytokine, acts as a potent inhibitor of Th1 effector mechanisms (21–23). There is some evidence that the capacity for cytokine production in individuals has a major genetic component (24). This has been ascribed to polymorphisms within the regulatory regions or signal sequences of cytokine genes. Several biallelic polymorphisms have been described within the TNF-α gene, including five in the promoter region at positions −1031(T/C), −863(C/A), −857(C/T), −308(G/A), and −238(G/A) base pairs from the transcription start site (25, 26). Similarly, the promoter region of the IL-10 gene contains three biallelic polymorphisms at positions −1082(A/G), −819(T/C), and −592(A/C) base pairs from the transcription start site, which produce three different haplotypes, GCC, ACC and ATA (27–29). The aim of the present study was to clarify whether the inheritance of TNF-α or IL-10 gene promoter polymorphisms could serve as a candidate for determining clinical outcomes of the disease caused by chronic HBV infection.

MATERIALS AND METHODS

A total of 221 patients who were positive for hepatitis B surface antigen (HBsAg) visited the clinics for liver disease in Nagasaki University Hospital or National Nagasaki Medical Center between August, 1999, and June, 2000. They were regularly followed, with measurements of serum ALT and HBV makers such as HBsAg, HBeAg, and anti-HBe using commercially available radioimmunoassay kits (Dainabot, Tokyo, Japan) every month, and with ultrasonography or computed tomography of the liver every 3 months. Of these, 213 patients who continued to be positive for HBsAg for more than 6 months and did not have any other types of liver disease such as chronic hepatitis C, alcoholic liver disease, autoimmune hepatitis, or primary biliary cirrhosis underwent analysis for the TNF-α and IL-10 gene promoter polymorphisms. In addition, 52 healthy volunteers (31 men and 21 women, aged 21–61 years, mean ± SD 31±7 yr), were studied as a control group. The study protocol was approved by the Ethics Comittees of both Nagasaki University Hospital and National Nagasaki Medical Center, and informed consent was obtained from each individual.

Of the 213 HBV carriers, 66 (33 men and 33 women, 18–85 yr of age, mean ± SD 51±18 yr) were considered to be asymptomatic carriers (ASC) based on sustained normalization of the serum levels of ALT together with seropositivity for anti-HBe throughout the study (Table 1). This group of patients was further classified into the two subgroups; the first included 20 patients who had a past history of a flare-up of hepatitis (chronic hepatitis-ASC), and the second included 46 patients who had never been found to have such clinical evidence (*i.e.*, definite ASC). On the other hand, 147 of the 213 HBV carriers were considered to have chronic progressive liver disease (CPLD) such as chronic hepatitis or cirrhosis, manifested by elevated ALT levels and by clinical or histological findings on examination of liver tissue during the follow-up period. This group comprised 105 men and 42 women, aged 18–85 yr (mean ± SD 49 ± 15 yr). Of the 147 patients with CPLD, 58 (39%) were found to have chronic hepatitis; 89 (61%) had cirrhosis, including 23 patients with cirrhosis and hepatocellular carcinoma; and 55 (37%) were positive for HBeAg. The serum levels of ALT at baseline were significantly higher in CPLD patients than in ASC. Six and 25 patients in the ASC and CPLD groups, respectively, had received IFN-α treatment before enrollment in the study.

Of 213 HBV carriers, 53 had undergone liver biopsy during the study period, and they were accessed for the degree of liver fibrosis using the METAVIR system (30). To ensure sufficient numbers in each category, the severity of fibrosis was classified into two categories: F0–F2 and F3–F4. Among 53 patients, fibrosis was classified as category F0–F2 in five and 16 patients in the ASC and CPLD groups, respectively, whereas it was classified as F3–F4 in one and 31 patients in each group (Table 1).

Genomic DNA was extracted from PBMC using a Qiagen DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions. The 5′-

flanking region of the TNF-α gene, spanning from −1462 to −457, and the 5′-flanking region of the IL-10 gene, spanning from −1351 to +19, were amplified by PCR. The amplification was performed for 35 cycles on 2 μl of the extracted DNA and the oligonucleotide primers (for TNF-α, sense; 5′-GAGGGGAATAATAGAAGAAC-3′ and antisense; 5′-ATGCAGGAAAAAGATAGAAC-3′, for IL-10, sense; 5′-GTTCCTC-CCAGTTACAGTCT-3′ and antisense; 5′-CTGTCTTGTGGTTTGGTTTT-3′) in a volume of 50 μl using a polymerase chain reaction (PCR) amplification kit (Takara Biomedicals, Tokyo, Japan). For determination of allelic polymorphisms in the TNF-α promoter gene at positions −1031, −863, and −857, and those in the IL-10 promoter gene at positions −1082 and −819, each PCR product was purified by a Qiagen PCR purification kit, and the DNA sequence analysis was carried out using an Applied Biosystems prism big dye terminator cycle sequencing ready-reaction kit and an Applied Biosystems Model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Allelic polymorphisms of the IL-10 promoter gene at position −592 were determined by the PCR-restriction fragment length polymorphism (RFLP) technique. Briefly, the PCR product was digested with RsaI restriction enzyme. One base-exchange substitution from C to A at position −592 creates the RsaI restriction site. Therefore, −592 C allele produces 8-, 42-, 469-, and 851-bp fragments with RsaI digestion, whereas −592 A allele produces 8-, 42-, 469-, 240-, and 611-bp fragments. Similarly, allelic polymorphisms in the TNF-α gene promoter at positions −308 and −238 were analyzed by the PCR-RFLP technique with modification of the methods of Grove *et al.* (31). Briefly, 194- and 152-bp fragments containing the TNF-α gene promoter at positions −308 and −238, respectively, were amplified by PCR with the oligonucleotide primers (for 194-bp fragment, sense 5′-AATAGGTTTT-GAGGGCCATG-3′ and antisense 5′-CCTTCTG-TCTCGGTTTCTTC-3′; for 152-bp fragment, sense 5′-AGAAGACCCCCCTCGGA-ACC-3′ and antisense 5′-ATCTGGAGGAAGCGGTAGTG-3′). The PCR products were digested with NcoI or MspI restriction enzymes. One base-exchange substitution from A to G at position −308 creates the NcoI restriction site. Thus, −308 G allele produces 20- and 174-bp fragments with NcoI digestion, whereas −308 A allele cannot produce such fragments. One base-exchange substitution from A to G at position −238 creates the MspI restriction site, resulting in 20- and 132-bp fragments with MspI digestion, whereas −238 A allele cannot create the MspI restriction site.

Statistical Analysis

Results are expressed as mean ± SD. Comparisons were made using Student's *t* test, Fisher's extract probability test, and the χ^2 test. Odds ratios (with 95% CI) were calculated to measure the relative risks in various subgroups of patients. All *p* values were two-tailed, and *p* values <0.05 were considered to indicate statistical significance.

RESULTS

TNF-α Gene Promoter Polymorphisms in HBV Carriers

Five biallelic polymorphisms in the TNF-α gene promoter were determined in this study (Table 2). The allelic distribution or allelic frequencies of the TNF-α gene promoter in HBV carriers did not differ from those in healthy volunteers. Taken together, no significant difference in the TNF-α gene promoter polymorphisms could be found between ASC and patients with CPLD.

IL-10 Gene Promoter Polymorphisms in HBV Carriers

Three biallelic polymorphisms and haplotype frequencies in the IL-10 gene promoter were analyzed (Table 3). The majority of HBV carriers as well as healthy volunteers had A allele at position −1082 in the IL-10 gene promoter. In addition, there was no significant difference in the frequencies of alleles or haplotypes in the IL-10 gene promoter between HBV carriers and healthy volunteers. However, among HBV carriers, the allelic frequency of T at position −819 in ASC was significantly higher than that in patients with CPLD ($p = 0.0157$), and the difference was recognized irrespective of HBeAg/anti-HBe status of patients with CPLD. Because linkage disequilibrium with the allele in the IL-10 gene promoter is present, in which T and C alleles at position −819 always correspond to A and C alleles at position −592, respectively, as reported previously (27–29), the allelic frequencies of A at position −592 were also higher in ASC than in patients with CPLD ($p = 0.0157$). Similarly, the frequencies of ATA haplotype were significantly higher in ASC than in patients with CPLD ($p = 0.0157$). Even after adjusting for individuals positive for anti-HBe, the difference in ATA haplotype frequency was statistically significant between the two groups ($p = 0.0169$). Conversely, patients with CPLD tended to have ACC haplotype and were found to have GCC haplotype more frequently than ASC ($p = 0.0498$).

Association of IL-10 Gene Promoter Polymorphisms With Disease Progression in HBV Carriers

For further analysis of the relationship between IL-10 gene promoter polymorphisms and disease progression in HBV carriers, the ASC group of patients was divided into two subgroups (definite ASC and chronic hepatitis-ASC, according to the absence or presence of a past history of a flare-up of hepatitis), whereas the CPLD group of patients was classified into patients with chronic hepatitis and those with cirrhosis, as mentioned in Materials and Methods. As shown in Table 4, the IL-10 gene promoter polymorphisms at positions −1082, −819, and −592, respectively, from the transcription start site, produced the following combinations of haplotypes in HBV carriers: ATA/ATA, characterized by a low capacity for IL-10 production; ACC/ATA or ACC,

Table 2. Allelic Distribution of TNF-α Gene Promoter

Polymorphism	Healthy Volunteers (N = 52)	Overall HBV Carriers (N = 213)	ASC/Patients With CPLD (N = 66)/ (N = 147)
	Number (%) of Subjects		
Genotype at position −1031			
T/T	36 (69)	148 (70)	43 (65)/105 (71)
T/C	14 (27)	61 (29)	21 (32)/40 (27)
C/C	2 (4)	4 (2)	2 (3)/2 (1)
Genotype at position −863			
C/C	38 (73)	153 (72)	44 (67)/109 (74)
C/A	13 (25)	56 (26)	21 (32)/35 (24)
A/A	1 (2)	4 (2)	1 (2)/3 (2)
Genotype at position −857			
C/C	35 (67)	137 (64)	45 (68)/92 (63)
C/T	15 (29)	67 (32)	19 (29)/48 (33)
T/T	2 (4)	9 (4)	2 (3)/7 (5)
Genotype at position −308			
G/G	50 (96)	207 (97)	64 (97)/143 (97)
G/A	2 (4)	6 (3)	2 (3)/4 (3)
A/A	0	0	0/0
Genotype at position −238			
G/G	49 (94)	201 (94)	61 (92)/140 (95)
G/A	3 (6)	12 (6)	5 (8)/7 (5)
A/A	0	0	0/0
		Allelic Frequency	
C at position −1031	0.17	0.16	0.19/0.15
A at position −863	0.14	0.15	0.17/0.14
T at position −857	0.18	0.20	0.17/0.21
A at position −308	0.02	0.01	0.02/0.01
A at position −238	0.03	0.03	0.04/0.02

and GCC/ATA or ACC with an intermediate capacity for IL-10 production; and GCC/GCC, with high capacity for IL-10 production. When the frequencies of ATA, ACC, and GCC haplotypes in the IL-10 gene promoter were compared in these subgroups of HBV carriers, ATA haplotype frequencies were significantly higher in definite ASC than in patients with chronic hepatitis ($p = 0.0499$) and decreased in parallel with disease progression in HBV carriers; thus, the difference in ATA haplotype frequency between definite ASC and patients with cirrhosis reached an OR of 2.19 (95% CI = 1.25–3.85, $p = 0.0066$). Similarly, the inheritance of ACC haplotype was inversely associated with definite ASC status, with an OR of 0.56 (95% CI = 0.31–1.00, $p = 0.0497$) compared to patients with cirrhosis. A similar trend was observed in GCC haplotype; in fact, it was noteworthy that GCC/GCC haplotype was found in two patients, both of whom had cirrhosis. Furthermore, a statistically significant relationship was also seen between IL-10 gene promoter polymorphisms and the stage of hepatic fibrosis, although patients who had undergone liver biopsy during the study period were limited (Table 5). ATA haplotype frequencies were significantly higher in patients with fibrosis stage F0–F2 than in patients with fibrosis stage F3–F4 (OR = 3.44, 95% CI = 1.45–8.17, $p = 0.0042$).

DISCUSSION

The host genetic factors involving genetic polymorphisms are believed to be responsible for clinical outcomes of infectious disease (32–34), because differences in the susceptibility to infection or severity of disease cannot solely be attributed to the virulence of an organism. For chronic viral hepatitis, genetic associations are likely to provide some clues to viral persistence and disease progression, and might lead to a new therapeutic approach. Recent studies have shown that several immunoregulatory cytokines such as IFN-γ and TNF-α inhibit HBV replication through the noncytolytic process (7, 17, 18). In contrast, IL-10 counteracts their effector mechanisms (21–23). Because the capacity for cytokine production in individuals largely depends on genetic polymorphisms (24), heterogeneity of the candidate gene in patients with HBV emerges as a probable biomarker for determining the disease phenotypes.

In the present study, five biallelic polymorphisms in the TNF-α gene promoter and three biallelic polymorphisms in the IL-10 gene promoter, which are known to alter their gene expression (25–29), were analyzed in 213 Japanese HBV carriers and 52 healthy volunteers as a control. The allelic frequencies of both gene promoters in HBV carriers did not differ from the frequencies in healthy volunteers or

Table 3. Allelic Distribution and Haplotype Frequencies of IL-10 Gene Promoter

Polymorphisms	Healthy Volunteers (N = 52)	Overall HBV Carriers (N = 213)	ASC/Patients With CPLD; HBeAg/Anti-HBe Status (N = 66)/(N = 147); (N = 55)/(N = 92)
	Number (%) of Subjects		
Genotype at position −1082			
A/A	48 (92)	201 (94)	65 (99)/136 (93);52 (95)/84 (91)
A/G	4 (8)	10 (5)	1 (2)/9 (6);3 (5)/6 (7)
G/G	0	2 (1)	0/2 (1);0/2 (2)
Genotype at position −819			
T/T	26 (50)	95 (45)	37 (56)/58 (40);22 (40)/36 (39)
T/C	20 (39)	91 (43)	24 (36)/67 (46);26 (47)/41 (45)
C/C	6 (12)	27 (13)	5 (8)/22 (15);7 (13)/15 (16)
Genotype at position −592			
A/A	26 (50)	95 (45)	37 (56)/58 (40);22 (40)/36 (39)
A/C	20 (39)	91 (43)	24 (36)/67 (46);26 (47)/41 (45)
C/C	6 (12)	27 (13)	5 (8)/22 (15);7 (13)/15 (16)
	Allelic Frequency		
A at position −1082	0.96	0.97	0.99/0.96; 0.97/0.95
T at position −819	0.69	0.66	*0.75/0.62; 0.64/0.61
A at position −592	0.69	0.66	*0.75/0.62; 0.64/0.61
	Haplotype (−1082/ −819/ −592) Frequency		
ATA	0.69	0.66	*0.74/0.62; 0.64/0.61
ACC	0.27	0.31	0.25/0.33; 0.29/0.34
GCC	0.04	0.03	†0.01/0.04; 0.00/0.05

* $p < 0.02$ compared with patients with CPLD irrespective of HBeAg/anti-HBe status.
† $p < 0.05$ compared with patients with CPLD.

those in the Japanese control population reported previously (25, 35). In addition, the inheritance of the TNF-α gene promoter polymorphisms could not be found to influence disease progression in HBV carriers. Several investigators suggest that TNF-α functions to suppress HBV gene expression in *in vitro* experiments or in animal models (7, 17, 18). Given these data, a recent report showed that the A allele at position −238 in the TNF-α gene promoter was closely linked to HBV persistence in a cohort of German patients (36). However, in Japan, as well as in other areas of Asia, vertical transmission of HBV in infancy is a major cause of chronic infection (3–5). Furthermore, the frequency of the −238 promoter variant was as low as less than 5% in the current study. Thus, ethnic differences in the mode of infection and the distribution of allelic polymorphisms may account for the lack of the correlation in our study.

Among three biallelic polymorphisms of the IL-10 gene promoter, more than 95% of individuals had A allele at

Table 4. Haplotype Distribution of IL-10 Gene Promoter in HBV Carriers

Haplotype (−1082/−819/−592) (Capacity for IL-10 Production)	Asymptomatic Carriers (ASC) (N = 66)		Patients With Chronic Progressive Liver Disease (N = 147)	
	Definite ASC (N = 46)	Chronic Hepatitis-ASC (N = 20)	Chronic Hepatitis (N = 58)	Cirrhosis (N = 89)
	Number (%) of Subjects			
ATA/ATA (Low)	27 (59)	10 (50)	22 (38)	36 (40)
ACC/ATA or ACC (Intermediate)	18 (39)	10 (50)	32 (55)	46 (52)
GCC/ATA or ACC (Intermediate)	1 (2)	0	4 (7)	5 (6)
GCC/GCC (High)	0	0	0	2 (2)
	Haplotype Frequency			
ATA (Low)	*0.77	0.68	0.65	0.61
ACC (Intermediate)	†0.22	0.33	0.32	0.34
GCC (High)	0.01	0.00	0.03	0.05

* $p < 0.05$ and $p < 0.01$ *vs* patients with chronic hepatitis and those with cirrhosis, respectively.
† $p < 0.05$ *vs* patients with cirrhosis.

Table 5. Haplotype Distribution of IL-10 Gene Promoter and METAVIR Score in HBV Carriers

Haplotype (−1082/−819/−592) (Capacity for IL-10 Production)	Stage of Fibrosis F0–F2 (N = 21)	F3–F4 (N = 32)
	Number (%) of Subjects	
ATA/ATA (Low)	12 (57)	10 (31)
ATA/ATA or ACC (Intermediate)	9 (43)	17 (53)
GCC/ATA or ACC (Intermediate)	0 (0)	4 (13)
GCC/GCC (High)	0 (0)	4 (3)
	Haplotype Frequency	
ATA (Low)	0.79*	0.52
ACC (Intermediate)	0.21	0.39
GCC (High)	0.00	0.10

* $p < 0.005$.

position −1082. Taken together, linkage disequilibrium with the allele in the IL-10 gene promoter was found as described previously (27–29). Accordingly, only three haplotypes, GCC, ACC, and ATA at positions −1082/−819/−592, were detected in this study. In HBV carriers, allelic frequencies of T and A at positions −819 and −592, respectively, as well as ATA haplotype frequencies in the IL-10 gene promoter were significantly higher in ASC than in patients with CPLD. Such results were obtained even after adjusting for individuals positive for anti-HBe. Conversely, GCC haplotype frequencies were significantly lower in ASC than in patients with CPLD. Moreover, the frequencies of ATA haplotype, in contrast to ACC or GCC haplotypes, decreased in association with disease progression, particularly progression to cirrhosis, in HBV carriers. As several lines of evidence indicate that ATA haplotype in the IL-10 gene promoter is relevant to a genetically low capacity for IL-10 production, whereas GCC haplotype is identified as a high IL-10-producing phenotype (27–29), it is conceivable that the difference in disease progression in patients with HBV depends in part on different capacities for IL-10 production resulting from the inheritance of the IL-10 gene promoter polymorphisms.

Nelson *et al.* (37) reported that a 3-month course of IL-10 therapy resulted in amelioration of hepatic inflammation and reduced the severity of fibrosis in patients with chronic hepatitis C, suggesting a reduction in immune response and fibrogenesis. Furthermore, they also showed that IL-10 therapy led to a decrease in serum TNF-α level and IFN-γ production from PBMCs. These cytokines play an important role in the elimination of HBV. Although IL-10 has both anti-inflammatory and antifibrotic properties, high levels of IL-10 production may increase viral replication in chronic HBV infection and result in enhancement of the immune response. Our finding that high IL-10–producing haplotypes were associated with more fibrosis is inconsistent with the above results. Such discrepancy may reflect differences in the immunopathogenesis of HBV and HCV infection.

IL-10 gene promoter polymorphisms have been implicated in evaluating the severity of several inflammatory diseases (29, 33, 34). Taken together, the recent investigation has shown that responsiveness to IFN-α treatment in patients with chronic hepatitis C is closely linked to ATA haplotype of the IL-10 gene promoter (28). Monocytes are the main source of IL-10 production after stimulation (23). However, Kupffer cells and stellate cells have also been shown to produce IL-10, and this site of production seems to contribute directly to the process of immune-mediated viral eradication and liver inflammation (38, 39). Our results suggest that patients who are genetically predisposed to a low capacity for IL-10 production have a relatively favorable outcome in chronic HBV infection. Both viral and host genetic factors are involved in viral pathogenesis of liver disease. Of these, analysis of the IL-10 gene promoter polymorphisms may have prognostic significance in patients with HBV, although ethnic differences can modulate the magnitude of the clinical usefulness of this tool.

ACKNOWLEDGMENTS

We thank Ms. Rumiko Hamada and Mr. Tatsuya Fujino in National Nagasaki Medical Center, and Ms. Yuuki Ishida and Ms. Masako Matsuo in Nagasaki University Hospital for invaluable assistance and technical support.

Reprint requests and correspondence: Katsumi Eguchi, M.D., First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan.
Received Sep. 10, 2001; accepted Feb. 4, 2002.

REFERENCES

1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997; 337:1733–45.
2. Andre FE, Zuckerman AJ. Protective efficacy of hepatitis B vaccines in neonates. J Med Virol 1994;44:144–51.
3. Okada K, Kamiyama I, Inomata M, et al. e Antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of

positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 1976;294:746–9.
4. Stevens CE, Beasley RP, Tsui J, et al. Vertical transmission of hepatitis B antigen in Taiwan. N Engl J Med 1975;292:771–4.
5. Matsuo A, Kusumoto Y, Ohtsuka E, et al. Changes in HBsAg carrier rate in Goto Islands, Nagasaki Prefecture, Japan. Lancet 1990;335:955–7.
6. West DJ, Calandra GB, Ellis RW. Vaccination of infants and children against hepatitis B. Pediatr Clin North Am 1990;37: 585–601.
7. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. Annu Rev Immunol 2001;19:65–91.
8. Dragosics B, Ferenci P, Hitchman E, et al. Long-term follow-up study of asymptomatic HBsAg-positive voluntary blood donors in Austria: A clinical and histologic evaluation of 242 cases. Hepatology 1987;7:302–6.
9. Villeneuve JP, Desrochers M, Infante-Rivard C, et al. A long-term follow-up study of asymptomatic hepatitis B surface antigen-positive carriers in Montreal. Gastroenterology 1994; 106:1000–5.
10. Hamasaki K, Nakata K, Nagayama Y, et al. Changes in the prevalence of HBeAg-negative mutant hepatitis B virus during the course of chronic hepatitis B. Hepatology 1994;20:8–14.
11. Moriyama K, Okamoto H, Tsuda F, et al. Reduced precore transcription and enhanced core-pregenome transcription of hepatitis B virus DNA after replacement of the precore-core promoter with sequences associated with e antigen-seronegative persistent infections. Virology 1996;226:269–80.
12. Kajiya Y, Hamasaki K, Nakata K, et al. A long-term follow-up analysis of serial core promoter and precore sequences in Japanese patients chronically infected by hepatitis B virus. Dig Dis Sci 2001;46:509–15.
13. Lin TM, Chen CJ, Wu MM, et al. Hepatitis B virus markers in Chinese twins. Anticancer Res 1989;9:737–41.
14. Thio CL, Carrington M, Marti D, et al. Class II HLA alleles and hepatitis B virus persistence in African Americans. J Infect Dis 1999;179:1004–6.
15. Hohler T, Gerken G, Notghi A, et al. HLA-DRB1*1301 and *1302 protect against chronic hepatitis B. J Hepatol 1997;26: 503–7.
16. Thursz MR, Kwiatkowski D, Allsopp CE, et al. Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N Engl J Med 1995;332:1065–9.
17. Guidotti LG, Ishikawa T, Hobbs MV, et al. Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes. Immunity 1996;4:25–36.
18. Guidotti LG, Ando K, Hobbs MV, et al. Cytotoxic T lymphocytes inhibit hepatitis B virus gene expression by a noncytolytic mechanism in transgenic mice. Proc Natl Acad Sci USA 1994;91:3764–8.
19. Guidotti LG, Guilhot S, Chisari FV. Interleukin-2 and alpha/beta interferon down-regulate hepatitis B virus gene expression in vivo by tumor necrosis factor-dependent and -independent pathways. J Virol 1994;68:1265–70.
20. Rico MA, Quiroga JA, Subira D, et al. Hepatitis B virus-specific T-cell proliferation and cytokine secretion in chronic hepatitis B e antibody-positive patients treated with ribavirin and interferon alpha. Hepatology 2001;33:295–300.
21. Fiorentino DF, Bond MW, Mosmann TR. Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J Exp Med 1989;170: 2081–95.
22. Fiorentino DF, Zlotnik A, Vieira P, et al. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J Immunol 1991;146:3444–51.
23. Mosmann TR. Properties and functions of interleukin-10. Adv Immunol 1994;56:1–26.
24. Westendorp RG, Langermans JA, Huizinga TW, et al. Genetic influence on cytokine production in meningococcal disease. Lancet 1997;349:1912–3.
25. Higuchi T, Seki N, Kamizono S, et al. Polymorphism of the 5'-flanking region of the human tumor necrosis factor (TNF)-alpha gene in Japanese. Tissue Antigens 1998;51:605–12.
26. Uglialoro AM, Turbay D, Pesavento PA, et al. Identification of three new single nucleotide polymorphisms in the human tumor necrosis factor-alpha gene promoter. Tissue Antigens 1998;52:359–67.
27. Turner DM, Williams DM, Sankaran D, et al. An investigation of polymorphism in the interleukin-10 gene promoter. Eur J Immunogenet 1997;24:1–8.
28. Edwards-Smith CJ, Jonsson JR, Purdie DM, et al. Interleukin-10 promoter polymorphism predicts initial response of chronic hepatitis C to interferon alfa. Hepatology 1999;30: 526–30.
29. Crawley E, Kay R, Sillibourne J, et al. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. Arthritis Rheum 1999;42:1101–8.
30. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology 1996;24:289–93.
31. Grove J, Daly AK, Bassendine MF, et al. Association of a tumor necrosis factor promoter polymorphism with susceptibility to alcoholic steatohepatitis. Hepatology 1997;26:143–6.
32. McGuire W, Hill AV, Allsopp CE, et al. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. Nature 1994;371:508–10.
33. Helminen M, Lahdenpohja N, Hurme M. Polymorphism of the interleukin-10 gene is associated with susceptibility to Epstein-Barr virus infection. J Infect Dis 1999;180:496–9.
34. Mozzato-Chamay N, Mahdi OS, Jallow O, et al. Polymorphisms in candidate genes and risk of scarring trachoma in a Chlamydia trachomatis–endemic population. J Infect Dis 2000;182:1545–8.
35. Ito M, Takahashi H, Fuse K, et al. Polymorphisms of tumor necrosis factor-alpha and interleukin-10 genes in Japanese patients with idiopathic dilated cardiomyopathy. Jpn Heart J 2000;41:183–91.
36. Hohler T, Kruger A, Gerken G, et al. A tumor necrosis factor-alpha (TNF-alpha) promoter polymorphism is associated with chronic hepatitis B infection. Clin Exp Immunol 1998;111:579–82.
37. Nelson DR, Lauwers GY, Lau JY, et al. Interleukin 10 treatment reduces fibrosis in patients with chronic hepatitis C: A pilot trial of interferon nonresponders. Gastroenterology 2000; 118:655–60.
38. Thompson K, Maltby J, Fallowfield J, et al. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. Hepatology 1998;28:1597–606.
39. Thompson KC, Trowern A, Fowell A, et al. Primary rat and mouse hepatic stellate cells express the macrophage inhibitor cytokine interleukin-10 during the course of activation in vitro. Hepatology 1998;28:1518–24.

Molecular Biology and Immunology in Hepatology
Edited by T. Tsuji, T. Higashi, M. Zeniya and K.-H. Meyer zum Büschenfelde

CHAPTER 3

Genetic Diversity and Pathophysiology of Hepatitis B Virus

Hiroshi Yatsuhashi and Michitami Yano

Institute for Clinical Research, National Nagasaki Medical Center, and WHO Collaborating Center for Reference and Research on Viral Hepatitis, Nagasaki, Japan

INTRODUCTION

The hepatitis B virus (HBV), discovered in 1966, infects more than 350 million people worldwide. One third of the global population has been acutely infected with HBV. Hepatitis B is a leading cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, accounting for 1 million deaths annually. Viral and host factors, such as age, immunosuppression, and sex contribute to the patient outcome [1]. Recent advances in polymerase chain reaction (PCR) and DNA sequencing technique made it possible to identify various HBV mutations. So far, increasing attention focused on the contribution of variant HBV strains to the clinical course of acute or chronic infection has been reported and discussed widely. Mutant HBV might display enhanced virulence with increased levels of HBV replication, resistance to antiviral therapies, facilitated cell attachment/penetration, or alteration of epitopes important in the host immune response [2]. This review addresses clinical aspects of genetic diversity and pathophysiology of HBV, including our data.

1. HBV-DNA

HBV belongs to a family of closely related DNA viruses called the hepadnaviruses. The viral genome of HBV is a partially double-stranded circular DNA of approximately 3200 base pairs that encodes four overlapping open reading frames. The four genes are core, surface, X, and polymerase. The core gene encodes the core nucleocapsid protein (important in viral packaging) and hepatitis B e antigen (HBeAg). The surface gene encodes pre-S1, pre-S2, and S protein (yielding large, middle, and small surface proteins, respectively). The X gene encodes the X protein, which has transactivating properties and may be important in hepatic carcinogenesis. The polymerase gene encodes a large protein with functions critical for packaging and DNA replication (including priming, RNA- and DNA-dependent DNA polymerase) [1,2] (Fig. 1).

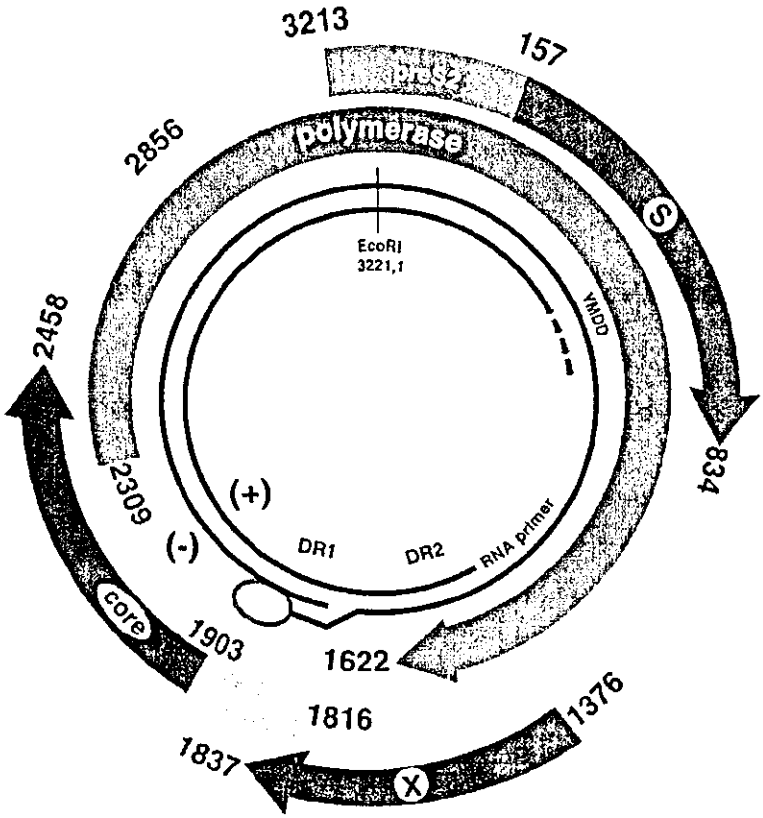

Fig. 1. Genome of HBV (genotype A). The inner circles represent the minus (−) and (+) DNA strands of the viral genome. The HBV polymerase is shown as an circle covalently bound to the 5′-end of the (−) DNA strand. The different open reading frames encoded by the genome, designated as S, core, polymerase, and X, are indicated by arrows. Nucleotide numbers designate the boundaries of each open reading frame with position 1 mapped at the *Eco*RI site. S, surface antigen; Y, tyrosine; M, methionine; D, aspartate; DR, direct repeat segment, used in viral replication

It is known that HBV replication cycle requires a reverse transcription step. HBV undergoes a higher rate of mutations than other DNA viruses, because the HBV P protein, like the reverse transcriptase of other viruses, lacks proofreading functions. Therefore, HBV exhibits a mutation rate more than 10-fold higher than other DNA viruses, the estimated mutation rate is approximately 1 nucleotide/10,000 bases/infection year [3].

2. MUTATIONS IN THE PRECORE/CORE REGION

The X open reading frame encodes the X protein that has transactivating activity and contains the basic core promoter (BCP). The BCP, mapping between nucleotides 1744 and 1804, controls the transcription of both precore messenger RNA (mRNA), which codes for the protein that is the precursor of the e antigen, and pregenomic RNA (pgRNA), which controls HBV replication. A variety of precore/core mutants have been described. The two well-defined precore mutations include a stop codon mutation at nucleotide (nt) 1896 and mutations in the basal core promoter (BCP) at nt 1762 and nt 1764.

2.1. Precore stop mutation

Mutations in the precore region of the HBV genome have been reported in many HBeAg-negative patients with chronic HBV infection. The predominant mutation involves a G-to-A change at nucleotide 1896 (A1896), which creates a premature stop codon at codon 28. This mutation prevents the translation of the precore protein and completely abolishes the production of HBeAg. Longitudinal studies found that A1896 emerges or is selected around the time of HBeAg seroconversion. These findings suggest that A1896 plays an important role in HBeAg clearance [4].

However, not all patients develop A1896 after HBeAg seroconversion; some patients retain wild-type precore sequence, while others have undetectable HBV DNA in serum by PCR. Furthermore, marked geographic differences in the prevalence of these precore stop mutant viruses have been noted. Only 10% of HBV isolates from US patients with fulminant hepatitis and 12–27% of isolates from US and European patients with chronic active hepatitis exhibit these precore stop mutations, whereas 47–60% of isolates from patients with chronic active hepatitis in Asia, Africa, Southern Europe, and the Middle East exhibit these stop mutations [5,6].

Recently, seven genotypes of HBV are recognized that are distinguished by differences of more than 8% in the entire nucleotide sequence of approximately 3,200 nt, and they are designated by capital letters from A to G [7]. HBV genotypes have distinct geographical distributions [8]. Overall, genotypes B and C are frequent in Asia, whereas genotypes A and D prevail in Western countries. Genotype E is restricted to Africa, and genotype F prevails in Central America. Genotype G was added to the alphabet list very recently, and its distribution is yet to be determined. It is now recognized that the occurrence of A1896 is restricted to HBV genotypes with T at nucleotide 1858. A change from G to A at nucleotide 1896 increases the stability of the stem–loop structure of the pregenome encapsidation sequence (e) when the opposite nucleotide at 1858 is a T (T1858), but this change disrupts a pre-existing C–G pair when the nucleotide at 1858 is a C (C1858) [9]. The restriction of A1896 to specific HBV genotypes accounts for its high prevalence in Asia and the Mediterranean basin, where the predominant HBV genotypes (B, C, and D) frequently have T1858, and its low prevalence in North America and Europe, where the predominant HBV genotype (A) almost always has C1858.

2.2. BCP mutations (X-ORF region)

The core promoter controls the transcription of the two core gene products: core and precore RNA. The core RNA is essential for viral replication because it encodes the major capsid protein and the viral polymerase; it also serves as the pregenomic RNA. The nonessential precore RNA encodes the precore protein, which is processed in the endoplasmic reticulum to produce the secreted HBeAg.

A pair of mutations in the BCP that is associated with a reduced level of HBeAg expression was first described in Japanese patients; an adenine (A) to thymine (T) transversion at position 1762 together with a guanine (G) to A transition at 1764 in the second AT-rich region of the BCP are often present in patients with chronic hepatitis B

and fulminant hepatitis B, and less often in asymptomatic carriers, immunosuppressed patients, and carriers without serological HBV markers [10,11].

In some patients, the TA changes were not associated with A1896, suggesting that mutations in the core promoter region alone may play a role in HBeAg clearance. This is supported by in vitro observations that the TA changes decrease transcription of precore mRNA and secretion of HBeAg [12]. However, TA changes have also been found in some HBeAg-positive patients, especially those with chronic hepatitis.

Recently, Orito et al. [13] have reported the BCP double mutation was significantly associated with genotype C, more severe disease (liver cirrhosis with or without hepatocellular carcinoma) and old age (more than 35 years), but it was not associated with sex, HBeAg, HBV-DNA,or the precore mutation (A1896) in a case-control study. Their study indicated that the occurrence of BCP double mutation was associated with genotype C and a longer duration of infection in aggravation of chronic hepatitis B. They also indicated that BCP double mutation was not associated with the HBeAg/anti-HBe status,unlike the preC mutation that was closely associated with anti-HBe.

From these considerations, all discussion on preC and BCP double mutation in patients with chronic HBV infection should carried out with regard to the association with HBV genotypes. The study of HBV genotypes is required to define the clinical significance of these mutations.

3. MUTATIONS IN PATIENTS WITH HBV-RELATED FULMINANT HEPATITIS

Approximately 1% of patients with acute HBV infection develop fulminant hepatitis. Fulminant hepatitis B is widely thought to be caused by a well-described HBeAg-negative mutant of HBV, which has a point mutation (G to A at nucleotide (nt) 1896) in the precore region that blocks synthesis of HBeAg. Omata et al. [14] found precore mutations in all of seven Japanese patients with fulminant hepatitis B. Similarly, Liang et al. [15] found precore mutations in all five patients with fulminant hepatitis in an outbreak of hepatitis B in Israel. However, subsequent studies in patients from the United States and France have indicated that these mutations are neither necessary nor sufficient for fulminant hepatitis B [16–18]. Sato et al. [11] reported an association between core promoter mutations (especially an A-to-T substitution at nt 1762 and a G-to-A transition at nt 1764) and fulminant hepatitis B in Japan. These mutations were uniformly present in patients with fulminant hepatitis without, as well as in a majority of patients with, the classic nt 1896 mutation.

In order to investigate the association of mutations in the precore and core promoter region of HBV with fulminant and severe acute hepatitis, we demonstrated our study results in Japanese patients with acute HBV infection, including 12 patients with fulminant hepatitis, 23 with severe acute hepatitis and 98 with acute self-limited hepatitis (Fig. 2). They were all admitted to five hospitals (National Nagasaki Medical Center, Osaka National Hospital, Shinsyu Medical University Hospital, Miyazaki Medical University Hospital and Kurume Medical University Hospital). We used the following diagnostic criteria for fulminant hepatitis as described by Trey et al. [19]: (1) the development of stage II–IV hepatic encephalopathy within 8 weeks of the onset of illness; (2) prothrombin time less than 40%; and (3) no known history of liver disease

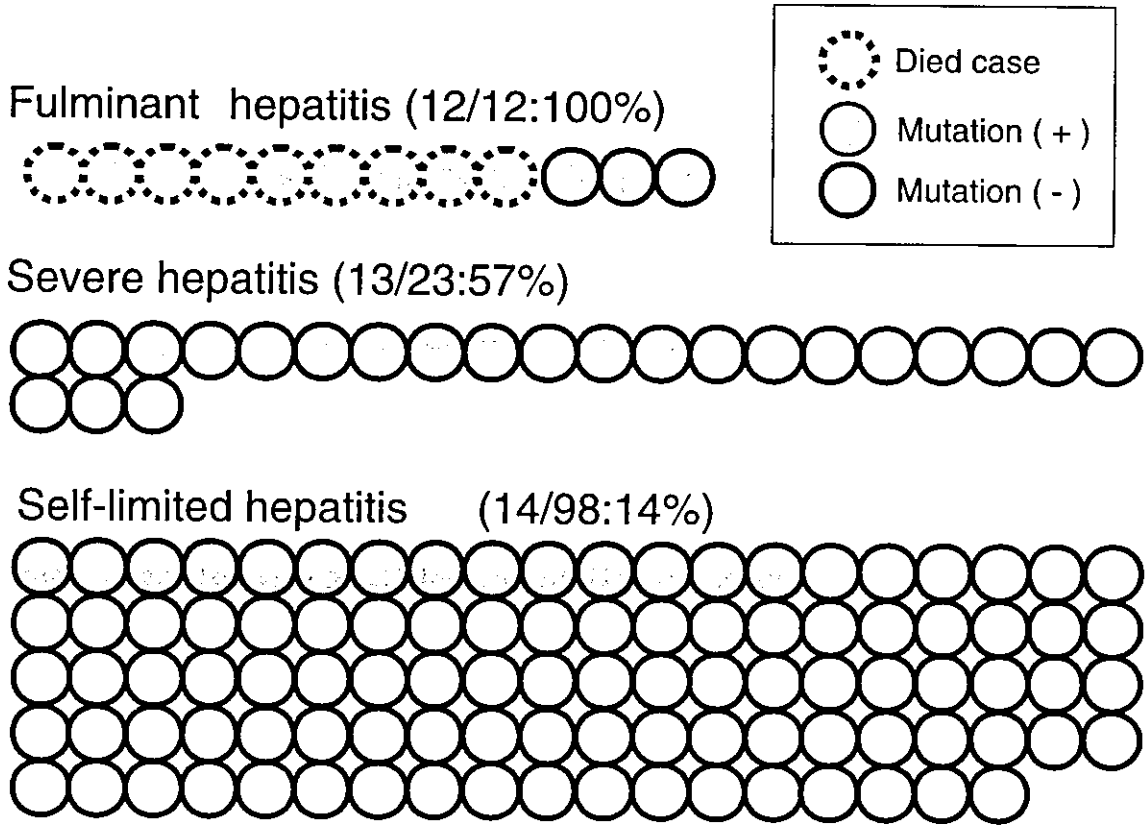

Fig. 2. Association of mutations in the precore and core promoter region of HBV with fulminant and severe acute hepatitis in Japanese patients with acute HBV infection, including 12 patients with fulminant hepatitis, 23 with severe acute hepatitis and 98 with acute self-limited hepatitis. Among 12 patients with fulminant hepatitis, nine patients died due to acute hepatic failure. The presence of HBV mutants was examined by using a point mutation assay (PMA) to detect a G-to-A transition at position 1896 in the precore region and an A-to-T transition at position 1762 and a G-to-A transition at position 1764 in the core promoter region.

(to rule out the possibility of chronic liver failure). The diagnostic criteria for severe hepatitis were: (1) stage I hepatic encephalopathy; and (2) prothrombin time less than 40%. The diagnosis of acute HBV infection was based on detection of high titers of IgM antibody to hepatitis B core antigen (anti-HBc) in the absence of any evidence of acute infection with other hepatitis viruses. Among 12 patients with fulminant hepatitis, nine died due to acute hepatic failure. All patients who survived were clear of hepatitis B surface antigen (HBsAg) after the episode of acute hepatitis. The presence of HBV mutants was examined by using a point mutation assay (PMA) to detect a G-to-A transition at position 1896 in the precore region and an A-to-T transition at position 1762 and a G-to-A transition at position 1764 in the core promoter region. PMA was HBV-DNA mutations detection PCR kit whose sensitivity was 100 copies/ml [20].

Mutations were detected in either the precore or core promoter region in all 12 patients (100%) with fulminant hepatitis B including nine patients who died. Among 23 patients with severe hepatitis, 13 (57%) had mutations of HBV. Among 98 patients with self-limited acute hepatitis B, 14 (14%) had mutations in either the precore or core promoter regions. Significant differences in the proportion of mutations in the precore