*Comparison of protein characteristics for HVR-1 and -2 in the E1/E2 region*

To examine the possibility that structural variation was generated in the E1/E2 region (a.a. 192 ~ 480) during disease progression in all five patients (Table 1), hydrophilicity, surface probability and antigenic indices were calculated from deduced a.a. sequences at each point. Figure 5 indicates the results of hydrophilicity (Fig. 5A) and antigenic index analyses (Fig. 5B) in patients A (acute infection) and C (chronic infection). These structural profiles displayed no significant changes during disease progression in patients A and C. The results of surface probability analysis in patients A and C likewise remained basically unchanged during disease progression. These three structural profiles demonstrate no significant changes in E1/E2 protein during the progression of HCV infection. Likewise, the remaining 3 patients (1 acute infection, 2 chronic infections; Table 1) displayed no significant changes in E1/E2 protein during disease progression. Moreover, the predicted secondary structure (chou-Fas) did not show any drastic changes between time points in any of the five patients (data not shown). Although some a.a. substitutions were observed in the E1/E2 region during disease progression in each patient, the major a.a. structure seems likely to have remained conserved in each case.

*Humoral immune responses to each synthetic peptide from HVR-1*

Synthetic HVR-1 peptides from chronic patients C and E were tested to characterize host immune responses during progression points using ELISA (points 1, 2, and 3; Fig. 1, Table 3). Patient C did not display antibody-positives against their own 3 HVR-1 peptides (C-1, C-2, C-3; Table 3) at any time point. In contrast, patient E displayed antibody-positives against their own 3 HVR-1 peptides (E-1, E-2, E-3; Table 3) at every time point (Table 3).

## Discussion

The present study characterized nucleotide sequences of the E1/E2 protein region during clinical course from sera of 2 patients with acute HCV infection and 3 patients with chronic HCV infection using direct DNA sequencing methods. Furthermore, amino acid sequences and protein structures of the E1/E2 protein region (a.a. 192 ~ 480) were deduced during disease progression.

Nucleotide sequence variation in the E1/E2 region was mainly observed in HVR-1 and -2 for the 2 acute phase patients, and in only HVR-1 for the 3 chronic phase patients during clinical course. In the E1 protein region, a.a. substitution speed was below 0.69% in all five patients (2 acute patients, 3 chronic patients). This result indicates the possibility that E1 and HVR-2 may not be involved in escape mutation for chronic infection.

Previous reports have suggested that HVR-1 could serve as a target for neutralization of antibody and generation of escape mutants from humoral immune

responses, potentially contributing to the establishment of persistent HCV infection [14, 16, 20]. In our experiment, host immune responses to HVR-1 peptide during the course of chronic infection differed substantially between 2 patients (Table 3). For patient C, no antibody responses against 3 HVR-1 peptides (C-1, C-2 and C-3; Table 3) were observed at any time point. These data suggest two possible explanations. One is that antibodies were not produced in these stages, while the other is that the positions of C-1, C-2 and C-3 peptides might not be included in linear epitopes, instead being included in conformational epitopes. Patient E displayed a consensus sequence in each HVR-1 peptide (peptide sequence, TARSAAGFT; Table 3). For this reason, sera from patient E might react positively for E-1, E-2 and E-3 peptides at each time point. These results indicate the possibility that HVR-1 might not represent a significant epitope region for neutralization of HCV escape mutants in some cases.

Our results indicate the existence of patient-specific conserved nucleotide sequences in the E1/E2 region during clinical course of all HCV patients (Fig. 2). This finding may be useful for identifying HCV vertical transmission and other infection pathways. Furthermore, the existence of these patient-specific nucleotide sequences indicate the possible adaptation of the virus in patients and escape from the host immune surveillance systems in the early phase of HCV infection.

Rate of amino acid substitution speed between each point (point 1 to 2 and point 2 to 3; Fig. 1) during clinical course was calculated as the monthly rate of a.a. substitutions per site (%; Fig. 4). The data indicate that high a.a. substitution speed in HVR-1 and -2 was present in the first phase of acute infection, and that a.a. substitution speed in HVR-1 was elevated in chronic patients during major transitions in viral RNA or ALT levels. This phenomenon should support the understanding of HCV adaptation to host immune pressures and the establishment of persistent HCV infection.

The acute and secondary structures of the E1/E2 protein region (a.a. 192 $\sim$ 380) from patients with hepatitis C displayed no significant change during clinical course. This observation suggests that HCV clones in hepatitis C patients may conserve a E1/E2 protein structure during persistent infection.

In conclusion, our observations suggest that the rapid substitution of amino acid sequences in the first phase of acute phase of infection may be involved the HCV adaptation to host immune pressures and the development of persistent HCV infection.

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## References


1. Abrignani S (1997) Immune responses throughout hepatitis C virus (HCV) infection: HCV from the immune system point of view. Springer Semin Immunopathol 19: 47–55
2. Agnello V, Abel G, Elfahal M, Knight GB, Zhang Q-X (1999) Hepatitis C virus and other *Flaviviridae* viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci USA 96: 12766–12771
3. Alter MJ, Margolis HS, Krawczynski K, Judson FN, Mares A, Alexander WJ, Hu PY, Miller JK, Gerber MA, Sampliner RE, Meeks E, Beach MJ (1992) The natural history of community acquired hepatitis C in the United States. N Engl J Med 327: 1899–1905
4. Chomczynski P, Sacchi N (1987) Single step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162: 156–159
5. Crotta S, Stilla A, Wack A, D'Andrea A, Nuri S, D'Oro U, Mosca M, Filliponi F, Brunetto RM, Bonino F, Abrignani S, Nicholas MV (2002) Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. J Exp Med 195: 35–41
6. Farci PA, Shimoda A, Wong D, Cabezon D, De Gioannis D, Strazzera A, Shimizu Y, Shapiro M, Alter HJ, Purcell RH (1996) Prevention of hepatitis C virus infection in chimpanzee by hyperimmune serum against the hypervariable region 1 of the envelope 2 protein. Proc Natl Acad Sci USA 93: 15394–15399
7. Farci P, Bukh J, Purcell RH (1997) The quasispecies of hepatitis C virus and the host immune response. Springer Semin Immunopathol 19: 5–26
8. Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpolder JC, Strazzera A, Chien DY, Munoz SJ, Balestrieri A, Purcell RH, Alter HJ (2000) The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science 288: 339–344
9. Higashi Y, Kakumu S, Yoshioka K, Wakita T, Mizokami M, Ohba K, Itoh Y, Ishikawa T, Takegami M, Nagai Y (1993) Dynamics of genome change in the E2/NS1 region of hepatitis C virus *in vivo*. Virology 197: 659–668
10. Itakura J, Nagayama K, Enomoto N, Sakamoto N, Tazawa J, Izumi N, Marumo F, Sato C (2001) CD81 nucleotide mutation in hepatocellular carcinoma and lack of CD81 polymorphism in patients at stages of hepatitis C virus infection. J Med Virol 63: 22–28
11. Garcia JE, Puentes A, Suarez J, Lopez R, Vera R, Rodriguez LE, Ocampo M, Curtidor H, Guzman F, Urquiza M, Patarroyo ME (2002) Hepatitis C virus (HCV) E1 and E2 proteins that specifically bind to HepG2 cells. J Hepatol 36: 254–262
12. Kao JH, Chen P-J, Lai M-J, Wang TH, Chen DS (1995) Quasispecies of hepatitis C virus and genetic drift of the hypervariable region in chronic type C hepatitis. J Infect Dis 172: 261–264
13. Kato N, Sekiya H, Ootsuyama Y, Nakazawa T, Hijikata M, Ohkoshi S, Shimotohno K (1993) Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. J Virol 67: 3923–3930
14. Kato N, Ootsuyama Y, Sekiya H, Ohkoshi S, Nakazawa T, Hijikata M, Shimotohno K (1994) Genetic drift in hypervariable region 1 of the viral genome in persistent hepatitis C virus infection. J Virol 68: 4776–4784
15. Lanford RE, Chavez D, Chsari FV, Sureau C (1995) Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. J Virol 69: 8079–8083

16. Lawa Z, Petrick J, Wong V-S, Alexander GJM, Allain JP (1997) Hepatitis C virus genomic variability in untreated and immunocompromised patients. Virology 228: 107–111
17. Levy S, Todd S, Maecker H (1998) CD81 (TAPA-1): a molecule involved in signal transduction and cell adhesion in the immune system. Annu Rev Immunol 16: 89–109
18. Monazahian M, Bohme I, Bonk S, Koch A, Scholz C, Grethe S, Thomssen R (1999) Low density lipoprotein receptor as a candidate receptor for hepatitis C virus. J Med Virol 57: 223–229
19. Nakatsuji Y, Matsumoto A, Tanaka E, Ogata H, Kiyosawa K (1992) Detection of chronic hepatitis C virus infection by four diagnostic systems: first-generation and second-generation enzyme-linked immunosorbent assay, second-generation recombinant immunoblot assay and nested polymerase chain reaction analysis. Hepatology 16: 300–305
20. Odeberg J, Yun Z, Sonnerborg K, Bijoro K, Uhlen M, Lundberg J (1997) Variation of hepatitis C hypervariable region 1 in immunocompromised patients. J Infect Dis 175: 938–943
21. Okada S, Akahane Y, Suzuki H, Okamoto H, Mishiro S (1992) The degree of variability in the amino acid terminal region of the E2/NS1 protein of hepatitis C virus correlates with responsiveness to interferon therapy in viremic patients. Hepatology 16: 619–624
22. Okamoto H, Kojima M, Okada S, Yoshizawa H, Iizuka H, Tanaka T, Munchmre E, Peterson DA, Ito Y, Mishiro S (1992) Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology 190: 894–899
23. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, AJ W, Houghton M, Rosa D, Grandi G, Abrignani S (1998) Binding of hepatitis C virus to CD81. Science 282: 938–941
24. Rice CM (1996) *Flaviviridae*: The viruses and their replication. In: Fields BN, Kniee DN, Howley PM (eds), Fields virology. Lippincott Raven Publ, Philadelphia, PA, pp 931–959
25. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, Watanabe Y, Koi S, Onji M, Ohta Y, Choo Q, Houghton M, Kuo G (1990) Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 87: 6547–6549
26. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406–425
27. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467
28. Weiner AJ, Brauer MJ, Rosenblat J, Rivhman KH, Tung J, Crawford K, Bonino F, Saracco G, Choo Q-L, Houghton M, Han JH (1991) Variable and hypervariable domains are found in the regions of HCV corresponding to the Flavivirus envelope and NS1 proteins and the pestivirus envelope glycoproteins. Virology 180: 842–848
29. Yamaguchi K, Tanaka E, Higashi K, Kiyosawa K, Matsumoto A, Furuta S, Hasegawa A, Tanaka S, Kohara M (1994) Adaptation of hepatitis C virus for persistent infection in patients with acute hepatitis. Gastroenterology 106: 1344–1348
30. Yoshioka K, Kakumu S, Wakita T, Ishikawa T, Itoh Y, Takayanagi M, Higashi Y, Shibata M, Morishima T (1992) Detection of hepatitis C virus by polymerase chain reaction and response to interferon α therapy: relationship to genotypes of hepatitis C virus. Hepatology 16: 293–299

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# A follow-up study to determine the value of liver biopsy and need for antiviral therapy for hepatitis C virus carriers with persistently normal serum aminotransferase

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***Background/Aims*: Long-term follow-up study was performed to identify the candidates for antiviral therapy for hepatitis C virus (HCV) infection among carriers with persistently normal aminotransferase (ALT ≤ 30 U/L) levels (PNAL).**

***Methods*: One hundred and twenty-nine HCV carriers with PNAL who underwent liver biopsy and had platelet count over 150,000/μl were entered and 69 were followed for over 5 years. Thirty-five patients underwent serial liver biopsies. Serum ferritin and thioredoxin levels were also determined.**

***Results*: Seventeen patients had normal liver histology, 10 had moderate chronic hepatitis and the remainder 102 had mild hepatitis. Serum ferritin and thioredoxin levels were normal. The mean follow-up period for the 69 patients was 8.5 years. Of these 69 patients, 10 had persistently normal ALT levels (group A), 39 had transient elevation of ALT (group B), and 20 changed to symptomatic chronic hepatitis (group C). The rate of progression of fibrosis for groups A, B, and C were 0.05, 0.04, and 0.08, respectively. Hepatocellular carcinoma was not diagnosed in any of the patients.**

***Conclusions*: Around 90% of HCV carriers with PNAL have normal to mild liver histology. This long-term follow-up study demonstrated that 30% of such carriers became candidates for antiviral therapy within 5 years.**



***Keywords*: Hepatitis C virus; Chronic hepatitis C; Asymptomatic HCV carrier; Normal serum ALT; Interferon**

## 1. Introduction

An estimated 170 million individuals are infected with hepatitis C virus (HCV) worldwide and chronic hepatitis C has recently become the leading cause of liver cirrhosis and hepatocellular carcinoma (HCC) in many countries including Japan. Most HCC develop in patients with advanced staged chronic hepatitis or cirrhosis, and rarely from mild chronic hepatitis type C.

It is thought that type C liver cirrhosis and HCC develop over 20–35 years following HCV infection [1], however, around 25% of patients with chronic HCV infection have normal serum aminotransferase (ALT) levels [2,3]. We reported previously that asymptomatic HCV carriers were predominant among females and that most of them had histologically minimal to mild chronic hepatitis [4]. In that paper, we defined asymptomatic HCV carriers as persistently HCV RNA positive patients with normal serum ALT levels (≤30 U/L) over 1 year. However, it has been reported that HCV carriers with normal serum ALT level had more advanced liver histology compared to HCV carriers with elevated serum ALT [5]. This discrepancy might be attributed to differences in the definition of the normal range of serum ALT used by various centers, however, it is very important to clarify whether HCV carriers with persistently normal ALT level (PNAL) are candidates for antiviral therapy.

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The current normal limit of serum ALT is 40 U/L, however, a recent report from an Italian group demonstrated that the healthy ranges for serum ALT were 30 U/L for men and 19 U/L for women, respectively [6], which are lower than the current values that have been used over the past 15 years. This criterion of normal serum ALT might be reasonable because a few cirrhotic patients have from 30 to 40 U/L of ALT [7].

In Japan, the number of HCC patients with HCV infection has increased since 1975. Antiviral treatment for chronic hepatitis C resulted in the inhibition of hepatic inflammation and progression of hepatic fibrosis and as a consequence the inhibition of the development of HCC [8–13]. Thus, inhibition of HCC is a very important issue in the treatment of patients with chronic hepatitis C. It remains controversial whether asymptomatic HCV carriers are candidates for antiviral therapy because of the low efficacy of treatment and flare-ups post treatment. However, taking into consideration the recent progress in antiviral therapy for chronic hepatitis C patients, the National Institute of Health Consensus Development Conference reported that patients with hepatitis C with normal serum ALT levels are candidates for interferon and ribavirin therapy [14]. Recently, a multicenter, randomized, controlled study for the treatment of patients with chronic hepatitis and persistently normal ALT levels with pegylated interferon alpha and ribavirin for 48 weeks led to eradication in 40% of patients infected with genotype 1b patients [15], which is similar to the results for symptomatic chronic hepatitis C patients [16,17]. However, most HCV carriers with PNAL have minimal to mild liver histology and their prognosis might be very good. Thus, there is some doubt, whether they are candidates for antiviral treatment to inhibit the progression of liver disease and hepatocarcinogenesis.

Recently, it has been reported that oxidative stress is an important factor in the development of HCV-related HCC [18–22] and the HCV core protein may generate oxidative stress via mitochondrial injury [23,24]. It is also demonstrated that iron overload generates oxidative stress, resulting in hepatic injury, and DNA damage and consequently this becomes an important factor for hepatocarcinogenesis [22,25,26].

We report here the biochemical and histological results of 8.5 years of follow-up of HCV carriers with PNAL. The data were analyzed according to the definitions of normal range ($\leq$30 U/L) of serum ALT and platelet count (PLT) over 150,000 μl/ml. We also analyzed the status of oxidative stress using serum ferritin and thioredoxin levels. These results demonstrate the importance of the normal range of serum ALT, oxidative stress and follow-up study to decide the indication for antiviral therapy of HCV carriers with PNAL.

## 2. Patients and methods

### 2.1. Eligibility and definition

This study was conducted from January 1990 to August 2004.

HCV carriers with persistently normal ALT levels (PNAL) were defined as those patients who were HCV RNA positive by reverse transcriptase polymerase chain reaction (RT-PCR), had normal serum ALT levels ($\leq$30 U/L) over a 12-months period and on least three different occasions and platelet count of over $15\times10^4$ μl/ml. Patients positive for hepatitis B surface antigen (HBsAg), previous interferon (IFN) treatment, a history of heavy alcohol abuse, anti-nuclear antibody (ANA) and anti-smooth muscle antibody (ASMA) positivity, patients with overt Diabetes mellitus and obesity (body mass index; over 30 kg/m$^2$) were excluded from this study.

The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki, and approved by the Ethics Committee of Kyoto Prefectural University of Medicine. Informed consent was obtained from every patient.

### 2.2. Quantification and determination of HCV RNA and genotyping

Frozen-stored sera from 129 individuals were tested. Serum HCV RNA levels was determined using the AMPLICOR GT HCV MONITOR (Roche Diagnostic Systems, Tokyo, Japan). The detection range of this assay was between 0.5 and 850 KIU/ml, and each sample was measured again after dilution with distilled water. HCV genotypes 1 and 2 were determined by a serologic genotyping assay [27]. Genotypes 1 and 2 in this assay correspond to genotype 1 (1a, 1b) and 2 (2a, 2b) proposed by Simmonds et al. [28].

### 2.3. Study design

Of the 129 patients who underwent liver biopsy, 69 patients enrolled in this study and followed over 5 years (8 males, 61 females). These patients received blood tests every 4 months for an initial 2 years and then received blood tests every 6 months when they remained still normal ALT. αfetoprotein (AFP) was measured every years in all patients, and all patients underwent ultrasonography every year to detect HCC.

All patients submitted to a liver biopsy using a Menghini needle guided by ultrasonography prior to entry. Formalin-fixed liver specimens were stained with hematoxylin and eosin for morphological evaluation, with Masson's trichrome stain for assessment of fibrosis, and with Perls' Prussian blue stain (from February 1998) for assessment of iron loading. Histological follow-up studies were carried out for 35 patients 3.4–13.4 years (mean: 6.8 years) after the first biopsy.

The histological findings of HCV carriers with PNAL were interpreted and scored according to the classification proposed by Desmet et al. [29] and Ishak et al. [30]. Steatosis is defined having fat droplets in over 10% of hepatocytes. The degree of iron loading was assessed using a Perls' score of 0 to 4+, based on the scoring system of MacSween et al. [31].

Fasting blood samples were collected in the morning. Serum ALT, blood glucose level, serum ferritin, platelet count (PLT), serum HCV RNA level and HCV genotype were examined in the laboratory of our university hospital, using the standard analytical method; the ULA ALT value was 30 U/L. Serum thioredoxin (TRX) levels were measured with a sensitive sandwich ELISA kit (Fujirebio, Inc., Tokyo, Japan) as described previously [26,32] and of the 129 patients 47 were available for this assay. Blood chemistry was done every 4–6 months during the follow-up period.

### 2.4. Statistical analysis

Data values are expressed as medians with interquartile ranges. We compared continuous variables using the Mann–Whitney *U*-test. The Kruskal–Wallis test was used for multiple group comparisons, and Spearman correction coefficients were used to examine the relationship between groups. Frequency analysis was performed with the $\chi^2$ test, and Fisher's exact test. *P* values of less than 0.05 were considered significant.

## 3. Results

### 3.1. Demographic and clinical features

The demographic and clinical features of the 129 HCV carriers with PNAL are shown in Table 1. Twenty-four were male and 105 were female. No significant differences were noted in age, serum ALT, PLT, and follow-up period between males and females. Serum ferritin levels were 76.1±53.4 ng/ml in male and 60.0±43.3 ng/ml in female. Serum HCV RNA levels were significantly ($P=0.0012$) higher in G1 compared with G2 (648.7±622.5 KIU/ml vs 356.2±628.8 KIU/ml (Table 1).

Characteristics of the 69 patients followed over 5 years are also shown in Table 1. Their mean follow-up period was 8.5±2.4 years.

Of the 105 female patients, 44 had serum ALT levels ≦19 U/L and 61 had serum ALT levels of 20–30 U/L at entry. There were no significant differences in their ages, platelet count, serum ferritin levels, serum HCV RNA levels, or BMI (Table 2).

Serum thioredoxin (TRX) levels in these patients were within the normal range, and significantly lower than those of patients with chronic hepatitis and cirrhosis (Table 3).

**Table 1**
**Characteristics of 129 HCV carriers with persistently normal ALT who underwent liver biopsy**

| | $N=129$ | Followed over 5 years ($N=69$) |
|---|---|---|
| Follow-up period (years) | 5.7±3.6 | 8.5±2.4 |
| Age (years) | 48 (21–77) | 45 (29–71) |
| Male ($N=24$) | 49.8±16.4 | 42.3±14.9 |
| Female ($N=105$) | 47.2±12.5 | 46.63±11.6 |
| Sex (M/F) | 24/105 | 8/61 |
| ALT (U/L) | 8–30 | 9–30 |
| Male ($N=24$) | 22.5±5.7 | 21.1±5.4 |
| Female ($N=105$) | 21.6±4.8 | 22.3±5.1 |
| PLT ($\times 10^4$/ml) | 15–31 | 15–31 |
| Male ($N=24$) | 20.3±4.4 | 20.9±5.3 |
| Female ($N=105$) | 21.8±4.4 | 21.2±4.0 |
| Ferritin (ng/ml) | 5–225 | 5–225 |
| Male | 76.2±53.5 | 84.6±59.2 |
| Female | 60.0±43.3 | 66.6±52.5 |
| HCV RNA (KIU/ml) | 6–3350 | 22–2100 |
| G1 ($N=58$) | 648.9±622.57* | 595.1±561.1** ($N=32$) |
| G2 ($N=45$) | 356.2±628.8 | 211.0±219.2 ($N=27$) |
| Mixed and unclassified | 6–1994 | |
| BMI (kg/m$^2$) | 16–27 | 16–27 |
| Male | 22.2±1.7 | 21.9±1.9 |
| Female | 21.3±2.2 | 21.0±2.4 |

Values were expressed as mean±SD. $P$ values were calculated by Mann–Whitney $U$-analysis with correction for tie. *$P=0.0012$ (G1 vs G2); **$P=0.0006$ (G1 vs G2).

**Table 2**
**Baseline of female patients between HCV carriers having ≦19 U/L of ALT and HCV carriers showing 20–30 U/L of ALT**

| | ALT≦19 (U/L) | 20<ALT≦30 (U/L) | $P$ value |
|---|---|---|---|
| Number of patient | 44 | 61 | |
| Age (y.o) | 44.9±12.5 | 48.8±12.2 | |
| ALT (U/L) | 16.0±2.4 | 24.3±2.9 | <0.0001 |
| PLT ($\times 10^4$/μl) | 22.0±4.4 | 21.6±4.3 | |
| HCV RNA (KIU/ml) | 400.2±555.1 | 500.7±541.1 | 0.3896 |
| BMI (kg/m$^2$) | 21.2±2.3 | 21.4±2.2 | |

Values were expressed as mean±SD. $P$ values were calculated by Mann–Whitney $U$-analysis with correction for tie.

### 3.2. Liver histology

The results of liver histology for the first biopsy are described in Table 4. Normal liver histology was noted in 17 (14%) subjects, 102 (79%) showed minimal to mild chronic hepatitis, 10 (8%) had moderate chronic hepatitis.

Steatosis was seen in nine patients (7%) and iron loading was noted in 6/50 (12%).

### 3.3. Follow-up study of laboratory data

Of the 69 patients followed over 5 years (mean±SD: 8.5±2.4 years), 10 (14%) had continuously normal ALT (group A), 39 (57%) showed transient elevation of ALT (group B), and 20 (29%) changed to chronic hepatitis with continuously abnormal serum ALT (group C) (Table 5). Of the 61 female patients, eight were group A, 34 were group B, and 19 were group C. There were no significant differences in age, ferritin levels, serum HCV RNA levels, or BMI among the three groups. However, serum ALT levels were significantly lower in group A compared with group B and C (Table 6). The number of patients having ALT levels ≦19 IU/L in these three groups were seven (7/8:87.5%) in group A, 12 (12/34:35.3%) in group B, and three (3/19:15.8%) in group C.

**Table 3**
**Serum thioredoxin (TRX) levels in 47 HCV carriers with PNAL at liver biopsy**

| | Serum thioredoxin (ng/ml) |
|---|---|
| HCV carriers with PANL ($n=47$) | 27.7 [9.1–38.5] |
| Chronic hepatitis ($n=124$) | 34.5 [8.6–135.6][a]+ |
| Liver cirrhosis ($n=24$) | 42.5 [21.4–97.2][a]++ |
| Control ($n=15$) | 24.9 [1.3–50.7][a] |

*$P=0.0012$ when compared with G2. The overall significance of differences between four groups according to non-parametric Kruskal–Wallis analysis of variance was $P<0.001$. Therefore, the significance of differences between groups was determined by Scheffe's method: +$P<0.01$; ++$P<0.001$, compared to HCV carriers with PNAL.

[a] These data were reported in J Hepatol 2000; 33: 616–622.

**Table 4**
**Liver histology of 129 carriers at the first biopsy**

| Grade | Stage of liver fibrosis | | | | Total number of patients |
|---|---|---|---|---|---|
| | F0 | F1 | F2 | F3 | |
| A0 | 17 (11) | 3 (1) | 0 | 0 | 20 (12) |
| A1 | 24 (21) | 75 (62) | 2 (2) | 0 | 101 (85) |
| A2 | 0 | 6 (5) | 2 (2) | 0 | 8 (7) |
| A3 | 0 | 0 | 0 | 0 | 0 |
| Total | 41 (32) | 84 (68) | 4 (4) | 0 | 129 (104) |

Numbers of female patients are given in parentheses.

The stage of liver fibrosis in the 22 female patients with ALT levels ≦19 IU/L at entry were F0 ($N=10$) or F1 ($N=12$). The frequency of stage F0 liver histology was slightly higher in group A and B patients compared with group C. However, there were no significant differences among the three groups.

Seven patients from group C had ALT levels over 100 U/L during the follow-up period and received antiviral therapy (five received interferon monotherapy and two received interferon plus ribavirin therapy), and five had a sustained virological response.

### *3.4. Follow-up study of liver histology*

Thirty-five patients submitted to repeat biopsies and five of them a third biopsy. Of the 35 patients, 5 were in group A, 16 in B, and 14 in C. The intervals between the first biopsy and the last biopsy in these three groups were 7.3±2.1 years (group A), 6.8±2.0 years (group B), and 6.1±2.3 years (group C). The changes in stage of live fibrosis are shown in Fig. 1 (group A), 2 (group B), and 3 (group C). Progression of fibrosis stage was noted in 2 of 5 in group A, 5 of 16 in group B, and 6 of 14 in group C, as shown in Figs. 1–3. The median rates of fibrosis progression per year for these three groups were 0.05, 0.04, and 0.08 fibrosis unit, respectively. There were no significant differences in the rate of fibrosis progression per year between group A and B, B and C, and A and C (A vs B; $P=0.6643$, B vs C; $P=0.0699$, A vs C; $P=0.3512$).

Of the 32 female patients who received serial biopsies, 10 had ALT levels ≦19 U/L at entry, in four of whom had F0 stage progress to F1. One F0 and five F1 patients showed no changes in their stages during the follow-up periods.

**Table 5**
**Changes of serum ALT in 69 patients followed over 5 years**

| | No. of patients |
|---|---|
| Persistently normal (group A) | 10 (14%) |
| Transient elevation (group B) | 39 (57%) |
| Continuous elevation (group C) | 20 (29%) |

Group A, continuously normal serum ALT during the follow-up period. Group B, serum ALT transiently over 31 U/L during the follow-up period. Group C, serum ALT became continuously abnormal during the follow-up period.

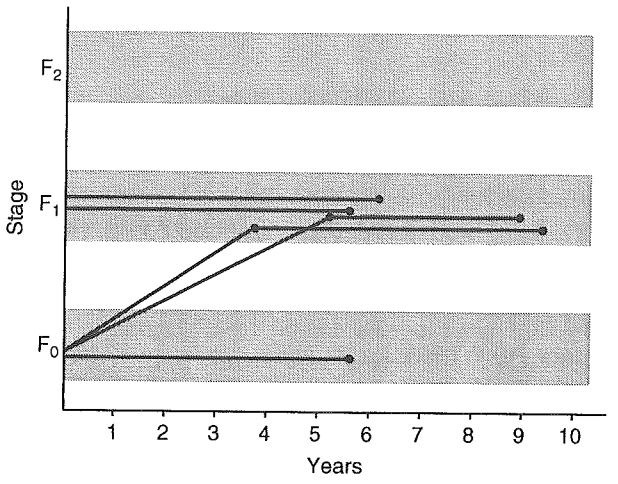


**Fig. 1. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels remained normal during the follow-up period. Five patients with persistently normal serum aminotransferase levels submitted to repeat biopsies and the stage of liver fibrosis progressed from F0 to F1 in two patients after 3.4 and 5 years.**

### *3.5. Follow-up study of AFP and ultrasonography*

Three patients in group C showed transient elevation of AFP over 20 ng/ml. No patients in groups A or B had elevations of serum AFP during their follow-up periods. HCC was not detected in any patients by ultrasonography and/or computed tomography. AFP titers in those three patients did not increase further.

## 4. Discussion

The present study demonstrated several characteristics of HCV carriers with persistently normal ALT levels (PNAL).

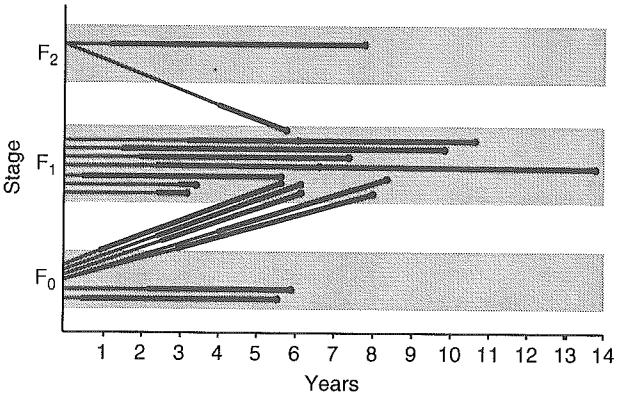


**Fig. 2. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels were transiently elevated during the follow-up period. Sixteen patients with transient elevation of serum aminotransferase levels submitted to repeat biopsies and the stage of liver fibrosis progressed from F0 to F1 after 5.3–8.1 years in five patients. One patient showed the regression of the stage of liver fibrosis from F2 to F1 after 5.5 years. The left side edge of the large bar indicates the initial recording of abnormal serum aminotransferase during follow-up period.**

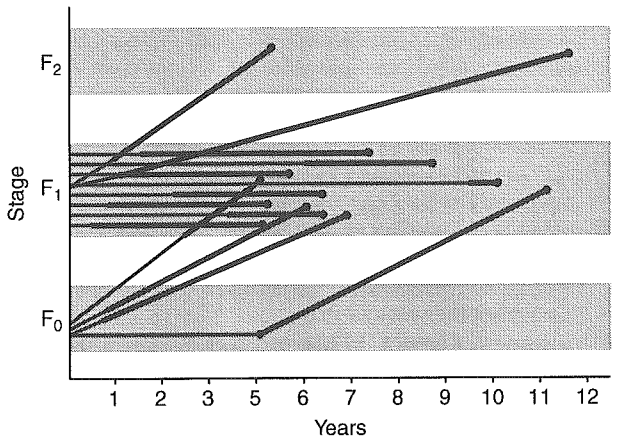


**Fig. 3. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels became persistently abnormal during the follow-up period. Fourteen patients who developed continuously abnormal serum aminotransferase levels submitted to repeat liver biopsies after 4.0–10.3 years. Progression of the stage of liver disease was noted in six patients, of whom four progressed from F0 to F1 and two from F1 to F2 after 4.0–10.3 years. The left side edge of the large bar indicates the initial recording of abnormal serum aminotransferase during follow-up period.**

Serum HCV RNA levels were similar to patients with symptomatic chronic hepatitis, however, the frequency of HCV genotype 2 was significantly higher in HCV carriers with PNAL than those with chronic hepatitis C (data not shown here, of 123 patients with chronic hepatitis C in our clinic 75% had genotype 1 and 22% were genotype 2). Females were predominant among the HCV carriers with PNAL compared with chronic hepatitis [4] which is similar to other reports [5,33–35]. Female HCV carriers with continuously normal ALT had significantly lower ALT levels at entry as shown in Table 6. Of the 105 female patients, 44 had ALT levels ≦19 U/L and showed mild liver injury compared with carriers with whose ALT levels were 20–30 U/L. However, the progression rate of fibrosis was not significantly different.

The serum ferritin and serum thioredoxin (TRX) levels in HCV carriers with PNAL showed normal ranges and were significantly lower than in chronic hepatitis C patients, as we have reported previously [26]. The frequency and grade of fatty liver and iron loading were quite low compared with chronic hepatitis C patients, also as reported previously [26]. Liver histology was minimal to mild and moderate chronic hepatitis was noted in only around 8% of subjects. Long-term follow-up study demonstrated that 29% of HCV carriers with PNAL developed chronic hepatitis with persistently high serum ALT within 5 years, 57% showed transient elevation of serum ALT, and 14% had continuously normal ALT. There are many reports concerning the natural course of liver fibrosis in chronic hepatitis C patients including patients with normal serum ALT level [5,33–41]. More than half of chronic hepatitis C patients show progression of stage of liver fibrosis from F1 to F2-4 within 10 years and it was previously reported that progression of liver fibrosis in HCV carriers with PNAL was more rapid compared with the present result [5]. The main reason for this discrepancy between the previous reports and the present result might be due to the difference in the definition of the normal range of serum ALT. Poynard et al. [37] reported that the median rate of progression of fibrosis per year was 0.1333 fibrosis unit, which was 1.5–3 times faster than the present results in HCV carriers with PNAL.

These results indicate that HCV carriers with PNAL are in a condition with less oxidative stress [26] and they have a lower risk of cirrhosis and hepatocarcinogenesis compared to chronic hepatitis patients [13,22].

It is well known that the rate of the development of hepatocellular carcinoma (HCC) is correlated with the progression of liver fibrosis; the stage of liver disease [9,11,13]. Sustained low serum ALT also lowers the rate of the development of HCC [9,13,42]. No HCC was detected during the follow-up period in any of the HCV carriers in this study, reflecting the results of previous clinical studies.

Peginterferon and ribavirin administration for 48 weeks resulted in sustained virological response in around 40% of patients with genotype 1 [15], however, this therapy is expensive and induces various side effects.

The present results indicate that most HCV carriers with persistently normal serum ALT have a good prognosis with a low risk of developing hepatocellular carcinoma. Antiviral treatment for these patients should take into consideration the follow-up results of blood chemistry and liver histology.

**Table 6**
**Characteristics of 61 female patients in groups A–C followed over 5 years**

| | Group A ($N$=8) | Group B ($N$=34) | Group C ($N$=19) |
|---|---|---|---|
| Age (y.o.) | 49.6±12.9 | 44.9±12.5 | 48.2±8.9 |
| BMI (kg/m$^2$) | 20.8±2.9 | 20.6±2.1 | 21.8±2.5 |
| Ferritin (ng/ml) | 73.4±33.7 | 59.3±56.8 | 76.8±47.1 |
| ALT (U/L)* | 15.8±3.2 | 22.4±4.6 | 23.9±4.9 |
| HCV RNA (KIU/ml)** | 186.5±141.8 | 474.6±486.0 | 454.0±575.2 |

Values were expressed as mean±SD. There were no significant differences in their age, BMI, ferritin, and HCV RNA levels in three groups. *Serum ALT level was significantly lower in group A compared with group B (group A vs group B; $P$=0.0045) and with group C (group A vs group C; $P$=0.0003), however, no significant difference was noted between group B and C ($P$=0.0758). **There were no significant differences in serum amount of HCV RNA between group A and B ($P$=0.3529) and group A and C ($P$=0.8676).

## References


[1] Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship between of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12: 671–675.

[2] Marcellin P, Levy S, Erlinger S. Therapy of hepatitis C: patients with normal aminotransferase levels. Hepatology 1997;26: 133S–1136.

[3] Tassopoulos NC. Treatment in patients with normal ALT levels. European association for the study of the liver (EASL) international conference on hepatitis C, Paris, February 26–27, 1999. J Hepatol 1999;30:956–961.

[4] Okanoue T, Yasui K, Sakamoto S, Minami M, Nagao Y, Itoh Y, et al. Circulating HCV RNA, HCV genotype, and liver histology in asymptomatic individuals reactive for anti-HCV antibody and their follow-up study. Liver 1996;16:241–247.

[5] Puoti C, Magrini A, Stati T, Rigato P, Montagnese F, Rossi P, et al. Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine aminotransferase levels. Hepatology 1997;26:1393–1398.

[6] Prati D, Taidoli E, Zanela A, Torre ED, Butelli S, Vecchio ED, et al. Updated definition of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med 2002;137:1–9.

[7] Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine transferase in chronic hepatitis. Relationship to cirrhosis. Gastroenterology 1988;95:734–739.

[8] Kasahara A, Hayashi N, Mochizuki K, Takayanagi M, Yoshioka K, Kakumu S, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka liver disease study group. Hepatology 1998;27: 1394–1402.

[9] Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C but not significantly in advanced stage: A retrospective study in 1148 patients. J Hepatol 1999;30:653–659.

[10] Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. Hepatology 1999;29:11–19.

[11] Yoshida H, Shiratori Y, Moriyama M, Arakawa Y, Ide T, Sata M, et al. Interferon therapy reduced the risk for hepatocellular carcinoma: national surveillance program of cirrhotic and noncirrhotic patients with chronic hepatitis C in Japan. IHIT study group. Inhibition of hepatocarcinogenesis by interferon therapyIHIT study group. Inhibition of hepatocarcinogenesis by interferon therapy. Ann Intern Med 1999;131:174–181.

[12] Tanaka H, Tsukuma H, Kasahara A, Hayashi N, Yoshihara H, Masuzawa M, et al. Effect of interferon therapy on the incidence of hepatocellular carcinoma and mortality of patients with chronic hepatitis C: retrospective cohort study of 738 patients. Int J Cancer 2000;87:741–749.

[13] Okanoue T, Itoh Y, Kirishima T, Daimon Y, Toyama T, Morita A, et al. Transient biochemical response in interferon therapy decreases the development of hepatocellular carcinoma for five years and improves the long-term survival of chronic hepatitis C patients. Hepatol Res 2002;23:62–77.

[14] Bacon BR. Treatment of patients with hepatitis C and normal serum aminotransferase levels. Hepatology 2002;36:S179–S184.

[15] Zeuzem S, Diago M, Gane E, Reddy R, Pockros P, Prati D, et al. Peginterferon alfa-2a (40 kilo Daltons) and ribavirin in patients with chronic hepatitis C and normal aminotransferase levels. Gastorenterology 2004;127:1724–1732.

[16] Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared to interferon alfa-2b plus ribavirin for the treatment of chronic hepatitis C: a randomized controlled trial. Lancet 2001;359: 958–965.

[17] Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales Jr FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. New Engl J Med 2002;347:975–982.

[18] Shimoda R, Nagashima M, Sakamoto M, Yamaguchi N, Hirohashi S, Yokota J, et al. Increased formation of oxidative DNA damage, 8-hydroxydeoxyguanosine, in human livers with chronic hepatitis. Cancer Res 1994;54:3171–3172.

[19] Yamashita T, Kaneko S, Hashimoto S, Sato T, Nagai S, Toyoda N, et al. Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma. Biochem Biophys Res Commun 2001;282: 647–654.

[20] Ichiba M, Maeta Y, Mukoyama T, Saeki T, Yasui S, Kanbe T, et al. Expression of 8-hydroxy-2′-deoxyguanosine in chronic liver disease and hepatocellular carcinoma. Liver Int 2003;23:338–345.

[21] Qadri I, Iwahashi M, Capasso JM, Hopken MW, Flores S, Schaack J, et al. Induced oxidative stress and activated expression of manganese superoxide dismutase during hepatitis C virus replication: role of JNK, p38 MAPK and AP-1. Biochem J 2004;378:919–928.

[22] Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, et al. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001;61:8697–8702.

[23] Moriya K, Nakagawa K, Santa T, Shintani Y, Fujie H, Miyoshi H, et al. Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocarcinogenesis. Cancer Res 2001;61:4365–4370.

[24] Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, et al. Mitochondrial in jury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 2002;122:366–375.

[25] Yano M, Hayashi H, Wakusawa S, Samae F, Takikawa T, Shiono Y, et al. Long term effects of phlebotomy on biochemical and histological parameters of chronic hepatitis C. Am J Gastroenterol 2002;97:133–137.

[26] Sumida Y, Nakashima T, Yoh T, Nakajima T, Ishikawa H, Mitsuyoshi H, et al. Serum thioredoxin levels as an indicator of oxidative stress in patients with hepatitis C virus infection. J Hepatol 2000;33:616–622.

[27] Tsukiyama-Kohara K, Yamaguchi K, Maki N, Ohta Y, Miki K, Mizokami M, et al. Antigenicities of group 1 and 2 hepatitis C virus polypeptides: molecular basis of diagnosis. Virology 1993;192: 430–437.

[28] Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, et al. A proposed system for the nomenclature of hepatitis C virus genotypes. Hepatology 1994;19:1321–1324.

[29] Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: Diagnosis, grading and staging. Hepatology 1994;19:1513–1520.

[30] Ishak K, Baptista L, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995; 22:696–699.

[31] MacSween RNM, Anthony PP, Sheuer PJ. Pathology of the liver. Edinburgh: Churchill Livingstone; 1987 p. 185.

[32] Sumida Y, Nakashima T, Yoh T, Furutani M, Hirohama A, Kakisaka Y, et al. Serum thioredoxin levels as a predictor of steatohepatitis in patients with nonalcoholic fatty liver disease. J Hepatol 2003;38:32–38.

[33] Healey CJ, Chapman RWG, Fleming KA. Liver histology in hepatitis C virus infection: a comparison between patients with persistently normal or abnormal transaminase. Gut 1993;37:274–278.

[34] Ohkoshi S, Tawaraya H, Kuwana K, Harada T, Watanabe M, Higuchi S, et al. A retrospective study of hepatitis C virus carriers in a local endemic town in Japan. Dig Dis Sci 1995;40:465–471.
[35] Puoti C, Castellacci R, Montagness F. Hepatitis C virus carriers with persistently normal aminotransferase levels: healthy people or true patients? Dig Dis Sci 2000;32:634–643.
[36] Yano M, Kumada H, Kage M, Ikeda K, Shimamatsu K, Inoue O, et al. The long-term pathological evolution of chronic hepatitis C. Hepatology 1996;332:1463–1466.
[37] Poynard T, Bedossa P, Opolon P. Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINVIR, and DOSVIRC. Lancet 1997;346:825–832.
[38] Takahashi M, Yamada G, Miyamoto R, Doi H, Endo H, Tsuji T. Natural course of chronic hepatitis C. Am J Gastroenterol 1993;88: 240–243.
[39] Ghany MG, Kleiner DE, Alter H, Doo E, Khokar F, Promart K, et al. Progression of fibrosis in chronic hepatitis C. Gastroenterology 2003; 124:97–104.
[40] Mathurin P, Moussalli J, Cardanei J-F, Thibault V, Charlotte F, Dumouchel P, et al. Slow progression rate of fibrosis in hepatitis C virus patients with persistently normal alanine transaminase activity. Hepatology 1998;27:868–872.
[41] Hui C-K, Belaye T, Montegrade K, Wright TL. A comparison in the progression of liver fibrosis in chronic hepatitis C between persistently normal and elevated transaminase. J Hepatol 2003;38: 511–517.
[42] Tarao K, Ohkawa S, Tamai S, Miyakawa K. Sustained low serum GPT level below 80 INU in HCV- associated cirrhotic patients by multiagents prevent development of hepatocellular carcinoma. Cancer 1994;73:1149–1154.

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# Pretreatment Prediction of Interferon-Alfa Efficacy in Chronic Hepatitis C Patients

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***Background & Aims:*** **Interferon has been used widely to treat patients with chronic hepatitis C infections. Prediction of interferon efficacy before treatment has been performed mainly by using viral information, such as viral load and genotype. This information has allowed the successful prediction of sustained responders (SR) and non-SRs, which includes transient responders (TR) and nonresponders (NR). In the current study we examined whether liver messenger RNA expression profiles also can be used to predict interferon efficacy.** ***Methods:*** **RNA was isolated from 69 liver biopsy samples from patients receiving interferon monotherapy and was analyzed on a complementary DNA microarray. Of these 69 samples, 31 were used to develop an algorithm for predicting interferon efficacy, and 38 were used to validate the precision of the algorithm. We also applied our methodology to the prediction of the efficacy of interferon/ribavirin combination therapy using an additional 56 biopsy samples.** ***Results:*** **Our microarray analysis combined with the algorithm was 94% successful at predicting SR/TR and NR patients. A validation study confirmed that this algorithm can predict interferon efficacy with 95% accuracy and a *P* value of less than .00001. Similarly, we obtained a 93% prediction efficacy and a *P* value of less than .0001 for patients receiving combination therapy.** ***Conclusions:*** **By using only host data from the complementary DNA microarray we are able to successfully predict SR/TR and NR patients for interferon therapy. Therefore, this technique can help determine the appropriate treatment for hepatitis C patients.**

Chronic hepatitis C is one of the major causes of chronic liver disease and can lead to cirrhosis and hepatocellular carcinoma. Interferon is the only effective drug for chronic hepatitis C patients, although better efficacy can be attained with modification of the regimen including the amount of interferon, the duration of treatment, and the use of a combination of pegylated-interferon and ribavirin.

Many studies have identified factors that can help predict the efficacy of interferon therapy such as hepatitis C virus (HCV) genotype[1] and viral loads.[2] Methods based on viral information are able to identify sustained responders (SR). However, this method places transient responders (TR) and nonresponders (NR) in the same category. Follow-up data clearly indicate that interferon treatment of patients in the TR group can lead to a reduction in the probability of tumor development compared with the NR group.[3,4] This suggests that the NR patients should be separated out first and that the TR group should be handled separately as an SR-like group. Furthermore, host factors may help the prediction of NR clinical outcome before treatment. Several candidates have been suggested that may be used to predict this effect including body mass index,[5] γ-glutamyltransferase/alanine transaminase levels,[6] the messenger RNA expression levels of the interferon receptor,[7,8] interferon-γ and tumor necrosis factor-α levels,[9] and the Th1/Th2 ratio[10]; however, there is no definitive evidence that any of these is a single dominant factor. Therefore, additional studies must be performed to identify host factors that can predict the efficacy of interferon therapy because complex changes in these host parameters may reflect variations in hepatic gene expression.

Complementary DNA (cDNA) microarrays can provide an enormous amount of data for identifying clusters

*Abbreviations used in this paper:* cDNA, complementary DNA; HCV, hepatitis C virus; MD, Mahalanobis distance; NR, nonresponder; SR, sustained responder; SSDB, standard space database; TR, transient responder.


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of predictive factors. For example, we previously have used custom-made cDNA microarrays to dissect gene expression patterns and to differentiate between patients infected with HCV and hepatitis B virus.[11,12] Other oligo-DNA chip approaches have proven to be very effective for identifying sets of genes expressed in vitro in response to interferon.[13] However, these approaches have not been useful for determining which treatment regimen should be used for each patient. In the current study we developed a cDNA microarray and a data analysis algorithm that can predict whether a patient will be an NR for interferon therapy based only on host messenger RNA expression and without the use of viral data.

## Materials and Methods

### Patients and Biopsy Samples

From 1993 to 2001, we collected liver biopsy samples from 99 HCV patients undergoing interferon monotherapy at Kyushu University Hospital. In addition, between 1999 and 2002, an additional 4 samples were collected from patients undergoing interferon monotherapy at Kanazawa University Hospital and 5 samples from Kyoto Prefectural University Hospital as part of a validation study. These patients received the standard 6-month protocol for interferon-alfa treatment. Thus, all patients received more than 468 MU of interferon-alfa monotherapy. Finally, between 2002 and 2003, 56 patients at Kanazawa University Hospital and Tokyo Metropolitan Komagome Hospital were treated with a 6-month regimen of interferon-alfa combined with 600–800 mg/day of ribavirin. Informed consent was obtained from all patients in accordance with the Helsinki protocol. Liver samples were obtained from these patients by biopsy procedure with a 14- or 16-gauge needle. The samples were snap-frozen in liquid nitrogen and stored at −80°C until use for RNA extraction. The viral genotype in pretreatment serum samples from these patients was determined as described previously,[14] the viral RNA copy number was tested using the HCV Amplicore kit (Roche Japan, Tokyo, Japan), and the viral serotype was assayed using an F-HCV-Gr enzyme-linked immunosorbent assay kit (Sysmex, Kobe, Japan). The patients were categorized into 3 groups: SR (patients with an absence of serum HCV RNA both during therapy and 6 months after the completion of therapy), NR (patients persistently positive for serum HCV RNA during therapy), and TR (patients negative for serum HCV RNA at the end of interferon treatment but positive after cessation of therapy).

### RNA Extraction, Complementary DNA Microarray, Data Collection, and Data Mining

The total RNA extraction procedure from biopsy samples and the low-density cDNA microarray together with a unique artificial reference RNA (Genomessage; JGS, Tokyo, Japan) used in these studies were described in our previous report.[15]

## Results

### Selection of Liver Biopsy Samples

RNA degradation is one of the main factors causing variability in data from cDNA microarrays. Because some of the biopsy samples used in this study were stored for more than 8 years, we examined the quality of the extracted RNA by microcapillary electrophoresis. Enough RNA (>2 μg) was obtained from only 69 of the 108 samples from patients treated with interferon monotherapy. We randomly divided these 69 samples into 2 groups of 31 and 38 samples for training and validation of the prediction algorithm, respectively. Based on the 28S/18S ratio, the RNA quality of these 69 samples was good. Of the 69 total patients, 47 were men and 22 were women, and the average age was 49 ± 12 years (range, 21–71 y). Table 1 summarizes the values of alanine transaminase, γ-glutamyltransferase, viral load, and genotypes for the 31 samples used for developing the prediction algorithm. Similarly, qualified RNA extracted from all 56 samples that had been obtained from patients receiving combination therapy were divided randomly into 2 groups of 33 and 23 samples. Of these patients, 46 were men and 10 were women, and the average age was 54 ± 8 years (range, 39–71 y). The characteristics of the group of 33 patients for establishment of SSDB are summarized in Table 2.

### Development of the Complementary DNA Microarray

To develop the cDNA microarray for the current study we first performed a serial analysis of gene expression on data from normal and hepatitis B and C patients for approximately 2000 genes. For this serial analysis of gene expression study we analyzed the results from our previous microarray analysis combined with publicly available data.[16,17] During this initial screening phase we tried to choose genes that could distinguish between hepatitis and normal samples. In addition, to focus on genes with meaningful signal levels, we omitted those with a low-frequency expressed tag in serial analysis of gene expression. These approaches are consistent with those of Chang et al[18] who, for statistical calculation, selected only the strong intensity signals from their GeneChip (Affymetrix; Santa Clara, CA) data. Furthermore, we omitted most sequences representing expressed sequence tags in the serial analysis of gene expression data. Finally, we selected genes whose functions have been well established. For example, 26 interferon-related genes were selected for the microarray. We also tried to select genes that have been reported previously to predict interferon efficacy, including interferon-alfa/β receptor,

**Table 1.** Characteristics of the Core Patients Used for the SSDB and Training

| Number | Age | Sex | Genotype | Viral load (*KIU/mL*) | Histology/stage and activity | ALT (*IU/L*) | γ-GT (*IU/L*) | Clinical outcome | Use |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 23 | F | 1b | 4.4 | F1A1 | 90 | 32 | SR | SSDB |
| 2 | 31 | M | 2a | 23 | F1A1 | 29 | 11 | SR | SSDB |
| 3 | 34 | F | 2a | 3.5 | F1A1 | 32 | 199 | SR | SSDB |
| 4 | 40 | M | 2a | 100 | F1A2 | 233 | 68 | SR | SSDB |
| 5 | 41 | M | 1b | 110 | F1A2 | 182 | 117 | SR | SSDB |
| 6 | 48 | M | 2a | 2.2 | F2A2 | 189 | 37 | SR | SSDB |
| 7 | 50 | M | 2b | 3.7 | F1A3 | 267 | 114 | SR | SSDB |
| 8 | 54 | F | 2a | 2.3 | F1A2 | 41 | 31 | SR | SSDB |
| 9 | 55 | M | 2a | 2.4 | F1A1 | 301 | 85 | SR | SSDB |
| 10 | 58 | M | 1b | 50 | F1A2 | 36 | 59 | SR | SSDB |
| 11 | 60 | M | 2b | 50 | F1A1 | 149 | 150 | SR | SSDB |
| 12 | 66 | M | 2a | 1.8 | F3A2 | 286 | 104 | SR | SSDB |
| 13 | 66 | M | 1b | 140 | F1A1 | 88 | 31 | SR | SSDB |
| 14 | 21 | M | 1a, 1b | 480 | F1A1 | 34 | 32 | NR | Training |
| 15 | 27 | M | 1b | 520 | F1A1 | 62 | 39 | NR | Training |
| 16 | 31 | F | 2a | 20 | F1A1 | 63 | 36 | SR | Training |
| 17 | 35 | M | 2a | 5.9 | F1A1 | 72 | 34 | SR | Training |
| 18 | 37 | F | 1b | 650 | F1A1 | 219 | 58 | NR | Training |
| 19 | 37 | M | 1b | 150 | F0A1 | 79 | 74 | TR | Training |
| 20 | 37 | M | 2b | 250 | F1A1 | 225 | 29 | TR | Training |
| 21 | 40 | M | 2a | 16 | F1A2 | 211 | 129 | SR | Training |
| 22 | 42 | M | 1b | 900 | F3A2 | 86 | 139 | TR | Training |
| 23 | 49 | M | 1b | 540 | F1A1 | 100 | 30 | TR | Training |
| 24 | 51 | F | 1b | 480 | F1A1 | 80 | 34 | NR | Training |
| 25 | 52 | M | 1b | 50 | F1A2 | 96 | 79 | SR | Training |
| 26 | 53 | M | 1b | 520 | F4A2 | 97 | 90 | NR | Training |
| 27 | 57 | M | 1b | 130 | F1A2 | 61 | 37 | TR | Training |
| 28 | 57 | M | 2a | 120 | F1A2 | 164 | 53 | SR | Training |
| 29 | 59 | F | 1b | 230 | F3A2 | 70 | 38 | NR | Training |
| 30 | 59 | M | 2b | 32 | F1A1 | 162 | 119 | NR | Training |
| 31 | 62 | F | 1b | 91 | F1A2 | 90 | 34 | NR | Training |

ALT, alanine transaminase; γ-GT, γ-glutamyltransferase.

tumor necrosis factor-α.[7–9] In addition, we incorporated clinical information to help select genes; specifically, we included iron transporter–related genes, such as transferrin and the transferrin receptor, because iron depletion has been shown to enhance the efficacy of hepatitis C treatment.[19] Finally, we included some genes (eg, housekeeping genes) as controls for the microarray. Together, 295 genes were selected originally for the low-density cDNA microarray. Furthermore, after we developed the low-density cDNA microarray, interferon-stimulated genes were analyzed systematically by using a different microarray.[20] Another 452 genes that were derived mainly from interferon-, tumor necrosis factor–, and extracellular matrix–related genes were added to a new cDNA microarray that included a total of 747 genes.

### Establishment of the Algorithm for Predicting Interferon Efficacy Based on Complementary DNA Microarray Data

As previously described,[15] we used a series of steps to make a reasonable prediction, including establishing a standard space database (SSDB), selecting characteristic parameters to differentiate groups of interest, setting variance–covariance, calculating the variance–covariance matrix, selecting a correlation/gene network, and, finally, calculating the Mahalanobis distance (MD) (the distance from the center of gravity can be determined for a new test sample using the SSDB), leading to a single parameter as a scale from multiple parameters. Thus, the SSDB dataset was selected from the SR patients that had clear clinical outcomes (13 members). This SSDB was trained by expanding it to different datasets, including SR/TR (10 members) and NR (8 members) data, to find genes that are expressed differentially between the 2 groups. The prediction probability of this stage was as follows: SR/TR (10 of 10; 100%), NR (7 of 8; 88%), with a $P$ value of less than .0005.

By using the new prediction algorithm we assessed the accuracy of prediction using the 38 validation samples (31 SR/TR and 7 NR). We calculated the MD and scaled MD from this established dataset for each patient to determine the distance from the established SSDB center of gravity. At this point the calculation does not incor-

**Table 2.** Characteristics of the Core Patients Used for the SSDB and Training in Combination Therapy

| Number | Age | Sex | Naive[a] | Serotype | Viral load (KIU/mL) | Histology/stage and activity | ALT (IU/L) | γ-GT (IU/L) | Clinical outcome | Use |
|---|---|---|---|---|---|---|---|---|---|---|
| 1056 | 62 | M | 0 | 1 | 595 | F4A3 | 199 | 87 | SR | SSDB |
| 1043 | 54 | M | 0 | 1 | 77 | F2A2 | 95 | 80 | SR | SSDB |
| 1042 | 39 | M | 0 | 2 | 850 | F1A1 | 59 | 89 | SR | SSDB |
| 1044 | 53 | M | 0 | 2 | 300 | F3A1 | 194 | 147 | SR | SSDB |
| 1052 | 53 | M | 1 | 2 | 440 | F1A1 | 97 | 80 | SR | SSDB |
| 1051 | 54 | M | 1 | 2 | 600 | F3A1 | 30 | 22 | SR | SSDB |
| 1048 | 52 | M | 1 | 1 | 580 | F1A0 | 81 | 37 | SR | SSDB |
| 1046 | 55 | M | 1 | 1 | 510 | F1A2 | 68 | 49 | SR | SSDB |
| 1040 | 37 | M | 1 | 1 | 360 | F1A1 | 45 | 90 | SR | SSDB |
| 1041 | 57 | M | 0 | 1 | 250 | F4A2 | 159 | 93 | SR | SSDB |
| 1050 | 62 | M | 0 | 2 | 690 | F1A2 | 118 | 96 | SR | SSDB |
| 1034 | 47 | F | 0 | 1 | 820 | F1A1 | 39 | 43 | TR | SSDB |
| 1026 | 57 | M | 1 | 1 | 550 | F3A2 | 106 | 14 | TR | SSDB |
| 1024 | 42 | M | 1 | 2 | 570 | F2A2 | 639 | 83 | TR | SSDB |
| 1022 | 60 | M | 1 | 1 | 610 | F1A1 | 56 | 209 | TR | SSDB |
| 1035 | 55 | F | 0 | 1 | 360 | F1A2 | 131 | 42 | TR | SSDB |
| 1025 | 58 | F | 0 | 1 | 850 | F1A1 | 58 | 35 | TR | SSDB |
| 1028 | 52 | M | 1 | 1 | 650 | F1A1 | 44 | 17 | TR | SSDB |
| 1029 | 46 | M | 1 | 1 | 850 | F1A1 | 40 | 30 | TR | SSDB |
| 1031 | 53 | M | 0 | 1 | 690 | F2A2 | 83 | 52 | TR | SSDB |
| 1033 | 61 | M | 0 | 1 | 850 | F2A2 | 64 | 46 | TR | SSDB |
| 1027 | 59 | M | 0 | 1 | 630 | F3A2 | 79 | 59 | TR | SSDB |
| 1023 | 61 | M | 1 | 2 | 300 | F3A3 | 67 | 61 | TR | SSDB |
| 1036 | 44 | M | 1 | 1 | 850 | F1A1 | 75 | 54 | TR | SSDB |
| 1020 | 64 | F | 0 | 2 | 850 | F1A2 | 358 | 76 | TR | SSDB |
| 1007 | 63 | M | 1 | 1 | 850 | F3A2 | 257 | 132 | NR | Training |
| 1009 | 49 | M | 1 | 1 | 620 | F3A1 | 346 | 274 | NR | Training |
| 1005 | 58 | M | 1 | 1 | 570 | F3A1 | 87 | 42 | NR | Training |
| 1015 | 53 | M | 0 | 1 | 850 | F1A1 | 37 | 65 | NR | Training |
| 1014 | 45 | M | 1 | 1 | 310 | F2A2 | 125 | 187 | NR | Training |
| 1013 | 57 | F | 1 | 1 | 440 | F3A2 | 57 | 35 | NR | Training |
| 1006 | 40 | M | 1 | 1 | > 850 | F3A1 | 244 | 237 | NR | Training |
| 1011 | 57 | M | 0 | 1 | > 850 | F2A2 | 90 | 48 | NR | Training |

ALT, alanine transaminase; γ-GT, γ-glutamyltransferase.
[a]0, first treatment; 1, retreatment.

porate any viral information such as genotype or viral load. The prediction probability of the validation stage was as follows: SR/TR (30 of 31; 97%) and NR (6 of 7; 86%). The $P$ value, calculated using the $\chi^2$ test, was less than .00001 for prediction accuracy. During the development of the algorithm we found several genes that were expressed differentially between the NR and SR/TR groups. The highlighted 75 genes according to $t$-test values are presented in Table 3. These could be separated into distinct groups such as interferon-, lipid metabolism–, complement-, and oxidoreductase-related genes. Because we used an artificial reference RNA as a control,[15] we were unable to determine whether the up- or down-regulation of these genes was meaningful biologically. $F$-test and $t$-test values for each gene, which represent the comparative expression levels between the SR/TR and NR groups, indicate only that the genes were expressed differentially between the SR/TR and NR groups.

Subsequently, we further analyzed samples from ribavirin combination therapy by using a DNA microarray containing 295 genes. The algorithm developed from the interferon monotherapy samples was ineffective for the samples from the combination therapy. Therefore, we tried to establish a new algorithm based on the data from this 295-gene DNA microarray but failed to obtain accurate prediction even using training samples from the combination therapy group (data not shown). To solve this problem we developed a new algorithm based on a new DNA microarray that included an additional 452 genes. The resulting algorithm allowed prediction of the outcome as follows: training (33 samples) stage: SR/TR (25 of 25; 100%) and NR (7 of 8; 88%); and validation (23 samples) stage: SR/TR (15 of 16; 94%) and NR (5 of 7; 71%), with prediction accuracies of 97% ($P < .0001$) and 87% ($P < .05$), respectively. Thus, the new microarray and algorithm could attain a high accuracy for prediction of treatment outcome using pretreatment liver

**Table 3.** Genes Differentially Expressed Between SR/TR and NR Groups in Monotherapy

| GenBank number | Gene name | *F*-test | *t*-test |
|---|---|---|---|
| U05340 | Cell division cycle 20 homolog (*S cerevisiae*)(CDC20) | .020 | .169 |
| BC008767 | Acyl-coenzyme A oxidase 1, palmitoyl (ACOX1) | .123 | .066 |
| AF279437 | Interleukin 22 | .005 | .201 |
| M36807 | Glycogen phosphorylase type IV | .007 | .250 |
| X03663 | Colony-stimulating factor 1 receptor | .254 | .096 |
| X02750 | Protein C (inactivator of coagulation factors Va and VIIIa) | .020 | .172 |
| BC000337 | Glucose-6-phosphate dehydrogenase | .146 | .066 |
| BC009345 | NADH dehydrogenase 1 | .082 | .233 |
| X00566 | Apolipoprotein A-1 | .183 | .063 |
| BC001188 | Transferrin receptor (p90, CD71) | .014 | .395 |
| J04026 | Thioredoxin | .050 | .160 |
| S57235 | CD68 antigen | .071 | .377 |
| M62403 | Insulin-like growth factor binding protein 4 | .010 | .113 |
| M65128 | FK506-binding protein 2 (13 kD) | .050 | .023 |
| M29145 | Hepatocyte growth factor | .115 | .034 |
| M11220 | Granulocyte-macrophage colony stimulating factor | .000 | .076 |
| M55654 | TATA box binding protein | .111 | .036 |
| X05360 | Cell division cycle 2, G1 to S and G2 to M | .193 | .043 |
| M21097 | CD19 antigen | .155 | .059 |
| J03171 | Interferon (α, β, and ω receptor 1 | .002 | .032 |
| U58196 | Interleukin enhancer binding factor 1 | .052 | .032 |
| Z12020 | p53-associated gene; Mdm2, transformed 3T3 cell double minute 2 | .014 | .121 |
| M93311 | Metallothionein-III (growth inhibitory factor [neurotrophic]) | .010 | .062 |
| X01992 | Intereferon γ | .001 | .156 |
| Y14736 | Immunoglobulin κ (light chain) variable 1D8 | .030 | .276 |
| M22538 | NADH dehydrogenase (ubiquinone) flavoprotein 2, 24 kilodaltons | .087 | .172 |
| X00955 | Apolipoprotein A-II | .019 | .292 |
| U94586 | NADH: ubiquinone oxidoreductase MLRQ subunit | .232 | .046 |
| X15949 | Interferon-regulatory factor-2 | .298 | .093 |
| M19154 | Transforming growth factor β-2 | .005 | .078 |
| X04688 | Interleukin 5 (colony-stimulating factor, eosinophil) | .050 | .173 |
| M14505 | Cyclin-dependent kinase 4 | .116 | .080 |

NOTE. Genes having *F*-test and *t*-test *P*-values <.4 and either value under .1 are listed.
Although *t*-test *P* values were calculated in Welch's method in cases in which the *F*-test *P* value <.05, the Student *t*-test method was applied in cases >.05.

samples. Interestingly, a different set of genes was highlighted in this study, including cyclophilin A and multidrug resistance protein 1 (Table 4).

## Discussion

In the current study we developed an algorithm for predicting the efficacy of interferon treatment in hepatitis C patients based only on host microarray data. Once the microarray dataset was normalized, we searched for the most suitable statistical method to differentiate it. We chose a statistical method based on the MD[15] because it allows the maximal flexibility in data dispersion. By using this method we were able to obtain a distinct separation of the NR and the SR/TR groups. In our algorithm we used a gene network system rather than a set of independent differentially expressed genes to generate the categories. Thus, none of the genes listed in Table 3 with *t*-test and *F*-test values alone was able to provide any definitive information for classification, and none of the genes could predict the classification independently. Despite this, we were able to validate our new algorithm using a separate validation group. We were able to predict SR/TR patients accurately in 30 of 31 cases (97%) and NR patients in 6 of 7 cases (86%). The *P* value for these predictions was less than .00001, which is acceptable for prediction purposes and suggests that the dataset from the low-density cDNA microarray can predict NR and non-NR patients with high accuracy without any viral information. Pretreatment classification and identification of NR patients is useful because they have a higher risk than TR or SR patients for developing hepatocellular carcinoma.[3,4] This same approach also allowed prediction of the efficacy of interferon/ribavirin combination therapy with high accuracy. A different gene set was required for the establishment, however, which reflects the different underlying mechanism of the drug action between the 2 treatment regimens.

Genes were selected for the SSDB based on differential expression in our cDNA microarray. Besides interferon-

**Table 4.** Gene List Highlighted as Differentially Expressed in Combination Therapy

| GenBank number | Gene name | *F*-test | *t*-test |
|---|---|---|---|
| X66362 | PCTAIRE-3 for serine/threonine protein kinase | .007 | .000 |
| U90551 | Histone (H2A II; histone 2A-like protein) | .054 | .002 |
| Y00285 | Insulin-like growth factor II receptor | .027 | .002 |
| X03884 | CD3 epsilon (T3 epsilon chain [20K] of T-cell receptor) | .045 | .007 |
| U12779 | MAP kinase-activated protein kinase 2 | .143 | .008 |
| Z33642 | Leukocyte surface protein V7; immunoglobulin superfamily, member 2 | .086 | .010 |
| U49837 | LIM protein (cystein-rich protein 3) | .001 | .011 |
| M77349 | BIGH3, TGF-β–induced product, TGF-β–induced 68 kilodalton | .141 | .013 |
| L16499 | Orphan homeobox protein; hematopoietically expressed homeobox | .017 | .013 |
| X78817 | ρ-GAP hematopoietic protein C1; ρ guanosine triphosphatase–activating protein 4 | .070 | .016 |
| AF159442 | Phospholipid scramblase 3 | .000 | .017 |
| J04164 | Interferon-inducible transmembrane protein 9–27 | .199 | .018 |
| L41351 | Serine protease 8 (prostatin) | .033 | .019 |
| U62437 | Nicotinic acetylcholine receptor β2 subunit prexursor | .052 | .020 |
| X58072 | GATA binding protein 3; transacting T-cell–specific transcription factor | .132 | .027 |
| X53414 | L-alanine: glyoxylate transaminase | .021 | .030 |
| Y00052 | Cyclophilin A (peptidylprolyl isomerase A; T-cell cyclophilin) | .152 | .034 |
| BC004490 | Fos | .002 | .035 |
| U03397 | Tumor necrosis factor–receptor superfamily, member 9 | .140 | .035 |
| Z47087 | Pol V elongation factor-like protein; S-phase kinase-associated protein 1A | .190 | .047 |
| M14758 | Multiple drug resistance protein 1; P-glycoprotein | .066 | .047 |
| U61397 | Ubiquitin-homology domain protein PIC1 (sentrin) | .021 | .050 |
| U16031 | Interleukin-4–induced transcription factor, signal transducer and activator of transcription 6 | .194 | .050 |
| BC032130 | Asialoglycoprotein receptor 1 | .032 | .057 |
| X05610 | Type IV collagen alpha (2) | .046 | .059 |
| D23661 | Ribosomal protein L37 | .035 | .066 |
| X69150 | Ribosomal protein S18 | .013 | .068 |
| M15400 | Retinoblastoma susceptibility | .040 | .104 |
| NM_001012 | Ribosomal protein S8 | .037 | .133 |
| M31627 | X-box binding protein-1 | .005 | .198 |

NOTE. Both *F*-test and *t*-test values less than .2 are listed.

related genes, the SSDB includes genes related to immune response, stress, metal transport, and lipid metabolism. The inclusion of genes controlled by the interferon signal cascade and related to the immune response is not surprising. In addition, genes associated with lipid metabolism are not unexpected because HCV has a high affinity for lipids.[21,22] Furthermore, lipoprotein receptors were reported as HCV receptor candidates.[23,24] In fact, the involvement of lipid metabolism–related genes is described in depth in a study of HCV clearance in the chimpanzee by GeneChip analysis.[25] Therefore, the lipid metabolism–related genes that we included in our analysis could be targets for future study and therapeutic intervention. Finally, the presence of iron transport–related genes in the SSDB corresponds with the use of blood depletion therapies to reduce liver inflammation in hepatitis patients. It also may be of interest to study how genes in the SSDB, including additional metal-related genes such as metallothioneins, play a role in interferon efficacy.

These findings suggest that the TR patients have an anti-HCV interferon response similar to that of the SR patients. Indeed, it is possible that these TR patients may have become SR patients if interferon treatment was administered for more than 6 months because there is a significant effect of treatment duration in the efficacy of interferon treatment for chronic hepatitis C.[26] This observation is consistent with a study of chimpanzee HCV cases based on oligo-chip data.[25] Furthermore, the fact that we could predict the NR group without any viral information suggests that, in these cases, the host has an unfavorable response to the interferon treatment, which also suggests that, as in the SR group, there is an interaction between the host and the virus. Understanding the host response to interferon in NR patients could provide interesting targets for the development of new treatments for HCV.

In conclusion, we have established a low-density cDNA microarray for predicting interferon efficacy in chronic hepatitis C patients. Based only on host messenger RNA expression profiles from pretreatment biopsy samples, we can categorize patients successfully into SR/TR and NR groups with over 90% accuracy.

## References

1. Yoshioka K, Kakumu S, Wakita T, et al. Detection of hepatitis C virus by polymerase chain reaction and response to interferon-

alpha therapy: relationship to genotypes of hepatitis C virus. Hepatology 1992;16:293–299.
2. Lau JY, Davis GL, Kniffen J, et al. Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. Lancet 1993;341: 1501–1504.
3. International Interferon-alpha Hepatocellular Carcinoma Study Group. Effect of interferon-alpha on progression of cirrhosis to hepatocellular carcinoma: a retrospective cohort study. Lancet 1998;351:1535–1539.
4. Kasahara A, Hayashi N, Mochizuki K, et al. Risk factors for hepatocellular carcinoma and its incidence after interferon treatment in patients with chronic hepatitis C. Osaka Liver Disease Study Group. Hepatology 1998;27:1394–1402.
5. Bressler BL, Guindi M, Tomlinson G, et al. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. Hepatology 2003;38:639–644.
6. Mihm S, Monazahian M, Grethe S, et al. Ratio of serum gamma-GT/ALT rather than ISDR variability is predictive for initial virological response to IFN-alpha in chronic HCV infection. J Med Virol 1999;58:227–234.
7. Yatsuhashi H, Yamasaki K, Aritomi T, et al. Quantitative analysis of interferon alpha/beta receptor mRNA in the liver of patients with chronic hepatitis C: correlation with serum hepatitis C virus-RNA levels and response to treatment with interferon. J Gastroenterol Hepatol 1997;12:460–467.
8. Mizukoshi E, Kaneko S, Yanagi M, et al. Expression of interferon alpha/beta receptor in the liver of chronic hepatitis C patients. J Med Virol 1998;56:217–223.
9. Dumoulin FL, Wennrich U, Nischalke HD, et al. Intrahepatic mRNA levels of interferon gamma and tumor necrosis factor alpha and response to antiviral treatment of chronic hepatitis C. J Hum Virol 2001;4:195–199.
10. Sobue S, Nomura T, Ishikawa T, et al. Th1/Th2 cytokine profiles and their relationship to clinical features in patients with chronic hepatitis C virus infection. J Gastroenterol 2001;36:544–551.
11. Honda M, Kaneko S, Kawai H, et al. Differential gene expression between chronic hepatitis B and C hepatic lesion. Gastroenterology 2001;120:955–966.
12. Shirota Y, Kaneko S, Honda M, et al. Identification of differentially expressed genes in hepatocellular carcinoma with cDNA microarrays. Hepatology 2001;33:832–840.
13. Der SD, Zhou A, Williams BR, et al. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. Proc Natl Acad Sci U S A 1998;95: 15623–15628.
14. Okamoto H, Sugiyama Y, Okada S, et al. Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673–679.
15. Daiba A, Inaba N, Ando S, et al. A low-density cDNA microarray with a unique reference RNA: pattern recognition analysis for IFN efficacy prediction to HCV as a model. Biochem Biophys Res Commun 2004;315:1088–1096.
16. Yamashita T, Hashimoto S, Kaneko S, et al. Comprehensive gene expression profile of a normal human liver. Biochem Biophys Res Commun 2000;269:110–116.
17. Yamashita T, Kaneko S, Hashimoto S, et al. Serial analysis of gene expression in chronic hepatitis C and hepatocellular carcinoma. Biochem Biophys Res Commun 2001;282:647–654.
18. Chang JC, Wooten EC, Tsimelzon A, et al. Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. Lancet 2003;362:362–369.
19. Fontana RJ, Israel J, LeClair P, et al. Iron reduction before and during interferon therapy of chronic hepatitis C: results of a multicenter, randomized, controlled trial. Hepatology 2000;31: 730–736.
20. de Veer MJ, Holko M, Frevel M, et al. Functional classification of interferon-stimulated genes identified using microarrays. J Leukoc Biol 2001;69:912–920.
21. Andre P, Komurian-Pradel F, Deforges S, et al. Characterization of low- and very-low-density hepatitis C virus RNA-containing particles. J Virol 2002;76:6919–6928.
22. Kono Y, Hayashida K, Tanaka H, et al. High-density lipoprotein binding rate differs greatly between genotypes 1b and 2a/2b of hepatitis C virus. J Med Virol 2003;70:42–48.
23. Agnello V, Abel G, Elfahal M, et al. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A 1999;96:12766–12771.
24. Scarselli E, Ansuini H, Cerino R, et al. The human scavenger receptor class B type I is a novel candidate receptor for the hepatitis C virus. EMBO J 2002;21:5017–5025.
25. Su AI, Pezacki JP, Wodicka L, et al. Genomic analysis of the host response to hepatitis C virus infection. Proc Natl Acad Sci U S A 2002;99:15669–15674.
26. Poynard T, Leroy V, Cohard M, et al. Meta-analysis of interferon randomized trials in the treatment of viral hepatitis C: effects of dose and duration. Hepatology 1996;24:778–789.

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# Differences of Hepatocellular Carcinoma Patients with Hepatitis B Virus Genotypes of Ba, Bj or C in Japan

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**Key Words**
Hepatocellular carcinoma, epidemiology · Subtypes Ba/Bj, hepatitis B · Hepatitis B virus, genotypes B/C

**Abstract**
Hepatitis B virus (HBV) genotypes B (HBV/B) and C (HBV/C) are prevalent in Asia. Recently HBV/B has been classified into two subtypes, HBV/Ba which is ubiquitously found in Asia, and HBV/Bj which is specific in Japan. In addition, the frequency of positive HBeAg has been reported to be higher in patients with HBV/Ba than those with HBV/Bj. However, little is known about the differences between patients with various genotypes who developed hepatocellular carcinoma (HCC). In 296 serum samples of HCC patients collected from all over Japan, HBV genotypes were determined with the restriction fragment length polymorphism. HBV/A was detected in 1.0%, HBV/Ba in 4.4%, HBV/Bj in 7.4%, and HBV/C in 86.5%. In the Tohoku district and Okinawa, HBV/Ba, HBV/Bj and HBV/C were found in 6.7, 40.0 and 48.9%, compared to 4.0, 1.6 and 93.2% in the other districts in Japan. HBV/Bj patients were more frequently found in the group older than 65 years while HBV/Ba patients were found in all age groups. The frequency of positive HBeAg in HBV/Bj patients was significantly low compared to that in the other patients. More than 60% of the patients with HCC had cirrhosis as the underlying liver diseases. However, in HBV/Ba patients aged 50 years or younger, 80% of them had chronic hepatitis, while 87.5% of those aged older than 50 years had cirrhosis. These data suggest that great differences exist among patients with HCC infected with different genotypes.



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## Introduction

In Japan, in more than two thirds of the patients with hepatocellular carcinoma (HCC) the disease is associated with hepatitis C virus (HCV). However, hepatitis B virus (HBV) is the major causative agent of HCC in Asian countries. All strains of HBV isolated from various countries can be classified into 8 HBV genotypes, HBV genotype A (HBV/A) to HBV/H, according to their phylogenetic relationships [1–3]. It has been reported that the clinical and virologic manifestations of patients with chronic HBV infection show significant differences among the different HBV genotypes [4–6]. In addition, specific distributions of HBV genotypes have been demonstrated among areas and countries [4, 7]. In south-east Asian countries, such as Japan, Taiwan, or China, HBV/B and HBV/C are prevalent [5, 7, 8].

In Japanese patients with HCC, the patients with HBV/B are rare and their mean age is high [7, 9]. However, in Taiwanese patients with HCC, a high proportion of younger patients have HBV/B. Until now, it is still unclear why younger Taiwanese patients with HBV/B develop HCC while Japanese patients with HBV/B rarely develop HCC, only in older age.

Recently, we demonstrated that HBV/B strains should be divided into two subtypes, HBV/Ba and HBV/Bj, according to their genetic relationship, and that HBV/Ba is found ubiquitously in Asian countries while HBV/Bj is found only in Japan [10, 11]. It was reported that HBeAg was found more frequently in patients with chronic infection with HBV/Ba than in those with chronic infection with HBV/Bj (32 vs. 9%) [12]. However, it is still unknown whether etiological and virologic differences are found between the HCC patients with HBV/Ba and HBV/Bj. Thus, in the patients with HCC, the difference between the subtypes of HBV/Ba and HBV/Bj might explain the etiological or clinical differences between Japan and Asia where HBV/Bj and HBV/Ba are endemic, respectively.

So, the aim of this study was to investigate the differences in the etiological, virologic and clinical characteristics among Japanese HCC patients with different HBV genotypes, such as HBV/Ba, HBV/Bj or HBV/C.

## Patients and Methods

*Patients with HCC*

Two hundred and ninety-six patients with HCC were consecutively collected from 19 hospitals throughout Japan during January 2001 to December 2002. All the patients were chronically positive for HBsAg, and negative for anti-HDV, anti-HCV and anti-HIV. The diagnosis of HCC was reached clinically with ultrasound, computerized tomography, magnetic resonance imaging, angiography, tumor markers and biopsy if possible. The diagnoses of chronic hepatitis (CH) and liver cirrhosis (LC) were principally done by liver biopsy. However, a proportion of patients with ascites, jaundice or severe thrombocytopenia were diagnosed by ultrasound, computerized tomography and liver function tests. The serum samples and clinical data were collected from these patients with written informed consent. This study was conducted according to the ethical guidelines in our hospitals.

*Virologic Assays*

In all serum samples, HBsAg (CLIA, Fujirebio, Japan, detection limit 0.13 ng/ml), HBeAg (CLIA, Fujirebio, Japan) and anti-HBe (CLIA) were tested. Serum HBV DNA was detected by nested polymerase chain reaction (PCR) with the primers derived from the S gene. The patients were not enrolled in this study if the serum HBV DNA was not detected by PCR. The HBV genotype was determined by restriction fragment length polymorphism as described previously [13]. In brief, the S gene of HBV DNA was amplified by nested PCR. Then the products were sequentially digested by the restriction enzyme, *Alw*I, *Ear*I, *Hph*I, *Nci*I and *Nla*IV, respectively. The HBV genotype was determined by the size of the digested PCR product which was electrophoresed on agarose gel. When the test results were inconclusive, the sequences of the S region were determined directly, then the genotype was decided by phylogenetic analysis [13, 14]. When patients were found to have HBV/B, the subtypes Ba and Bj were determined by restriction fragment length polymorphism [11]. In brief, at nucleotide position 1838 in the pre-core region, only A was found in patients with HBV/Ba while only G was found in those with HBV/Bj. The restriction enzyme detection system was established targeting the discrimination of this difference in nucleotides with the restriction enzyme, *Spe*I and *Mse*I after the pre-core region was amplified by PCR.

*Statistical Analysis*

The data were statistically analyzed by Student's t test, nonparametric Mann-Whitney test, and $\chi^2$ test where appropriate. A p value of $<0.05$ was regarded as statistically significant.

## Results

*HBV Genotypes and Clinical Findings*

Of the 296 patients, 223 were male and 73 were female. The mean age was 55.1 ± 10.8 (range 26–81) years. The clinical findings are shown in table 1. Thirty-five percent of the patients were positive for HBeAg. Regarding the HBV genotypes, 3 patients (1.0%) were HBV/A, 13 (4.4%) HBV/Ba, 22 (7.4%) HBV/Bj, 256 (86.5%) HBV/C, and 2 (0.7%) of mixed genotype (HBV/B and C). The clinical findings by HBV genotype are shown in table 2. There were no significant differences in the mean levels of total bilirubin, AST and ALT among patients with different HBV genotypes. However, the mean ALP level and γ-