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.

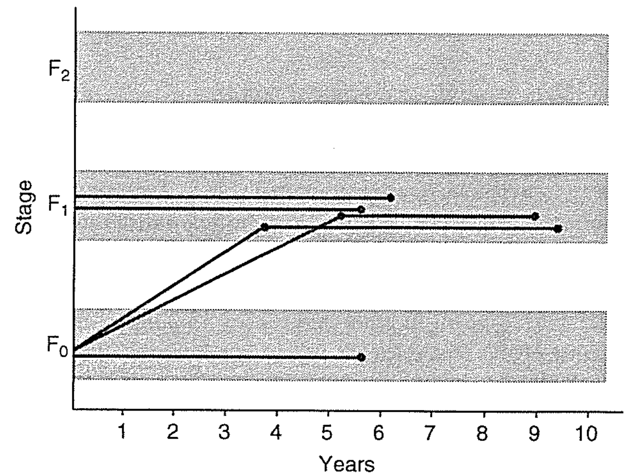

Fig. 1. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels remained normal during the follow-up period. Five patients with persistently normal serum aminotransferase levels submitted to repeat biopsies and the stage of liver fibrosis progressed 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).

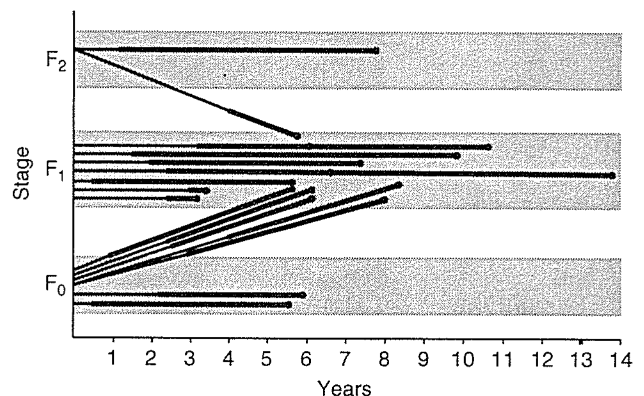

Fig. 2. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels were transiently elevated during the follow-up period. Sixteen patients with transient elevation of serum aminotransferase levels submitted to repeat biopsies and the stage of liver 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.

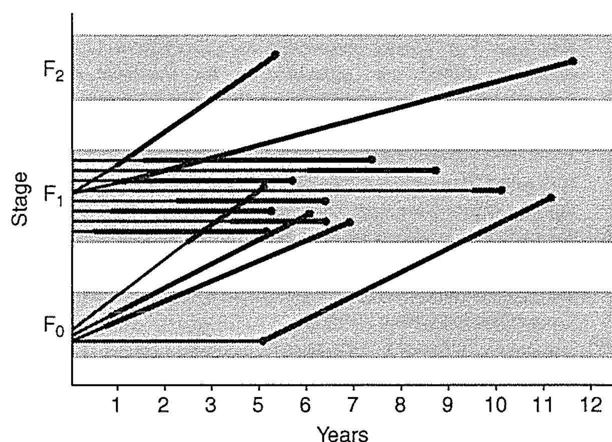

Fig. 3. Follow-up study of liver histology on asymptomatic hepatitis C virus carriers whose alanine aminotransferase levels became persistently abnormal during the follow-up period. Fourteen patients who developed continuously abnormal serum aminotransferase levels submitted to repeat liver biopsies 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).

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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 χ^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.

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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, *AlwI*, *EarI*, *HphI*, *NciI* and *NlaIV*, 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, *SpeI* and *MseI* 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 χ^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-

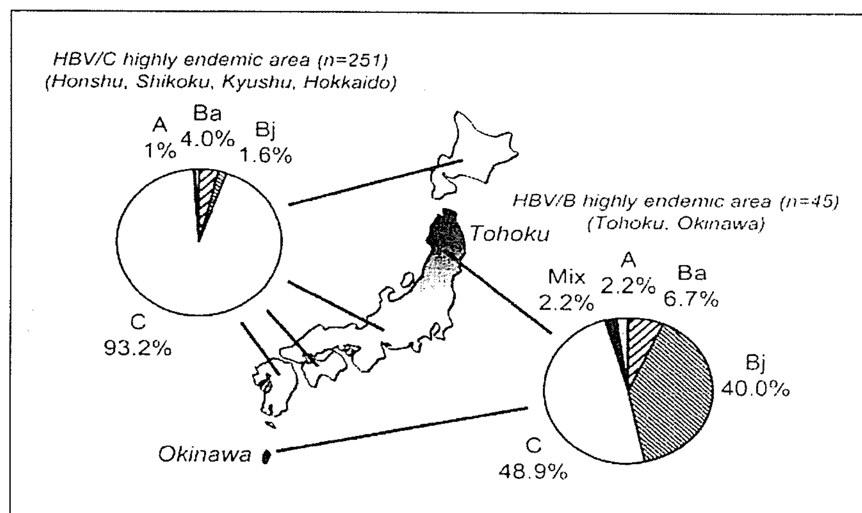

Fig. 1. The geographic distribution of HBV genotypes in Japan. In the Tohoku district, the northern area of mainland Japan, and Okinawa, the most southern islands, 48.9% of HCC patients were HBV/C, 6.7% were HBV/Ba, and 40.0% were HBV/Bj. In contrast, in other parts of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 93.2% were HBV/C, 4.0% were HBV/Ba and 1.6% were HBV/Bj.

Table 1. Characteristics of 296 HBsAg-positive Japanese patients with HCC collected from all over Japan

Male:female	223:73
Age, years	55.1 ± 10.8[a]
Total bilirubin, mg/dl	1.5 ± 1.9
AST, IU/l	78.5 ± 103.9
ALT, IU/l	63.0 ± 69.8
ALP, IU/l	321.1 ± 225.4
γ-GTP, IU/l	108.4 ± 174.4
HBeAg, % positive	35.0
Anti-HBe, % positive	64.8
HBV genotype	
HBV/A	3 (1.0%)
HBV/Ba	13 (4.4%)
HBV/Bj	22 (7.4%)
HBV/C	256 (86.5%)
Mix	2 (0.7%)

[a] Mean ± SD.

Table 2. Clinical findings of the HCC patients with HBV genotypes of Ba, Bj or C

	HBV genotype		
	Ba	Bj	C
Age, years	55.4 ± 12.9	66.6 ± 10.6	54.0 ± 10.7
	p < 0.01 (Ba vs. Bj)	p < 0.01 (Bj vs. C)	
Total bilirubin, mg/dl	1.0 ± 0.4	1.2 ± 0.7	1.5 ± 2.0
AST, IU/l	173.9 ± 352.6	51.6 ± 42.1	82.6 ± 113.4
ALT, IU/l	102.4 ± 162.9	33.9 ± 16.8	66.5 ± 74.9
ALP, IU/l	147.7 ± 126.6	209.8 ± 95.4	343.9 ± 238.0
	p < 0.05 (Ba vs. C)		
γ-GTP, IU/l	78.6 ± 55.9	63.1 ± 45.9	110.5 ± 186.7
		p < 0.05 (Bj vs. C)	

GTP level of the HBV/C patients was significantly higher than those with HBV/Ba and HBV/Bj, respectively ($p < 0.05$).

Geographic Distribution of HBV Genotypes

The geographic distribution of HBV genotypes was area-specific in Japan (fig. 1). This specific distribution of HCC patients was in accord with that of all the patients including asymptomatic carriers, CH and LC patients, as described previously [7]. Namely, in the Tohoku district, the northern area of the Japanese mainland, and Okinawa, the most southern islands, 22 (48.9%) of HCC patients were HBV/C, 3 (6.7%) were HBV/Ba, and 18 (40.0%) were HBV/Bj. In contrast, in other areas of Japan, Hokkaido, Honshu, Shikoku and Kyushu, 234 (93.2%) were HBV/C, 10 (4.0%) were HBV/Ba, and 4 (1.6%) were HBV/Bj ($p < 0.01$).

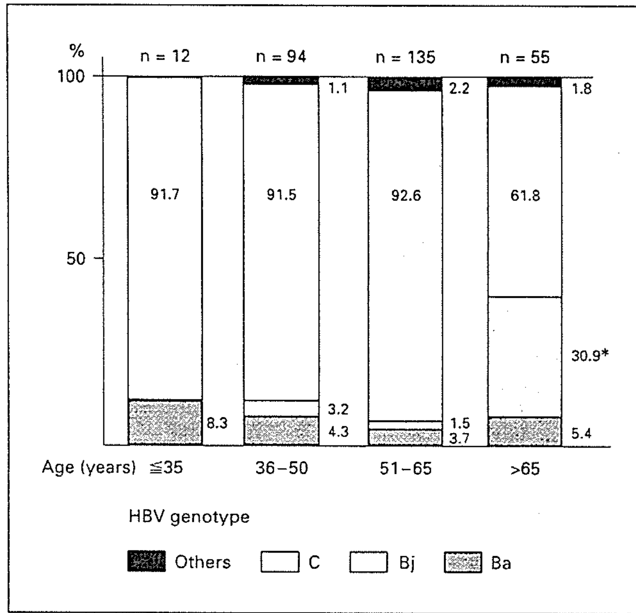

Fig. 2. The distribution of HBV genotypes in each age group. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of patients had HBV/C while 30.9% had HBV/Bj (* $p < 0.01$, group aged older than 65 years vs. other age groups). More patients with HBV/Ba were in the younger aged group, although the number of patients with HBV/Ba was small in all the groups.

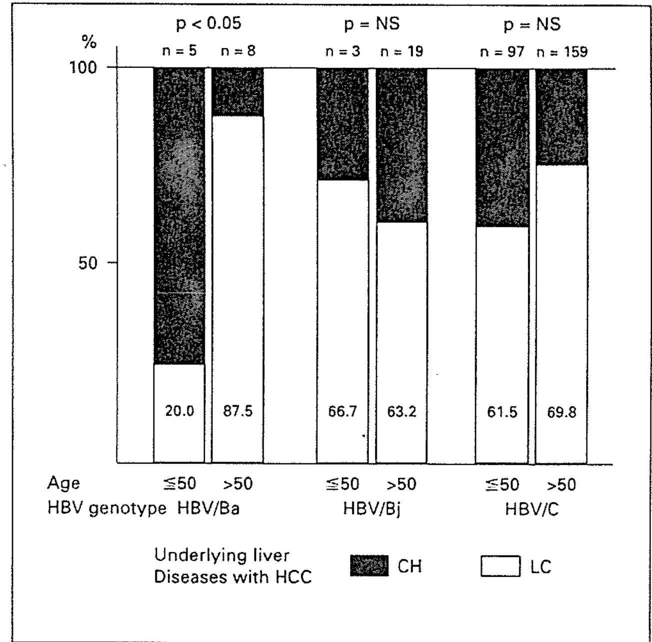

Fig. 4. The underlying liver diseases, chronic hepatitis (CH) or liver cirrhosis (LC), in HCC patients. In patients with HBV/Ba, only 25.0% of the group aged 50 years or younger had LC, while 85.7% of the group aged older than 50 years had LC ($p < 0.01$). However, in patients with HBV/Bj or HBV/C, the ratios of the underlying liver diseases were approximately identical even when compared by age.

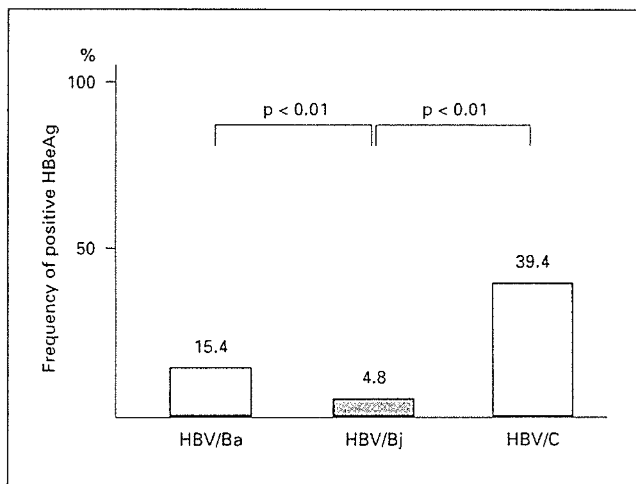

Fig. 3. The frequency of patients with positive HBeAg in each HBV genotype. The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$).

Mean Age and Frequency of Positive HBeAg among Patients with Each Genotype

The mean age of HBV/Bj patients (66.6 ± 10.6 years) was significantly higher than those with HBV/Ba (55.4 ± 12.9 years, $p < 0.01$) and HBV/C (54.0 ± 10.7 years, $p < 0.01$; table 2). The distribution of HBV genotypes in each age group is shown in figure 2. In groups aged 35 years or younger, 36–50 years, and 51–65 years, more than 90% of HCC patients had HBV/C. On the other hand, in the group aged older than 65 years, only 61.8% of the patients had HBV/C while 30.9% had HBV/Bj ($p < 0.01$, group aged older than 65 years vs. other age groups). HBV/Ba tended to be found in the younger age group although the number of patients with HBV/Ba was small in all groups.

The frequency of positive HBeAg was 4.8% in patients with HBV/Bj, compared with 39.4% in those with HBV/C (Bj vs. C, $p < 0.01$), and 15.4% in those with HBV/Ba (Bj vs. Ba, $p < 0.01$; fig. 3).

Underlying Liver Diseases

All HCC patients had underlying chronic liver diseases, such as CH or LC. We compared the underlying liver diseases among those aged 50 years or younger and those aged older than 50 years by HBV genotype (fig. 4). In 13 patients with HBV/Ba, only 1 (20.0%) of the 5 patients aged 50 years or younger had LC, while 7 (87.5%) of the 8 patients aged older than 50 years had LC ($p < 0.05$). However, in patients with HBV/Bj or HBV/C, the ratios of underlying liver diseases were approximately identical even when compared by age.

Discussion

The clinical and virologic features of patients with chronic HBV infection are specific according to their HBV genotypes [4, 15]. However, to date, there has been no report on the relationship between the HBV genotypes of Ba, Bj and C, and the clinical characteristics of HCC patients. We therefore analyzed the relationship between the clinical characteristics of Japanese HCC patients identified throughout Japan, and their HBV genotypes, including the HBV subtypes of Ba and Bj. In this study, we demonstrated that HBV/Ba (4.4%), HBV/Bj (7.4%) and HBV/C (86.5%) were found in Japanese HCC patients, and that there were distinct clinical differences among the three HBV genotypes, in geographic distribution, age distribution, and the frequency of positive HBeAg.

Of the Japanese patients with chronic HBV infection, including asymptomatic carriers, CH, LC and HCC, 1.7% were HBV/A, 12.2% HBV/B, 84.7% HBV/C, 0.4% HBV/D, and the others 1.0%, as reported previously [7]. In this study, we collected 296 serum samples from patients with HCC throughout Japan. In addition, we recently developed a new method for detecting HBV/Ba and HBV/Bj with restriction fragment length polymorphism [11]. Thus, we showed that 1.0% was HBV/A, 4.4% HBV/Ba, 7.4% HBV/Bj, 86.5% HBV/C, and mixed genotype 0.7% in Japanese HCC patients. This prevalence in HCC patients is almost identical to that in all patients with chronic HBV infection [7]. In addition, the geographic distribution of HBV/B and HBV/C in HCC patients is also identical to that in all patients. However, when we analyzed the HBV subtypes of HBV/Ba and HBV/Bj in patients with HBV/B, a high proportion of patients with HBV/Bj is found in the highly endemic HBV/B area, the Tohoku district and Okinawa, while the prevalence of HBV/Ba is approximately identical between the highly endemic HBV/C area, the other areas of Japan, and the highly endemic HBV/B area. Thus, HBV/Bj is specifically distributed in the Tohoku district and Okinawa.

As reported previously, HBV/Ba is ubiquitous in all Asian countries including Japan, although HBV/Bj is specific to Japan and is not found in other countries [11]. In Okinawa, it is reported that a high proportion of patients with chronic HBV infection have HBV/B and a good prognosis compared with patients with HBV/C [16, 17]. In contrast, in Taiwan, close to Japan, a higher proportion of patients aged 50 years or younger with HBV/B have HCC and CH [15]. The underlying liver diseases in those who developed HCC were compared among each HBV genotype group. In the HBV/Ba group, up to 75% of the patients aged 50 years or younger had CH as the underlying liver disease, compared with patients aged over 50 years. On the other hand, in the group with HBV/Bj or HBV/C, more than 60% of the patients had LC regardless of their age. The mean age of the patients with HBV/Ba in Japan is more than 10 years younger than those with HBV/Bj. So, more younger patients with HBV/Ba tend to have CH than the other patients. However, the molecular mechanism is unclear why patients with HBV/Ba develop HCC at a younger age and often have CH.

It is unclear why Japanese patients with HBV/B have a good prognosis while Taiwanese patients with HBV/B often have more advanced liver diseases, such as HCC. The frequency of patients positive for HBeAg in the HBV/Ba and HBV/C groups was higher than in the HBV/Bj group. So, the viral activity of HBV may be higher in patients with HBV/Ba or HBV/C than those with HBV/Bj. Thus, these differences in subtypes of HBV/Ba and Bj could be one of the reasons why the discrepancy in prognosis exists between Japanese and Taiwanese patients with HCC.

The differences in DNA sequences between HBV/Ba and HBV/Bj can be characterized in the core gene [10]. It has been reported that HBV/Ba, not HBV/Bj, recombines with HBV/C in the core gene. The product of the core gene is reported to be a cytotoxic T-cell epitope [18], suggesting that patients with HBV/Ba and HBV/C may be exposed to severe immune responses for destroying hepatocytes compared with those with HBV/Bj. In addition, patients with HBV/Ba more often have core promoter mutations at nucleotide 1762/1764 than those with HBV/Bj [11], which is associated with more advanced liver diseases [6, 19]. Taken together, these facts may indicate a poor prognosis in patients with HBV/Ba compared to those with HBV/Bj.

In the patients with HBV/C, the mean ALP and γ-GTP levels were higher than those with the other genotypes. In this study, there may exist some bias of regarding the tumor size of HCC between patients with HBV/C and the other patients. It is considered that more patients with a rather large size of HCC were found in the patients with HBV/C, resulting in elevation in ALT and γ-GTP levels.

To investigate the hepatocarcinogenesis and risk factors of HCC, it is important to study the differences in host, environmental and viral factors. The various genetic alterations, such as mutations of cancer-associated genes or loss of some chromosomes, are found in the HCC cells [20]. However, the genetic polymorphism varies among populations [21]. The differences in host genomes are still unknown between Japanese and other Asian populations. The association of environmental factors, such as air, water and food contaminated with some chemical agents, and HCC is still unclear, although aflatoxin affects the mutation of p53 in HCC [22]. However, with respect to the viral factors, a survey of the distribution of HBV genotypes or subtypes will be important clues for solving these problems.

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Clinical Studies

Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance

Tanaka E, Matsumoto A, Suzuki F, Kobayashi M, Mizokami M, Tanaka Y, Okanoue T, Minami M, Chayama K, Imamura M, Yatsuhashi H, Nagaoka S, Yotsuyanagi H, Kawata S, Kimura T, Maki N, Iino S, Kiyosawa K, HBV Core-Related Antigen Study Group. Measurement of hepatitis B virus core-related antigen is valuable for identifying patients who are at low risk of lamivudine resistance.
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Abstract: *Objective:* The clinical usefulness of hepatitis B virus core-related antigen (HBVcrAg) assay was compared with that of HBV DNA assay in predicting the occurrence of lamivudine resistance in patients with chronic hepatitis B. *Patients:* Of a total of 81 patients who were treated with lamivudine, 25 (31%) developed lamivudine resistance during a median follow-up period of 19.3 months. *Results:* The pretreatment positive rate of HBe antigen, or pretreatment levels of HBVcrAg or HBV DNA did not differ between patients with and without lamivudine resistance. Levels of both HBVcrAg and HBV DNA decreased after the initiation of lamivudine administration; however, the level of HBVcrAg decreased significantly more slowly than that of HBV DNA. The occurrence of lamivudine resistance was significantly less frequent in the 56 patients whose HBV DNA level was less than 2.6 log copy/ml at 6 months of treatment than in the remaining 25 patients. The cumulative rate of lamivudine resistance was as high as 70% within 2 years in the latter group, while it was only 28% in the former group. Lamivudine resistance did not occur during the follow-up period in the 19 patients whose HBVcrAg level was less than 4.6 log U/ml at 6 months of treatment, while it did occur in 50% of the remaining patients within 2 years. *Conclusion:* These results suggest that measurement of HBV DNA is valuable for identifying patients who are at high risk of developing lamivudine resistance, and that, conversely, measurement of HBVcrAg is valuable for identifying those who are at low risk of lamivudine resistance.

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Key words: chronic hepatitis B – HBV core-related antigen – HBV DNA – lamivudine resistance

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Lamivudine, a nucleoside analogue that inhibits reverse transcriptases, was first developed as an anti-viral agent against human immunodeficiency virus (HIV). It was later also found to be effective against hepatitis B virus (HBV) because HBV is a member of the Hepadnaviridae family of viruses, which use reverse transcriptases in their replication process (1, 2). Lamivudine was found to inhibit the replication of HBV, reduce hepatitis, and improve histological findings of the liver in long-term treatment (3–5). Furthermore, it has been shown that lamivudine treatment improves the long-term outcome of patients with chronic hepatitis B (6, 7). However, there are a number of problems with lamivudine therapy, such as relapse of hepatitis because of the appearance of YMDD mutant viruses and the reactivation of hepatitis after discontinuation of the treatment (8–11).

The concentration of HBV DNA in serum decreases and usually becomes undetectable during lamivudine administration, but it rapidly increases when HBV becomes resistant to lamivudine. Thus, the measurement of HBV DNA is useful for monitoring the anti-viral effects of lamivudine. However, a negative result of HBV DNA in serum does not necessarily indicate a good outcome of lamivudine therapy, because lamivudine resistance may occur even if HBV DNA levels remain undetectable during therapy (11–13). Recently, a chemiluminescence enzyme immunoassay (CLEIA) was developed in our laboratory for the detection of hepatitis B virus core-related antigen (HBVcrAg) (14, 15). The assay reflects the viral load of HBV in a similar manner to that used in assays, which detect HBV DNA. HBVcrAg consists of HBV core and e antigens; both proteins are transcribed from the precore/core gene and their first 149 amino acids are identical (16–18). The HBVcrAg CLEIA simultaneously measures the serum levels of hepatitis B core (HBc) and e (HBe) antigens, using monoclonal antibodies, which recognize common epitopes of these two denatured antigens. In the present study, we analyzed the clinical significance of the HBVcrAg assay in monitoring the anti-viral effects of lamivudine treatment.

Patients and methods

Patients

A total of 81 patients with chronic hepatitis B, who received lamivudine therapy, were enrolled in the present study. These were 58 men and 23 women with a median age of 49 years (range 24–79 years). The 81 patients were selected retrospectively from six medical institutions in Japan (Shinshu University Hospital, Toranomon Hospital, Nagoya City University Hospital, Kyoto Prefectural University Hospital, Hiroshima University Hospital, National Nagasaki Medical Center). Eight to 25 patients who met the following three criteria were selected consecutively in each institution: the first, a daily dose of 100 mg lamivudine was administered for at least 6 months in a period from 1999 to 2004; the second, histologically confirmed for chronic hepatitis without liver cirrhosis; and the third, serum samples at several time points available for testing. All patients were naive for lamivudine therapy. Chronic hepatitis B was defined as positive hepatitis B surface (HBs) antigen for more than 6 months with elevated levels of serum transaminases. The HBV genotype was A in two patients, B in three and C in 76. Serum HBV DNA was detectable in all patients, and HBe antigen was positive in 51 (63%) of the 81 patients just before lamivudine administration. The median follow-up period was 19 months with a range from 6 to 50 months. Follow-up of patients ended when lamivudine administration was discontinued. Written informed consent was obtained from each patient.

The occurrence of lamivudine resistance was defined as a rapid increase in serum HBV DNA levels with the appearance of the YMDD mutations during lamivudine administration. Using this criteria, resistance appeared in 27 (33%) of the 81 patients. The median period from the start of lamivudine administration to the occurrence of resistance was 12 months with a range from 4 to 37 months.

Serological markers for HBV

HBs antigen, HBe antigen and anti-HBe antibody were tested using commercially available enzyme immunoassay kits (Abbott Japan Co., Ltd., Tokyo, Japan). Six major genotypes (A–F) of HBV can be detected using the method reported by Mizokami et al. (19), in which the surface gene sequence amplified by polymerase chain reaction (PCR) is analyzed by restriction fragment length polymorphism. The YMDD motif, that is, lamivudine resistant mutations in the active site of HBV polymerase, was detected with an enzyme-linked mini-sequence assay kit (HBV YMDD Mutation Detection Kit, Genome Science Laboratories Co., Ltd., Tokyo, Japan) (20).

Serum concentration of HBV DNA was determined using Amplicor HBV monitor kit (Roche, Tokyo, Japan), which had quantitative range from 2.6 to 7.6 log copy/ml. Sera containing