

Supportive Role Played by Precore and PreS2 Genomic Changes in the Establishment of Lamivudine-Resistant Hepatitis B Virus

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Background. Hepatitis B virus (HBV) establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation. However, both lamivudine-resistant viruses with and those without rtL180M can exist in clinical settings. To elucidate the differences between viruses with and those without rtL180M, we conducted full-length sequencing analysis of HBV derived from patients with type B chronic hepatitis showing lamivudine resistance.

Methods. The full-length HBV DNA sequences derived from 44 patients showing lamivudine resistance were determined by polymerase chain reaction direct sequencing. Viral replicative competence was examined by in vitro transfection analysis using various HBV-expressing plasmids.

Results. Throughout the HBV genome, a precore-defective A1896 mutation and a short deletion in the preS2 gene were detected more frequently in viruses without rtL180M than in those with it (64% vs. 17% [$P < .005$] and 50% vs. 10% [$P < .01$], respectively). In vitro transfection analysis revealed that the level of reduction in intracellular viral replication caused by the introduction of lamivudine resistance-associated mutations was lower in precore-defective and preS2-deleted viruses than in wild-type virus.

Conclusions. Both the precore-defective mutation and the preS2 deletion may play a supportive role in the replication of lamivudine-resistant HBV, which may be a reason for there being no need for the compensatory rtL180M mutation in lamivudine-resistant HBV possessing the precore and preS2 genomic changes.

Therapeutic concepts for hepatitis B virus (HBV) infection have been strikingly modified by the introduction of nucleos(t)ide analogues. Nucleos(t)ide analogues, such as lamivudine, adefovir dipivoxil, entecavir, tenofovir disoproxil fumarate, emtricitabine, telbivudine, and clevudine, have been shown to lead to suppression of viral replication and improvement of liver diseases in chronic HBV infection [1–10]. However, the effective-

ness of nucleos(t)ide analogues is debilitated by the emergence of drug-resistant mutant virus. Treatment with lamivudine has been shown to lead to a higher rate of emergence of drug-resistant virus than with other newly developed nucleos(t)ide analogues, such as adefovir dipivoxil and entecavir [11–14]. The incidence of lamivudine resistance has been reported to be 24% at 1 year and 70% at 4 years of therapy [11].

Lamivudine resistance is known to be caused by a point mutation within the reverse transcriptase (rt) domain of the HBV polymerase gene, either rtM204V or rtM204I [15–17]. In addition, an rtL180M mutation has been shown to be frequently found together with the rtM204V/I mutation associated with lamivudine resistance [15–17]. Previous studies using in vitro transfection with the HBV-expressing plasmid have demonstrated that the rtM204V/I mutation principally confers lamivudine resistance but results in a decrease in viral replicative activity [18, 19]. It has also been shown that

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the rtL180M mutation has no relevance to lamivudine resistance in itself but restores the reduced replicative activity caused by the lamivudine resistance-associated rtM204V/I mutation [20]. In light of these findings, the rtL180M mutation has been recognized as being compensatory for the support of replication in lamivudine-resistant HBV. Among patients with type B chronic hepatitis (CH-B) showing lamivudine resistance, almost all rtM204V mutations have been detected in conjunction with the rtL180M mutation, and rtM204I mutations have been found either in isolation or together with the rtL180M mutation [15–17]. Thus, the compensatory rtL180M mutation is not always necessary for generating replicative-competent lamivudine-resistant HBV in the clinical setting. Virus without the rtL180M mutation is speculated to possess specific features in the genome that support viral replicative activity, compared with virus with the mutation. However, differences between lamivudine-resistant viruses with and those without the rtL180M mutation have not been elucidated.

To clarify this, we determined the nucleotide sequences of full-length HBV DNA in 44 patients with CH-B who showed lamivudine resistance, by means of the direct sequencing method. Differences in the whole HBV genome were comprehensively investigated in relation to the presence or absence of the rtL180M mutation.

METHODS

Patients. The subjects were 44 consecutive patients with CH-B (37 males and 7 females) who received lamivudine therapy and became refractory to it at Osaka University Hospital and National Hospital Organization Osaka National Hospital. At the beginning of therapy, all patients tested positive for hepatitis B surface antigen (HBsAg) and were positive for HBV DNA by a branched DNA assay (Quantiplex HBV DNA; Chiron) or a polymerase chain reaction (PCR)-based assay (Amplicor HB Monitor; Roche Diagnostics). All patients were negative for antibodies to hepatitis C virus and HIV; none showed evidence of alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury. Eight patients (18%) had previously received interferon (IFN) therapy.

All patients were treated with 100 mg of lamivudine daily, and liver function tests and monitoring of HBV markers were conducted during follow-up. In 16 patients (36%), natural IFN- α therapy (Sumiferon; Sumitomo Pharmaceuticals) was administered in combination with lamivudine for the initial 24 weeks (total dose, 432 million units). For all 44 patients, the lamivudine-resistant rtM204V/I mutation was detected by a PCR enzyme-linked minisequence assay (Sumitomo Metal Industries) [21] after an initial reduction and subsequent increase in HBV DNA during therapy. All serum samples for sequencing analysis of full-length HBV DNA were collected after the emergence of the lamivudine-resistant mutant virus and were stored

at -80°C until use. The serum sampling points ranged from 0.8 to 5.5 years (median, 2.7 years) after the commencement of lamivudine therapy. In addition, pairwise serum samples obtained before therapy were used to determine the nucleotide sequences in portions of HBV DNA as baseline controls for 23 patients (52%).

Patient characteristics at the point of analysis were as follows. Age ranged from 25 to 74 years (median, 51 years). Hepatitis B e antigen (HBeAg) was found in 31 patients (70%), and antibody to HBeAg developed in all 13 HBeAg-negative patients (30%). Serum HBV DNA levels ranged from 3.5 to $>7.6 \log_{10}$ copies/mL (median, 7.2 \log_{10} copies/mL). Serum alanine aminotransferase (ALT) levels ranged from 11 to 393 IU/L (median, 66 IU/L). Chronic hepatitis was diagnosed in 34 patients (77%), cirrhosis in 6 (14%), and hepatocellular carcinoma in 4 (9%), on the basis of liver biopsy and/or abdominal imaging procedures. Informed consent was obtained from all patients.

Genomic analysis of full-length HBV nucleotide sequences. From the serum sample, full-length HBV DNA was amplified by PCR and directly sequenced as described elsewhere [22]. The full-length HBV DNA sequences derived from the 44 patients with lamivudine-resistant CH-B (GenBank accession numbers AB367392–AB367435) were aligned together with the 12 representative HBV strains of various genotypes by means of CLUSTALW software. Phylogenetic tree analysis was then conducted [23, 24].

Plasmid and transfection. The HBV-expressing plasmid pHBC was derived from the genotype C2 HBV strain adr4 (GenBank accession number X01587) [25]. pHBC was constructed by inserting the 1.2-fold HBV genome into pBluescriptIIISK⁺. pHBC-PC and pHBC- Δ PS2, which were generated by site-directed mutagenesis, possessed the precore-defective A1986 mutation and the short deletion of 45 bp (nt 11–55) within the preS2 gene. Further site-directed mutagenesis was done to introduce rtM204V and rtL180M, rtM204I and rtL180M, or rtM204I alone into pHBC, pHBC-PC, and pHBC- Δ PS2. pCMV-SEAP was a secreted alkaline phosphatase-expressing plasmid.

For transfection, 3×10^5 Huh7 cells were seeded on a 35-mm-diameter culture dish and transfected with 1 μg of various HBV-expressing plasmids and 0.06 μg of pCMV-SEAP, using FuGENE6 reagent (Roche Diagnostics). On day 5, the culture supernatant and cell lysate were collected. Transfection efficiency was evaluated by measuring the secreted alkaline phosphatase activity.

Detection of viral progeny DNA and antigen in HBV-expressing cells. For detection of intracellular HBV DNA, cells were lysed in buffer containing 50 mmol/L Tris-Cl (pH 7.5), 1 mmol/L EDTA, and 1% Nonidet-P40. After a 15-min incubation on ice, nuclei were removed by brief centrifugation. Then, the sample was incubated at 37°C for 30 min in the presence of 0.1 mg/mL DNaseI and 10 mmol/L MgCl_2 . After the reaction was stopped by adding EDTA, the sample was subjected to over-

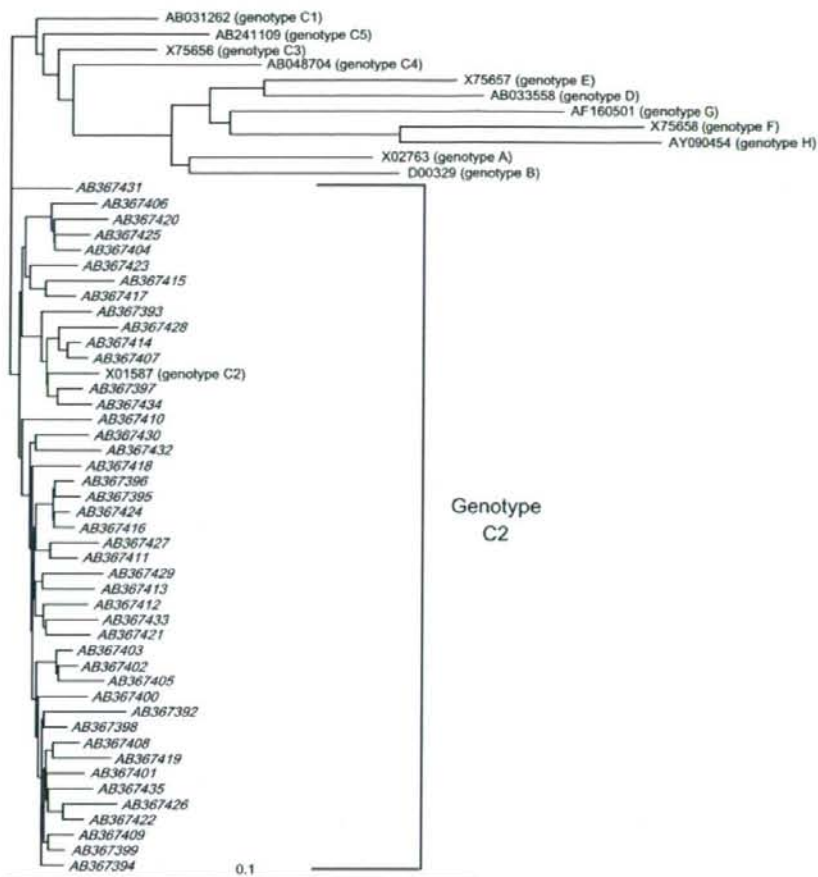


Figure 1. Phylogenetic tree analysis including 44 lamivudine-resistant hepatitis B virus (HBV) strains obtained in the present study and 12 representative HBV strains of various genotypes. All HBV strains are represented as GenBank accession nos., and the 44 lamivudine-resistant HBV strains are indicated by italics.

night incubation at 37°C in buffer containing 1% sodium dodecyl sulfate and 0.5 mg/mL proteinase K, followed by phenol/chloroform extraction and ethanol precipitation. The DNA sample was subjected to Southern blot analysis to detect HBV DNA, using a nonradioactive detection system (Alkphos Direct; GE Healthcare Life Sciences). Finally, the signals were analyzed quantitatively using image analyzing software (ImageJ; version 1.38). To detect extracellular HBV DNA, the transfection was scaled up to the 60-mm-diameter culture dish. After clarification by centrifugation at 8300 g for 30 min, the culture medium (3 mL) was centrifuged through a 20% sucrose cushion at 192,000 g for 4 h, using the Beckmann SW55Ti rotor. Then, DNA was extracted from the pellet and subjected to Southern blot analysis as described above. HBsAg and HBeAg in the culture medium were measured by chemiluminescent immunoassay.

Statistical analysis. Statistical analysis was performed by the χ^2 test, Fisher's exact test, and the Mann-Whitney *U* test. The

results for the in vitro transfection study were examined by 1-way analysis of variance, and pairwise comparison was done by Fisher's protected least significant difference test. $P < .05$ was considered to indicate statistical significance.

RESULTS

Patient clinical characteristics and lamivudine resistance-associated mutations. All 44 HBV strains obtained from the patients with lamivudine-resistant CH-B comprised 3161–3230 nt in length and belonged to genotype C2, the most prevalent type in Japan (figure 1). As for lamivudine resistance-associated mutations in these strains, the rtM204V mutation was observed in 16 strains (36%), whereas the remaining 28 strains (64%) had the rtM204I mutation. The compensatory rtL180M mutation was found in 30 strains (68%). All 16 strains with rtM204V and 14 (50%) of the 28 strains with rtM204I possessed the rtL180M

Table 1. Clinical features of patients with lamivudine-resistant type B chronic hepatitis, according to the mutational status of rt180 and rt204.

Clinical feature	rt180 status			rt204 status		
	rtL180M positive (n = 30)	rtL180M negative (n = 14)	P	rtM204V (n = 16)	rtM204I (n = 28)	P
Age, years	48 (25–74)	55 (27–71)	NS	48 (25–74)	51 (27–71)	NS
Sex, M/F, no.	25/5	12/2	NS	13/3	24/4	NS
Liver disease, chronic hepatitis/cirrhosis/ hepatocellular carcinoma, no.	26/3/1	8/4/2	NS	13/2/1	21/5/2	NS
ALT level, IU/L	66 (11–331)	67 (25–393)	NS	85 (17–261)	54 (11–393)	NS
HBeAg, positive/negative, no.	23/7	8/6	NS	13/3	18/10	NS
HBV DNA level, log ₁₀ copies/mL	7.5 (3.5 to >7.6)	7.1 (3.6 to >7.6)	NS	7.5 (3.8 to >7.6)	7.1 (3.5 to >7.6)	NS
Previous IFN therapy, no. (%)	6 (20)	2 (14)	NS	3 (19)	5 (18)	NS
Combination therapy with IFN, no. (%)	10 (33)	6 (43)	NS	4 (25)	12 (43)	NS
Duration of lamivudine administration until point of analysis, years	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS	2.9 (1.5–5.5)	2.2 (0.8–4.8)	NS

NOTE. Data are median (range) values, unless otherwise indicated. HBeAg, hepatitis B e antigen; IFN, interferon; NS, not significant.

mutation, in agreement with previous reports with respect to the emergence pattern of the rtM204V/I and rtL180M mutations [15–17].

Various patient clinical characteristics were first correlated with the presence or absence of the rtL180M mutation or with the alternative of the rtM204V or rtM204I mutation in our 44 patients with CH-B (table 1). No differences were observed between patients with and those without the rtL180M mutation with respect to age, sex ratio, disease severity, ALT level, HBeAg positivity, serum HBV DNA level, frequency of previous IFN therapy, frequency of combination therapy with IFN, and total duration of lamivudine administration until the point of analy-

sis. Also, there were no significant differences concerning these 9 characteristics between patients with virus having the rtM204V mutation and those with virus having the rtM204I mutation.

Genomic changes throughout the HBV genome associated with lamivudine resistance-associated mutations. Next, the genomic changes, which were significantly correlated with the occurrence of rtL180M or the preference for rtM204V or rtM204I, were investigated for the 44 HBV strains derived from the patients. As shown in table 2, 8 mutations and 1 deletion were identified as viral genomic changes significantly associated with the presence or absence of rtL180M. Among them, the A1896 mutation, which forms the in-frame stop codon in the

Table 2. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with and those without the rtL180M mutation.

Viral genomic changes	Consensus nucleotide ^a	Amino acid substitution	rtL180M, no. (%)		P
			Positive (n = 30)	Negative (n = 14)	
Mutation					
A373	C	Pol-L428M (rtL82M)	0 (0)	3 (21)	<.05
T619	C	None	0 (0)	3 (21)	<.05
G739	T	Pol-M550V (rtM204V), surface-I95R	16 (53)	0 (0)	<.001
T/C/A741	G	Pol-M550I (rtM204I), surface-W96L/S/stop	14 (47)	14 (100)	<.001
A1896	G	Precore-W28stop	5 (17)	9 (64)	<.005
T2102	C	None	0 (0)	3 (21)	<.05
A/G2660	C	Pol-N118K	0 (0)	3 (21)	<.05
A2860	T	PreS1-S5T, pol-V184D ^b	0 (0)	4 (29)	<.01
Deletion					
6–54-bp deletion within nt 1–55	...	Truncation of 2–18 amino acids in preS2 ^c	3 (10)	7 (50)	<.01

^a Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

^b One patient had the pol-V184Q amino acid substitution due to a mutation in the adjacent nucleotide position.

^c Detailed patterns of the preS2 deletion are shown in figure 2.

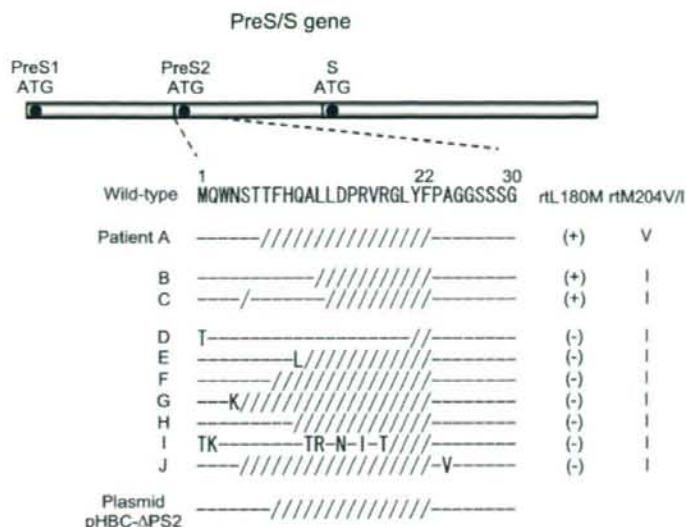


Figure 2. Patterns of the short deletion in the preS2 gene observed in lamivudine-resistant hepatitis B virus (HBV) strains. Ten of the 44 patients (patients A–J) had virus with the deletion in the preS2 gene of various patterns. The top sequence represents the amino acid sequence of the genotype C2 HBV DNA strain adr4 [25] as a representative strain. As for sequences derived from the patients, residues identical to the top sequence are indicated by dashes, whereas deletions of amino acid residues are shown by slashes. All deletions were found within the codon positions 5–22 of the preS2 gene. The bottom sequence represents the deletion pattern of the plasmid (pHBC-ΔPS2) used for in vitro transfection analysis (see figure 3), which expresses HBV DNA with the short deletion in the preS2 gene.

precure gene and results in the disability of HBeAg synthesis [26, 27], was found more frequently in viral strains without rtL180M than in those with it (64% vs. 17%; $P < .005$). Viral strains lacking rtL180M possessed the short deletion in the preS2 gene more frequently than those with rtL180M (50% vs. 10%; $P < .01$). The lengths of the deletion ranged from 12 to 54 bp, and all deletions were located within codon positions 5 to 22 of the preS2 gene (figure 2). Significant differences were also seen in the occurrences of 5 additional mutations—A373, T619, T2102, A/G2660, and A2860—between strains with and those without rtL180M. The detection rate of these 5 mutations was generally low among the lamivudine-resistant HBV strains obtained in

this study. The G739 and T/C/A741 mutations are the causes of the rtM204V and rtM204I amino acid changes, and the occurrences of these mutations differed between viral strains with and those without rtL180M ($P < .001$), as described above.

Throughout the HBV genome, 5 mutations were significantly associated with the preference for the rtM204V or rtM204I mutation in the 44 lamivudine-resistant HBV strains (table 3). Of them, 3 mutations—C565, A853, and C1568—were found more frequently in strains with rtM204V than in those with rtM204I, but the frequencies of these mutations were considerably low in our lamivudine-resistant HBV strains. The occurrence of the A667 mutation, which accounts for rtL180M, was

Table 3. Differences in the viral genome between lamivudine-resistant hepatitis B virus (HBV) strains with the rtM204V and rtM204I mutations.

Mutation	Consensus nucleotide*	Amino acid substitution	No. (%)		P
			rtM204V (n = 16)	rtM204I (n = 28)	
C565	T	None	4 (25)	0 (0)	<.05
T/C646	A	Pol-V519L (rtV173L)	5 (31)	0 (0)	<.005
A667	T	Pol-L528M (rtL180M)	16 (100)	14 (50)	<.001
A853	C	None	3 (19)	0 (0)	<.05
C1568	T	Pol-L826P	3 (19)	0 (0)	<.05

* Consensus nucleotides are derived from the genotype C2 HBV strain adr4 (GenBank accession no. X01587) [25].

Table 4. Changing pattern of the precore defective A1896 mutation and short deletion in the preS2 gene from the pretreatment baseline to development of lamivudine resistance in relation to the presence or absence of the rL180M mutation.

Type of mutation	Pattern of mutation		rL180M, no.	
	Before therapy	After therapy ^a	Positive (n = 15)	Negative (n = 8)
Precore-defective A1896 mutation	-	-	8	2
	+	+	4	4
	-	+	1	2
	+	-	2	0
Short deletion in the preS2 gene	-	-	12	5
	+	+	0	1
	-	+	2	2
	+	-	1	0

^a After development of lamivudine-resistant mutant virus.

higher in viral strains with rtM204V than in those with rtM204I ($P < .001$), as shown above. The T/C646 mutation, which causes the rtV173L change, was detected in 5 strains (31%) with rtM204V, compared with none of those with rtM204I ($P < .005$). It has been reported that the rtV173L mutation was detected together with the rtM204V and rL180M mutations and was considered to be associated with lamivudine resistance [17, 28]. Our finding concerning the rtV173L mutation agreed with those of previous reports.

According to these observations, the relevance of the precore-defective A1896 mutation and the preS2 deletion to the absence of rL180M was the most distinctive feature of the lamivudine-resistant HBV strains on screening of the whole genome. We therefore directed our attention to these precore and preS2 genomic changes and further investigated their role in the establishment of lamivudine-resistant virus.

Serial changes in the precore mutation and the preS2 deletion in lamivudine-resistant virus before and after lamivudine therapy. Serial changes in the precore-defective A1896 mutation, the short deletion in the preS2 gene, and the drug resistance-associated rtM204V/I, rL180M, and rtV173L mutations were investigated in the 23 (52%) of 44 patients with CH-B whose serum samples obtained before lamivudine therapy were available (table 4). Of the 11 patients with virus having the precore-defective mutation after the development of lamivudine resistance, 8 had virus that already possessed the mutation before therapy. Thus, the precore-defective mutation was generally a preexisting genomic change in most patients showing lamivudine resistance. On the other hand, of the 5 patients with virus that had the deletion in the preS2 gene after the development of drug resistance, 4 had virus that did not possess the deletion before therapy. The frequent detection of the preS2 deletion in lamivudine-resistant virus compared with virus before therapy indicates that this deletion may be coselected with drug resistance-associated mutations during the establishment of lamivudine-resistant mutant virus. As for the lamivudine-resistant rtM204V/I, rL180M, and rtV173L mutations, they were

not detected in any of the 23 viruses before lamivudine therapy, as expected.

Effect of the precore mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV in vitro. We further conducted in vitro transfection analysis to explore the influence of the precore-defective mutation and the preS2 deletion on the replicative competence of lamivudine-resistant HBV. Three plasmids that expressed wild-type virus, precore-defective virus, and virus with the preS2 deletion were prepared. Next, plasmids with rtM204V plus L180M, rtM204I plus L180M, and rtM204I alone were synthesized in each of the 3 HBV-expressing backbone constructs. The level of intracellular HBV DNA was examined in cells transfected with these HBV-expressing plasmids. As shown in figure 3A and 3B, the introduction of lamivudine resistance-associated mutations into the virus with the wild-type backbone led to a decrease in viral replication (lanes 1–4). In addition, the replicative competence of the drug-resistant virus lacking rL180M tended to be lower than that of the virus having rL180M, although the difference was not statistically significant. As for the precore-defective virus, its replicative activity at baseline was higher than that of the wild-type virus (lanes 1 and 5). The decline in HBV replication due to the insertion of drug resistance-associated mutations was also observed for the virus with the precore-defective backbone. However, unlike for the virus with the wild-type backbone, the replicative activity of the precore-defective virus with lamivudine-resistant mutations was maintained at a considerable level (lanes 5–8). As for the virus with the preS2-deleted backbone, a reduction in viral replication due to the introduction of lamivudine resistance-associated mutations was also seen, but the degree of the reduction was not as great as that in the wild-type virus (lanes 9–12). Thus, both the precore-defective mutation and the preS2 deletion possessed activity supporting the viral replicative competence of lamivudine-resistant HBV, although the activity with the preS2 deletion was not as strong as that with the precore-defective mutation. The

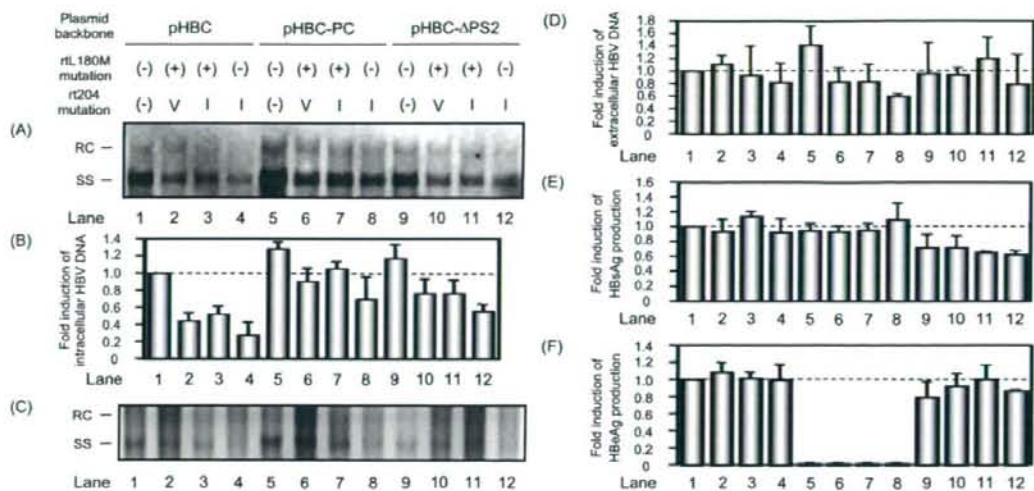


Figure 3. Levels of intracellular and extracellular progeny viral DNA and viral antigen production in cultured cells transfected with wild-type, precore-defective, or preS2-deleted hepatitis B virus (HBV)-expressing plasmids with or without lamivudine resistance-associated mutations. *A*, Representative result of Southern blot analysis to detect the intracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *B*, Quantitative analysis of the level of intracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean \pm SD values. Statistically significant differences ($P < .05$) are as follows: lane 1 vs. 2–4, 1 vs. 5, 2 vs. 6 and 10, 3 vs. 7 and 11, 4 vs. 8 and 12, 5 vs. 6 and 8, and 9 vs. 10–12. *C*, Representative result of Southern blot analysis to detect extracellular progeny HBV DNA in cells transfected with various HBV-expressing plasmids. *D*, Quantitative analysis of the level of extracellular progeny HBV DNA. The progeny HBV DNA level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 4 times, and results are shown as mean \pm SD values. A statistically significant difference was not observed by 1-way analysis of variance. *E*, Levels of hepatitis B surface antigen (HBsAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBsAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean \pm SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 5 vs. 9, 3 and 7 vs. 11, and 4 and 8 vs. 12. *F*, Levels of hepatitis B e antigen (HBeAg) in culture medium of cells transfected with various HBV-expressing plasmids. The HBeAg level for transfection with pHBC was considered to be 1, and the fold activities for transfection with the mutant HBV-expressing plasmids were calculated. The experiment was done 3 times, and results are shown as mean \pm SD values. Statistically significant differences ($P < .05$) are as follows: lanes 1 and 9 vs. 5, 2 and 10 vs. 6, 3 and 11 vs. 7, and 4 and 12 vs. 8. RC, relaxed circular HBV DNA; SS, single-stranded HBV DNA.

tendency appeared to be more evident in the drug-resistant virus without the rtL180M mutation. This may be a reason for the compensatory rtL180M mutation not being necessary during the establishment of lamivudine resistance in the HBV strain having the precore and preS2 genomic changes.

When the level of extracellular HBV DNA was examined in cells transfected with various HBV-expressing plasmids (figure 3C and 3D), no significant differences were observed among wild-type, precore-defective, and preS2-deleted viruses with respect to the reduction of viral secretion caused by the introduction of the lamivudine resistance-associated mutation. The discrepant results between the intracellular and extracellular viral DNA levels likely occurred because the extracellular viral DNA assay was less sensitive to minute changes in viral replication than the intracellular viral DNA assay.

As for the levels of production of HBsAg and HBeAg, the virus with the preS2-deleted backbone produced less HBsAg than did the viruses with the wild-type and precore-defective backbones

(figure 3E). The wild-type and preS2-deleted viruses secreted HBeAg, whereas the precore-defective virus did not (figure 3F). The lamivudine resistance-associated mutations did not affect the production levels of HBV antigens.

DISCUSSION

HBV establishes lamivudine resistance via the resistance-causative rtM204V/I mutation and the replication-compensatory rtL180M mutation [15–20]. The present study aimed to investigate the genomewide peculiarity of lamivudine-resistant HBV. In particular, we elucidated the differences between viruses with and those without the compensatory rtL180M mutation. For this purpose, we conducted full-length sequencing analysis of lamivudine-resistant viruses derived from patients with CH-B by means of the PCR direct sequencing method. In some patients, the results were also confirmed by the PCR-subcloning method (data not shown). As a result, the precore-defective

A1896 mutation and the short deletion in the preS2 gene were identified as genomic changes significantly associated with the occurrence of the rtL180M mutation. These 2 viral genomic changes were found to be highly relevant to the observation that the rtL180M mutation was not needed for the establishment of the lamivudine-resistant mutant virus. This suggests that the precore-defective mutation and the preS2 deletion may function as surrogates for the compensatory rtL180M mutation and assist replication of lamivudine-resistant HBV. In the serial analysis of the mutations examined before and after lamivudine therapy, the preS2 deletion tended to be coselected with the drug resistance-associated mutation after therapy, although this tendency was not seen in the case of the precore-defective mutation. This also indicates that the preS2 deletion may have some advantage for establishment of lamivudine-resistant HBV.

We further conducted *in vitro* transfection analysis to verify the possible supportive role played by the precore and preS2 genomic changes in replication of lamivudine-resistant virus. The intracellular viral DNA was measured as a marker of viral replicative competence. In the wild-type virus, lamivudine resistance-associated mutations reduced viral replicative competence, and the rtL180M mutation compensated for viral replication to a certain degree. This agreed with previous findings of some other investigators [18–20]. On the other hand, the reduction in the viral replication level caused by the lamivudine-resistant mutations was lower in the precore-defective and preS2-deleted viruses than in the wild-type virus. Even the lamivudine-resistant virus without the rtL180M mutation maintained a substantial level of replicative activity in the viruses with precore and preS2 genomic changes. Thus, our results contribute evidence for a supportive role of both precore and preS2 genomic changes in the replicative competence of lamivudine-resistant HBV. This tendency was not evident in the case of the extracellular viral DNA assay, which may have been due to this assay's lower ability to detect slight changes in viral replicative activity.

As for the functional role played by the precore-defective A1896 mutation in the replication competence of lamivudine-resistant HBV, enhanced replicative activity of virus with lamivudine resistance caused by introduction of the precore-defective mutation has been reported for the recombinant HBV-expressing baculovirus system using the genotype D HBV strain [29]. Another previous *in vitro* transfection analysis using the genotype A HBV strain revealed that experimental insertion of the precore-defective mutation together with the T1858 mutation compensated for the replication competence of the virus possessing lamivudine-resistant mutations [30]. Our experimental result using the genotype C2 HBV strain is consistent with these previous findings. In addition, we showed in the present study that the preS2 deletion may also play a supportive role in the replication yield of lamivudine-resistant HBV, although the enhancement of viral replication caused by the preS2

deletion was not as strong as that caused by the precore-defective mutation.

It remains unclear why the precore-defective mutation leads to an increase in the viral replication of drug-resistant HBV. Previous *in vitro* transfection analyses have shown that the precore-defective mutation had no influence on viral replicative competence [29–31]. However, in our transfection analysis using the genotype C2 HBV strain, the replicative competence of the precore-defective virus tended to be higher than that of the wild-type virus, even when viruses without the lamivudine resistance-associated mutations were compared. It has recently been shown that the precore-defective mutation caused an elevation in viral replication in the particular HBV strain of genotype B1 [32]. According to this, the precore-defective mutation may in some way enhance HBV replication irrespective of the lamivudine resistance.

As for the involvement of the preS2 deletion in the replicative advantage of lamivudine-resistant HBV, the deletion results in truncation of the polymerase protein as well as the surface protein. Such truncation of the polymerase protein may increase the enzymatic activity and replication capacity of drug-resistant virus. As another possibility, the surface protein with the preS2 deletion may link to incomplete envelopment and subsequent intracellular accumulation of immature viral particles, resulting in an elevated intracellular HBV DNA level. However, this is improbable, because viral envelopment and secretion may be achieved efficiently in preS2-deleted virus as well as wild-type and precore-defective viruses, as was shown in the extracellular viral DNA assay.

In summary, our findings indicate that a precore-defective A1896 mutation and a short deletion in the preS2 gene may support viral replicative activity and substitute for the compensatory rtL180M mutation. Both the precore-defective mutation and the preS2 deletion have been shown to be frequently found during chronic HBV infection [26, 27, 33]. It is noteworthy that such naturally occurring frequent genomic changes in HBV significantly affect the establishment of drug-resistant viral strains. The lamivudine-resistant rtM204V/I mutation has also been reported to be completely or partially involved in resistance to other nucleos(t)ide analogues (emtricitabine, telbivudine, entecavir, and clevudine) [8, 9, 14, 34]. Our findings reveal novel aspects about the establishment of drug-resistant virus possessing the rtM204V/I and rtL180M mutations during the antiviral treatment of patients with CH-B.

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Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A in patients with chronic liver diseases and changes during transcatheter arterial embolization for hepatocellular carcinoma

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Soluble forms of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) are increased in the sera of patients with malignancy and impair the antitumor immune response by downregulating expression of their cognate immunoreceptor natural killer group 2, member D (NKG2D). Recently, soluble MICA/B were reported to appear even in some premalignant diseases, raising questions about the impact of soluble MICA/B produced from tumors on the expression of NKG2D. The present study examined soluble MICA/B in chronic liver disease and hepatocellular carcinoma (HCC) and their involvement in the immune-cell expression of NKG2D during transcatheter arterial embolization for HCC. The levels of soluble MICA/B were significantly higher in chronic liver disease and HCC patients than in healthy volunteers. The progression of liver disease and that of the tumor were independent determinants for soluble MICA/B levels. Immunohistochemistry revealed that MICA/B were expressed not only in HCC tissue but also on hepatocytes in cirrhotic livers. The transcatheter arterial embolization therapy significantly decreased serum levels of soluble MICA, but not soluble MICB, and increased the NKG2D expression on natural killer cells and CD8-positive T cells; there was an inverse correlation between changes in soluble MICA levels and in NKG2D expression. In conclusion, although soluble MICA/B are produced from both HCC and premalignant cirrhotic livers, therapeutic intervention for HCC can reduce the levels of soluble MICA and thereby upregulate the expression of NKG2D. Cancer therapy may have a beneficial effect on NKG2D-mediated antitumor immunity. (*Cancer Sci* 2008; 99: 1643–1649)

MHC class I-related chain A and B, glycoproteins expressed on the cellular membrane, are ligands for NKG2D expressed on a variety of immune cells.⁽¹⁾ In contrast to classical MHC class I molecules, MICA/B are expressed rarely on normal cells but frequently on tumor cells, including colon cancer, prostate cancer, HCC, and brain tumors.^(2–5) The engagement of MICA/B and NKG2D strongly activates NK cells and costimulates T cells, enhancing their cytolytic ability and cytokine production.⁽⁶⁾ Thus, the MICA/B–NKG2D pathway is an important mechanism by which the host immune system recognizes and kills transformed cells.⁽⁷⁾ In addition to those membrane-bound forms, MICA/B are also cleaved proteolytically from tumor cells and appear as soluble forms in sera of patients with malignancy.^(8–10) The levels of NKG2D expression tend to be decreased in patients with high levels of soluble MICA/B.⁽⁴⁾ In addition, sera from those patients can downregulate NKG2D expression *in vitro*.^(5,11) These data

suggest that soluble MICA/B in the circulation downregulate NKG2D expression and disturb NKG2D-mediated antitumor immunity, raising the possibility that cancer therapy might reduce the serum levels of soluble MICA/B and thereby improve the NKG2D-related immune environment. However, this possibility has not been addressed directly by examining soluble MICA/B and NKG2D expression in a cohort of patients before and after cancer therapy. Furthermore, recent reports by Holdenrieder *et al.* demonstrating that soluble MICA/B are increased not only in malignant disease but also in some benign diseases, such as of the gastrointestinal tract, gynecologic organs, and lungs, raise questions about the impact of cancer therapy on modulating soluble MICA/B levels.^(12,13)

Hepatocellular carcinoma is one of the leading causes of cancer death worldwide. Chronic liver disease caused by hepatitis virus infection and non-alcoholic steatohepatitis leads to a pre-disposition for HCC; liver cirrhosis, in particular, is considered to be a premalignant condition.^(14,15) With regard to treatment, surgical resection or percutaneous techniques such as ethanol injection and radiofrequency ablation are considered to be choices for curable treatment of localized HCC, whereas TAE is a well-established technique for unresectable HCC.⁽¹⁶⁾ We reported previously that soluble MICA could be detected in sera of HCC patients.⁽¹⁷⁾ However, the clinical significance of the soluble forms of NKG2D ligands in liver disease has not yet been established in a comprehensive manner, because the previous study was conducted on a small number of patients, did not include patients with premalignant conditions such as liver cirrhosis, and did not analyze its closely related molecule MICB. Furthermore, influences of therapeutic intervention on soluble NKG2D ligands in patients have been unclear. In the present study, we examined soluble MICA and soluble MICB in sera from a large number of patients with chronic liver diseases and HCC and their impact on NKG2D expression on immune cells during TAE therapy for HCC.

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Abbreviations: APC, allophycocyanin; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; MFI, mean fluorescence intensity; MICA/B, major histocompatibility complex (MHC) class I-related chain A and B; NK, natural killer; NKG2D, natural killer group 2, member D; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Table 1. Control and patient characteristics

Characteristic	Healthy control	Chronic hepatitis	Liver cirrhosis	HCC
Number	104	141	104	232
Sex (male/female)	49/55	78/63	60/44	177/55*
Age (years)	62 ± 15	55 ± 13**	61 ± 12	68 ± 9***
Etiology				
HBV/HCV	–	27/107	12/78	37/187
Alcohol/NASH	–	0/5/	2/1/	4/0/
AIH/PBC/others	–	2/0/0	1/6/4	0/0/3
Child-Pugh (A/B/C)	–	–	34/27/26	131/84/17****
TNM stage (I/II/III/IV)	–	–	–	59/68/64/39

AIH, autoimmune hepatitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cirrhosis; TNM, tumor node metastasis.

* $P < 0.05$ vs control, hepatitis, and cirrhosis by χ^2 -test; ** $P < 0.05$ vs control, cirrhosis, and HCC by ANOVA and post hoc Bonferroni test; *** $P < 0.05$ vs control, hepatitis, and cirrhosis by ANOVA and post hoc Bonferroni test; **** $P < 0.05$ vs cirrhosis by χ^2 -test.

Materials and Methods

Stock sera from patients with chronic liver disease and HCC. We used frozen stock sera obtained from consecutive patients with chronic liver disease who had been registered at our institute from February 2002 to April 2006. They included 141 patients with chronic hepatitis, 104 patients with liver cirrhosis, and 232 patients with HCC. The differential diagnosis between chronic hepatitis and liver cirrhosis was basically from liver biopsy ($n = 98$), but for those who had not undergone biopsy the diagnosis was based on clinical findings from the aspartate aminotransferase/platelet ratio index (APRI) score.⁽¹⁸⁾ Diagnosis of HCC was based on unequivocal clinical and imaging data. The control group consisted of 104 healthy volunteers of an age range similar to the liver cirrhosis group. Table 1 summarizes the control and patient characteristics of age, sex, etiology of liver disease, Child-Pugh classification, and TNM staging of HCC. Child-Pugh classification is a well-established index for progression of liver disease in cirrhotic patients where A, B, and C indicate compensated cirrhosis, mildly decompensated cirrhosis, and severely decompensated cirrhosis, respectively. The TNM staging adopted in the present study was that modified by the Liver Cancer Study Group of Japan.⁽¹⁶⁾

Detection of soluble MICA/B by ELISA. Serum levels of soluble MICA and soluble MICB were determined differentially by commercially available ELISA kits (R & D Systems, Minneapolis, MN, USA). In preliminary experiments, we determined the median intra-assay variation ($n = 5$) to be between 3.5 and 5.6% for soluble MICA and between 2.4 and 7.8% for soluble MICB, and the median interassay variation ($n = 5$) to be between 12.8 and 18.9% for soluble MICA and between 15.2 and 18.7% for soluble MICB.

Detection of MICA/B on liver tissues by immunohistochemistry. The human liver tissues examined were one normal liver, three from those at fibrosis stages 1 and 2 of chronic hepatitis, five from liver cirrhosis (fibrosis stage 4) patients, and five from HCC patients. Paraffin-embedded liver sections were deparaffinized, heat-inactivated by a microwave oven and then subjected to immunohistochemical staining using the ABC procedure (Vector Laboratories, Burlingame, CA, USA). The primary antibody used was 6D4 monoclonal antibody, which recognizes the $\alpha 1$ and $\alpha 2$ domains of MIC molecules shared by both MICA and MICB.⁽²⁾ To confirm the specificity of the staining, the 6D4 antibody was incubated with recombinant MICA (R & D Systems) for 2 h and then applied to liver sections in parallel with staining of the primary antibody as the absorption test.

Table 2. Characteristics of hepatocellular carcinoma patients

Characteristic	TAE-treated group	Non-treated group
Number	38	21
Sex (male/female)	28/10	17/4
Age (years)	75 ± 11	74 ± 8
Etiology (HBV/HCV)	2/36	1/21
Child-Pugh (A/B/C)	29/9/0	16/5/0
TNM stage (I/II/III/IV)	4/20/14/0	2/11/8/0

HBV, hepatitis B virus; HCV, hepatitis C virus; TAE, transcatheter arterial embolization; TNM, tumor node metastasis.

Detection of membrane-bound and soluble forms of MICA/B on cultured cells. HepG2 hepatoma cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Human non-transformed hepatocytes were purchased from Cambrex Bio Science (Charles City, IA, USA) and cultured according to the manufacturer's instructions. For detection of membrane-bound MICA/B, a single-cell suspension was stained with PE-labeled 6D4 monoclonal (R & D Systems) antibody, fixed with 2% paraformaldehyde, and then subjected to flow cytometric analysis. The culture supernatants were subjected to analysis of soluble forms of MICA and MICB using the above-mentioned ELISA assay.

Patients with HCC and TAE therapy. Thirty-eight patients with HCC admitted to our institution for TAE therapy were enrolled prospectively in the present study. TAE was carried out by the standard procedure using an emulsion of farmorubicin and lipiodol followed by gelatin sponge particles. Blood samples were collected before and 2 weeks after TAE therapy. Twenty-one patients with HCC, matching the TAE group with respect to TNM stage and Child-Pugh score, were also enrolled as controls (Table 2). Blood samples were collected twice at a 2-week interval. Written informed consent was received from all patients and the study protocol was approved by the Ethical Committee of Clinical Research at Osaka University Hospital.

Natural killer cell analysis. PBMC were isolated from heparinized venous blood by a standard procedure. PBMC were stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD56 antibody, and PE-labeled anti-NKG2D antibody. They were also stained with FITC-labeled anti-CD3 antibody, APC-labeled anti-CD8 antibody, and PE-labeled anti-NKG2D antibody. All antibodies were purchased from Becton Dickinson (San Jose, CA, USA). NKG2D expression on NK cells (defined as CD56-positive and CD3-negative cells) and CD8-positive T cells (defined as CD3-positive and CD8-positive cells) were analyzed by flow cytometry. As a control, corresponding fluorescence-labeled irrelevant antibodies were used. As most NK and CD8-positive T cells express NKG2D, the levels of expression were evaluated by the mean fluorescence intensity of the stained cells.

Statistics. Values were expressed as the median and interquartile range as a box plot, and the 10th and 90th percentiles as a horizontal bar. For comparison of more than two groups, the Kruskal-Wallis rank sum test was used. If the Kruskal-Wallis test was significant, post hoc multiple comparisons were carried out using the Steel-Dwass procedure. Differences between pretreatment and post-treatment values were tested by paired t -test. $P < 0.05$ was considered statistically significant.

Results

Soluble MICA and soluble MICB in chronic liver disease and HCC. Soluble MICA and soluble MICB were assessed in sera from patients with chronic hepatitis, liver cirrhosis, and HCC as well as healthy volunteers. There was a stepwise increase in the levels of both soluble MICA and soluble MICB from hepatitis

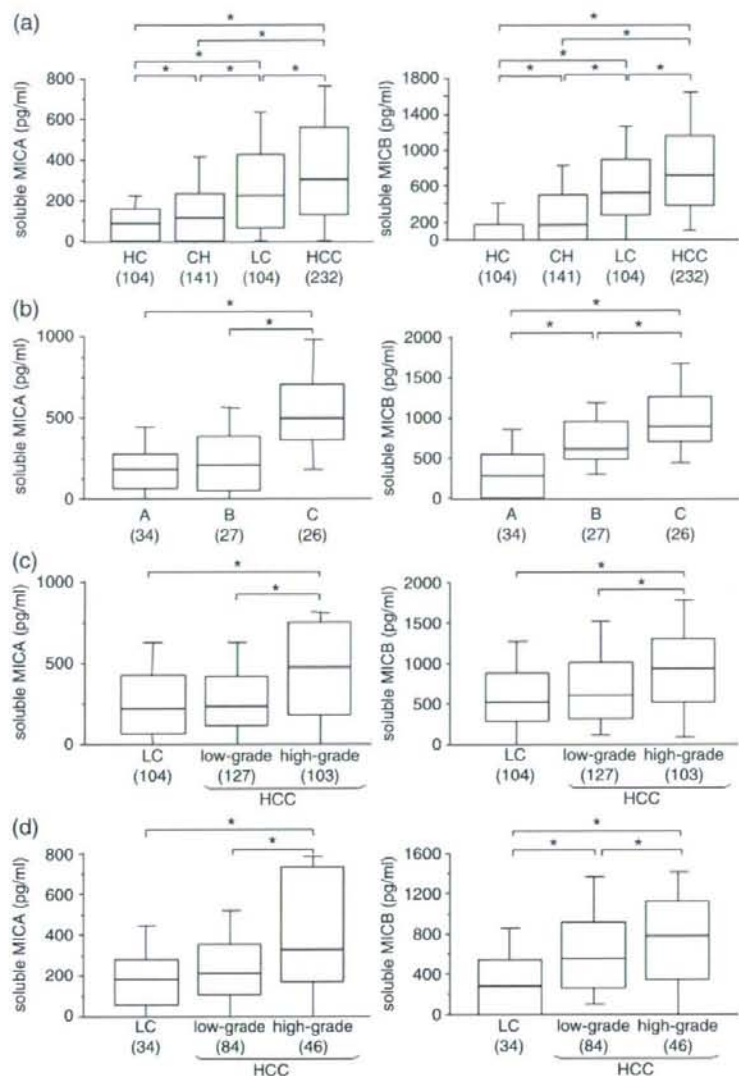


Fig. 1. Serum levels of soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) in chronic liver disease and hepatocellular carcinoma (HCC). (a) Soluble MICA and soluble MICB levels in serum samples of healthy controls (HC), chronic hepatitis (CH), liver cirrhosis (LC), and HCC. (b) Soluble MICA and soluble MICB are associated with the progression of liver disease. Data on cirrhotic patients were stratified based on Child-Pugh classification. (c, d) Soluble MICA and soluble MICB are associated with the progression of tumors. (c) Data on cirrhosis and HCC patients were classified into three groups: patients with absence of HCC (cirrhosis), patients with low-grade HCC (tumor node metastasis [TNM] stage I and II), and patients with high-grade HCC (TNM stage III and IV). (d) To exclude the possibility of progression of liver disease being involved in increase in soluble MICA/B, soluble MICA/B levels were compared among the three groups of Child-Pugh classification A. Data are represented as box plots (median values, 10th, 25th, 75th, and 90th percentiles). The number in parentheses indicates the number of patients in each group. * $P < 0.05$ by Kruskal-Wallis test and post hoc Steel-Dwass test.

to HCC (Fig. 1a). Although the difference between hepatitis patients and healthy volunteers was modest, both of the levels were clearly higher in patients with liver cirrhosis and HCC than in normal volunteers or hepatitis patients. To examine whether the progression of liver disease in cirrhotic patients affects the levels of soluble MICA/B, cirrhotic patients were stratified based on Child-Pugh classification. The levels of both soluble MICA and MICB were increased significantly with the progression of liver disease (Fig. 1b).

Hepatocellular carcinoma often develops from cirrhotic liver and most patients with HCC included in the present study had complications from cirrhosis. To examine whether the development and progression of HCC contributes to increasing soluble MICA/B, patients with liver cirrhosis and those with HCC were classified into three groups: those with an absence of HCC, low-grade HCC (TNM stage I/II) and high-grade HCC (TNM stage III/IV). There was no significant difference in soluble MICA or soluble MICB between patients without HCC and

low-grade HCC patients. However, the high-grade HCC patients showed significantly higher levels of soluble MICA or soluble MICB than patients without HCC or the low-grade HCC patients (Fig. 1c). To exclude the possibility of the progression of liver disease affecting the increases in soluble MICA/B in high-grade HCC, we selected and analyzed only the Child-Pugh A patients. In this subgroup of patients, the levels of soluble MICA/B were also significantly higher with high-grade HCC than with low-grade HCC or the absence of HCC (Fig. 1d). Thus, the progression of liver disease and that of the tumor independently affects the levels of soluble MICA or soluble MICB.

MICA/B expression in liver tissues and production of soluble MICA/B. The increase in soluble MICA/B in cirrhotic patients suggests that MICA/B may be expressed in cirrhotic livers. We therefore examined MICA/B expression by immunohistochemistry in various human tissues including normal liver, chronic hepatitis (F1 and F2 stage), liver cirrhosis, and HCC (Fig. 2a). MICA was detected clearly in four of five HCC tissues, agreeing with a

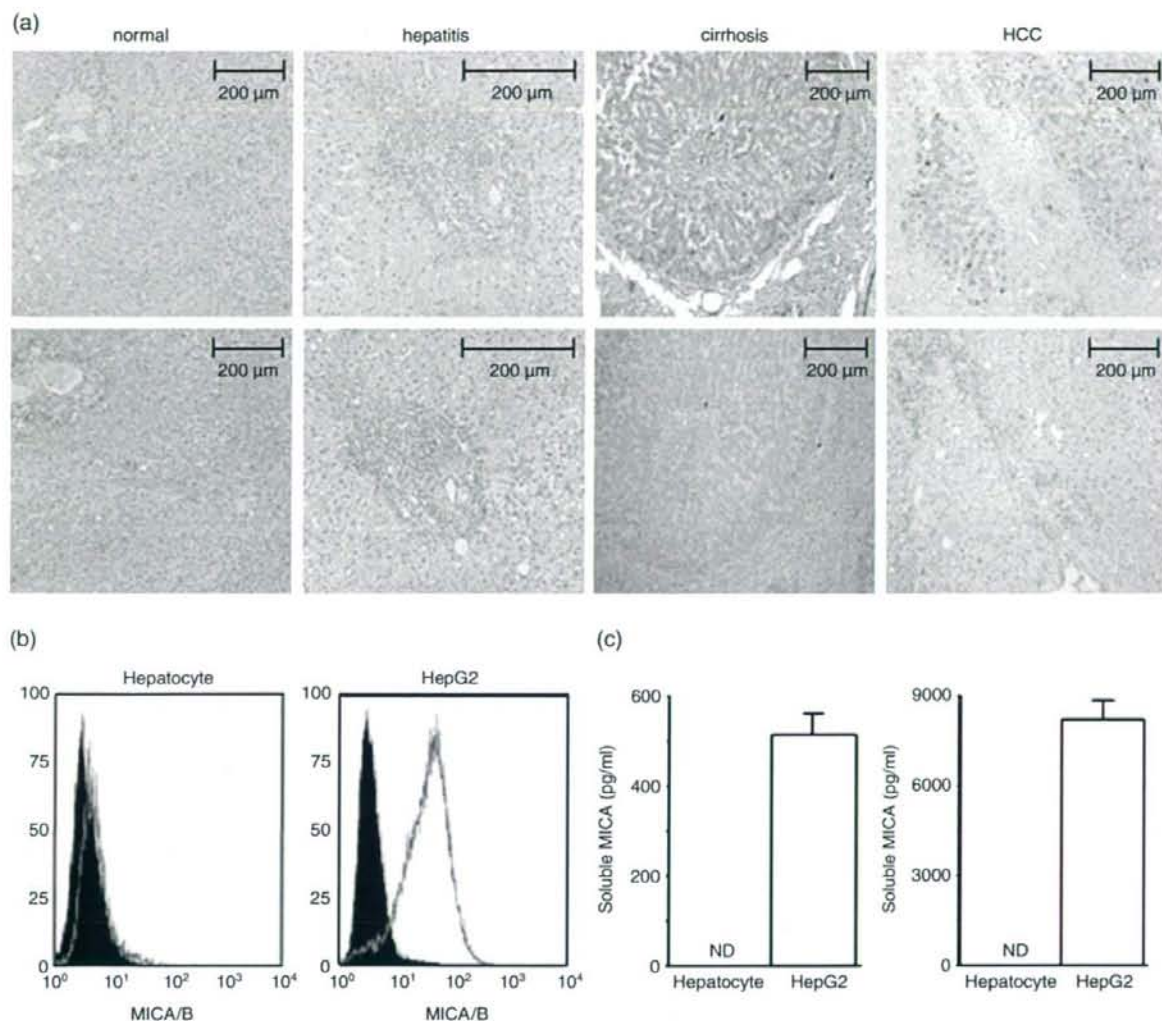


Fig. 2. Expression of major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) and production of their soluble forms. (a) Immunohistochemical detection of MICA/B in liver tissues. Representative staining with anti-MICA/B monoclonal antibody (6D4) is shown for normal liver, chronic hepatitis (F1 stage), liver cirrhosis (F4 stage), and hepatocellular carcinoma (HCC) (upper panel). As a control, 6D4 monoclonal antibody was preabsorbed with recombinant MICA and applied to the neighboring corresponding sections (lower panel). (b) Flow cytometric analysis of surface expression of MICA/B on HepG2 hepatoma cells and non-transformed hepatocytes. Open and closed histograms represent the staining of anti-MICA/B antibody (6D4) and control antibody, respectively. (c) Soluble MICA and soluble MICB released from HepG2 hepatoma cells and non-transformed hepatocytes. Cells were seeded in a subconfluent condition and cultured for 48 h. The culture supernatants were applied for analysis of soluble MICA and soluble MICB by enzyme-linked immunosorbent assay. ND, not detected.

previous report.⁽³⁾ Importantly, hepatocytes in four of five cirrhotic livers were positive for MICA/B, whereas MICA/B were not detected in hepatocytes from normal liver or liver at the early stage of chronic hepatitis.

We also examined the expression of MICA/B on normal hepatocytes and HepG2 hepatoma cells. Flow cytometric analysis revealed that HepG2 cells expressed MICA/B on the cell surface (Fig. 2b). Both soluble forms of MICA and MICB were detected in the supernatant of HepG2 cells cultured for 48 h (Fig. 2c). In contrast, non-transformed hepatocytes expressed MICA/B faintly and soluble MICA/B could not be detected in their culture supernatant. This observation supported the idea that both soluble MICA and soluble MICB are produced from MICA/B-expressing hepatic cells.

Downregulation of soluble MICA levels by TAE. The above findings suggest that soluble MICA/B are produced from cirrhotic livers as well as HCC. In addition, the progression of the tumor is an important determinant of soluble MICA/B independent of the progression of liver disease. We then asked the question of whether therapeutic intervention of HCC would reduce the levels of soluble MICA or soluble MICB and affect the levels of NKG2D expression on immune cells. We prospectively analyzed the levels of soluble MICA/B and NKG2D expression in 38 HCC patients before and 2 weeks after TAE therapy. As a control, 21 HCC patients who did not receive TAE therapy but were matched to the TAE group with respect to clinical characteristics were analyzed over a 2-week interval.

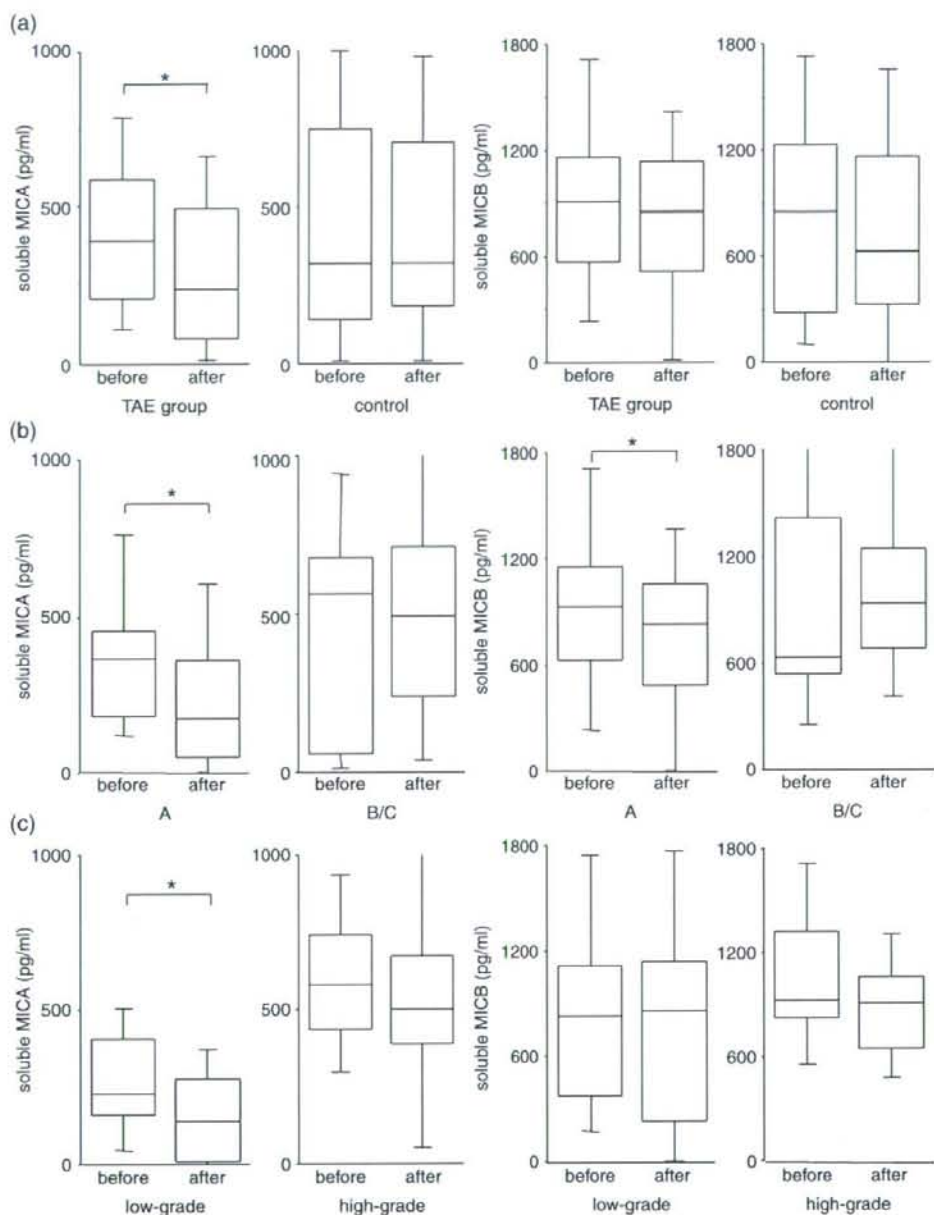


Fig. 3. Soluble major histocompatibility complex (MHC) class I-related chain A and B (MICA/B) during transcatheter arterial embolization (TAE) therapy. (a) Soluble MICA and soluble MICB were measured for 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as controls, with soluble MICA/B being measured twice with a 2-week interval. (b) TAE-treated patients were divided into two groups: Child-Pugh A ($n = 29$) and Child-Pugh B and C ($n = 9$). (c) TAE-treated patients were divided into two groups: low-grade hepatocellular carcinoma (HCC) ($n = 24$) and high-grade HCC ($n = 14$). * $P < 0.05$ by paired t-test.

In the TAE-treated group, the levels of soluble MICA were decreased significantly 2 weeks after TAE therapy compared with those before TAE (Fig. 3a). In contrast, TAE did not affect the levels of soluble MICB. Neither the levels of soluble MICA nor those of soluble MICB changed during the 2-week interval in HCC patients not receiving TAE therapy. As the progression of liver disease and that of the tumor affects the levels of soluble

MICA/B, TAE-treated patients were divided according to their Child-Pugh stage or tumor stage. The levels of soluble MICA decreased significantly after TAE therapy in Child-Pugh A patients but not in Child-Pugh B and C patients (Fig. 3b). Interestingly, Child-Pugh A patients showed a significant decrease even in soluble MICB levels after TAE therapy but Child-Pugh B and C patients did not. As for tumor stage, a significant decrease in

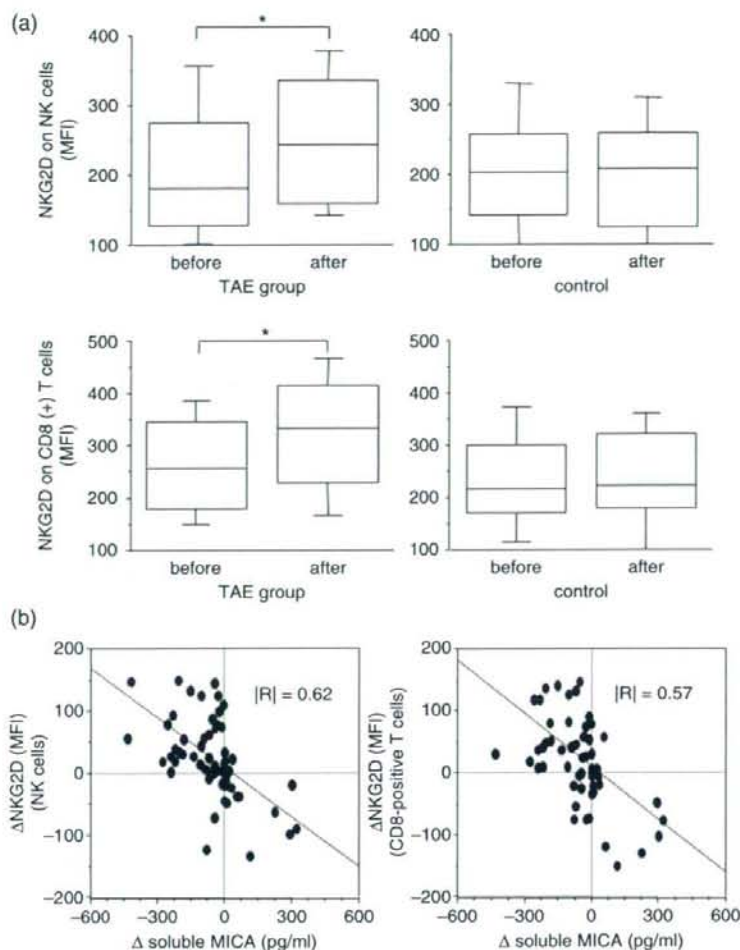


Fig. 4. Natural killer group 2, member D (NKG2D) expression during transcatheter arterial embolization (TAE) therapy. (a) NKG2D expression on natural killer (NK) cells and CD8-positive T cells. NKG2D expression on immune cells was analyzed in 38 patients before and 2 weeks after TAE therapy. Twenty-one patients who did not receive TAE therapy served as a control by measuring NKG2D expression for 2-week interval. NKG2D expression on each cell type was evaluated by mean fluorescence intensity (MFI). * $P < 0.05$ by paired t-test. (b) Correlation between change of soluble MICA and that of NKG2D expression on NK cells or CD8-positive T cells.

soluble MICA levels after TAE therapy was found in low-grade HCC but not in high-grade HCC (Fig. 3c). The levels of MICB did not change in the low-grade or high-grade HCC groups.

Upregulation of NKG2D expression by TAE. The number of PBMC as well as NK and T-cell subsets did not change over the 2-week interval in both the control and TAE-treated patients (data not shown). However, the levels of NKG2D expression on NK and CD8-positive T cells increased significantly upon TAE therapy, but not in the control group (Fig. 4a). To examine the involvement of soluble MICA in NKG2D expression, we analyzed the relationship of changes between soluble MICA and NKG2D expression in HCC patients. Change in soluble MICA was correlated inversely with changes in NKG2D expression on NK and CD8-positive T cells (Fig. 4b). There was no significant correlation between changes in soluble MICB and NKG2D expression (data not shown).

Discussion

In the present study, we demonstrated that soluble MICA/B increases with the progression of chronic liver disease as well as the progression of HCC. Increases in soluble MICA/B in advanced stages of tumors have been reported in some malignancies.⁽¹²⁾ However, little is known about soluble MICA/B in the premalignant

condition. Recently, Holdenrieder *et al.* examined soluble MICA/B levels in benign as well as malignant diseases from heterogeneous organs.^(12,13) They found that benign diseases, such as gastrointestinal tract adenoma, pulmonary infectious disease, and gynecologic benign tumors, showed intermediate levels of soluble MICA/B between healthy controls and malignant disease. Our present findings not only agree with theirs, but also provide evidence that soluble MICA/B increases in premalignant conditions such as liver cirrhosis.

Malignant disease is known to lead frequently to the expression of MICA/B.⁽²⁾ In contrast, their expression in premalignant tissues has not been fully elucidated. In the present study, MICA/B were found to be expressed in liver cirrhosis as well as HCC tissues, but not in the early stages of chronic hepatitis or in normal liver. This finding is consistent with the tendencies observed for serum-soluble MICA/B levels in chronic liver disease and HCC. Analysis of cultured cells also revealed that MICA/B expressed on hepatoma cells is released spontaneously into the culture supernatant as soluble forms, supporting the idea that MICA/B expressed in the liver may be released into the circulation. In contrast, MICA/B were not expressed on nor released from cultured non-transformed hepatocytes, which is consistent with the *in vivo* immunohistochemical finding. An issue to be resolved is the underlying mechanism by which non-transformed

hepatocytes express and release MICA/B in pathological conditions such as liver cirrhosis. Recently, it was reported that non-transformed pulmonary epithelial cells can express MICA/B under oxidative stress-inducing conditions.¹⁹⁹ It was also reported that MICA/B are upregulated in non-tumor cell lines by genotoxic stress.²⁰⁰ It has been speculated that oxidative and genotoxic stresses may accumulate in hepatocytes in chronic diseased liver. Thus, it is possible that those stresses may contribute to MICA/B expression in chronic diseased liver. Further study is needed to clarify this issue.

MICA/B expression in the premalignant condition raises the question of which contributes more to the production of soluble MICA/B, malignant tissues or non-malignant tissues. To address this question we analyzed the levels of soluble MICA/B in HCC patients before and after therapeutic intervention. Among treatments for HCC, TAE is a well-established technique for unresectable, advanced HCC.¹⁶⁶ To include HCC patients who show relatively high levels of soluble MICA/B, we chose a cohort of patients who received the TAE therapy in the present study. The data indicated that the levels of soluble MICA, but not those of soluble MICB, decreased after TAE therapy. It is not clear why soluble MICB did not change during TAE therapy. One possibility is that soluble MICB production from non-tumor livers may be relatively high compared with that of soluble MICA. In our subpopulation analysis, Child-Pugh A patients showed a significant decrease in soluble MICB levels after TAE therapy. In general, TAE therapy is more effective for Child-Pugh A patients than Child-Pugh B or C patients because the former is better able to tolerate the large dose of lipiodol emulsion and gelatin sponge that is necessary for efficient antitumor effect. Indeed, Child-Pugh A patients in our cohort showed a larger decrease in α -fetoprotein levels after TAE therapy than Child-Pugh B and C patients, although the difference did not reach a significant level (our unpublished data). Thus, TAE therapy might reduce the levels of soluble MICB when it achieves substantial antitumor effect. Most importantly, the data also indicated that NKG2D expression on immune cells was clearly ameliorated with TAE therapy. Furthermore, there was an inverse correlation between a reduction in soluble MICA and upregulation of NKG2D, suggesting the link between soluble MICA and NKG2D expression in cancer patients.

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Human Neutrophil Peptides 1-3 Are Useful Biomarkers in Patients with Active Ulcerative Colitis

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Background: A specific useful biomarker for diagnosing ulcerative colitis (UC) has not yet been described. This study employed proteomics to identify serum protein biomarkers for UC.

Methods: Ninety-four blood samples were isolated from patients and controls (including 48 UC, 22 Crohn's disease [CD], 5 colorectal cancer, and 6 infectious colitis patients and 13 healthy subjects). Serum samples were analyzed using the SELDI-TOF/MS ProteinChip system. After applying the samples to ProteinChip arrays, we assessed differences in the proteomes using Ciphergen ProteinChip software and identified candidate proteins, which were then characterized in immunoassays.

Results: Preliminary analysis using the ProteinChip system revealed significant peak-intensity differences for 27 serum proteins between 11 patients with UC and 7 healthy subjects. Among these proteins, 3 proteins (with mass/charge ratios of approximately 3400) were identified as human neutrophil peptides 1-3 (HNP 1-3). The presence of HNP 1-3 in the patient sera was confirmed using immunoassays. Enzyme-linked immunosorbent assays demonstrated that the mean plasma concentration of HNP 1-3 was significantly higher in patients with active UC ($n = 28$) than in patients whose UC was in remission ($n = 20$) or patients with CD ($n = 22$), infectious colitis, or healthy subjects, and tended to be higher than in patients with colon cancer. In addition, the plasma concentration of HNP 1-3 in patients that responded to corticosteroids-based therapy

decreased after treatment, whereas it was not changed in nonresponders.

Conclusions: HNP 1-3 is a novel biomarker that may be useful for diagnosing patients with active UC and predicting treatment outcomes.

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Key Words: biomarkers, inflammatory bowel disease, ulcerative colitis, human neutrophil peptides 1-3, SELDI-TOF/MS, proteomics

Genetic and environmental factors contribute to the disease process of inflammatory bowel disease (IBD), including ulcerative colitis (UC).^{1,2} The presence of active inflammation of the gut in patients with UC is associated with an acute-phase reaction and the migration of leukocytes to the gut. This, in turn, promotes the production of a large number of proteins.³ Determination of inflammatory activity is important for the comprehensive assessment of patients with UC and for the tailoring of therapy.⁴ Many clinical activity indices are used to stratify patients with UC. For example, the UC Disease Activity Index (UCDAI)⁵ is a widely used measure of clinical parameters of disease activity. These indices, however, only provide indirect assessments of disease activity. Whereas albumin, hemoglobin, the erythrocyte sedimentation rate (ESR), and acute-phase protein levels are commonly used biological parameters for assessing UC, there are no accurate markers to assess the inflammatory activity observed with histopathologic or endoscopic analyses.⁶

Proteomic array technology, in which a ProteinChip system is coupled with surface-enhanced laser desorption ionization/time-of-flight/mass spectrometry (SELDI-TOF/MS) for the profiling of serum or plasma, is a powerful tool that allows the identification of new biomarkers for malignant tumors and autoimmune diseases.^{7,8} This technology is a rapid and sensitive technique, in which the detected peak intensities for some proteins correlate with concentrations determined using enzyme-linked immunosorbent assay (ELISA). Novel blood biomarkers which are identified by this proteomics, may provide clinicians with more accurate parameters to assess inflammatory activity in UC.

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Host defense processes, which rely on both innate and adaptive immune mechanisms, are critical for the development of IBD.^{1,2} Innate immunity participates in the activation of antigen-specific adaptive immune responses, including the production of antimicrobial peptides/proteins. In mammals, defensins, a class of antimicrobial peptides, can be divided into 2 major groups: α -defensins and β -defensins.⁹ Six types of α -defensins have been identified, 4 of which are produced predominantly by neutrophils and phagocytes and stored in the granules of these cell types (denoted human neutrophil peptides 1–4; HNP 1–4). The remaining 2 α -defensins are localized in Paneth cell granules (denoted human α -defensins 5 and 6; HD 5 and 6). Although the amino-acid sequences of HNP 1, HNP 2, and HNP 3 are very similar, the sequence of HNP 4 is different than those of HNP 1–3. HD 5 is expressed by metaplastic Paneth cells in the colons of patients with UC or CD. The expression levels of HD 5 in blood, however, have not been examined; there are currently no data evaluating HNP 1–3 expression in patients with IBD.

In this study we clearly demonstrate serum profiling with increased levels of HNP 1–3 in the sera of patients with UC using a proteomics-based approach. We also compared the protein levels of HNP 1–3 in plasma samples from patients with UC and Crohn's disease (CD), before and after treatment for UC, and in patients in which treatment was effective or not effective. These analyses will contribute to our understanding of the pathogenesis of UC and aid in the discovery novel biomarkers to assess disease activity and therapeutic responses.

MATERIALS AND METHODS

Patients

After obtaining written informed consent, we analyzed a total of 94 blood samples from patients with IBD, colorectal cancer (CRC), infectious colitis, and control subjects. Forty-eight patients were diagnosed with UC (20 females and 28 males; median age, 39 years; age range, 12–72 years) and 22 with CD (11 females and 11 males; 29 years; 16–57 years). The control group contained 13 healthy subjects (5 females and 8 males; median age 30 years; age range, 24–34 years) and 5 with CRC (1 female and 4 males; median age 62 years; age range, 52–80 years) and 6 with infectious colitis (3 females and 3 males; median age 42 years; age range, 17–77 years). The study protocol was approved by the Ethics Committee of the Kagoshima University Graduate School of Medical and Dental Sciences (Kagoshima, Japan) and the Faculty of Medicine at the University of Miyazaki (Miyazaki, Japan). All IBD patients were diagnosed using established endoscopic, radiological, histological, and clinical criteria. The inactive or remission phase of UC was defined as a UCDAI score less than or equal to 2, whereas the active phase was defined as a UCDAI score greater than or equal to 3.⁵ Twenty and 28 patients with UC were identified as inactive-phase and

active-phase patients, respectively. All of the patients with active-phase UC were treated with oral corticosteroids, whereas 23 received leukocytapheresis therapy (LCAP) (Table 1). Furthermore, 4 of the active UC patients did not respond to treatment and eventually underwent a total colectomy. Fourteen patients with CD had high disease activities based on an International Organization for the Study of Inflammatory Bowel Disease (IOIBD) score of 2 or greater¹⁰ and were regarded as active-phase patients. Eight patients that had lower IOIBD scores (0 or 1) were defined as inactive-phase patients. All 5 CRC patients were diagnosed with Duke's A group cancers by endoscopic, radiological, and histological examinations. All 6 patients with infectious colitis had diarrhea and fever, and were diagnosed based on clinical findings.

SELDI-TOF/MS

We used chips with cationic surfaces for analysis (CM10; Bio-Rad Laboratories, Hercules, CA). Serum samples were denatured in urea buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 2% ampholites), and then diluted 1:9 in binding/washing buffer (50 mM sodium acetate, pH 4). After washing the chip twice in binding/washing buffer, we applied 100 μ L of diluted serum to each chip spot. Samples were incubated for 40 minutes and washed 3 times. After rinsing the chips once in water, 0.5 μ L CHCA (α -cyano-4-hydroxycinnamic acid; Nacal Tesque, Kyoto, Japan) was applied twice to each spot and allowed to air-dry. Arrays were analyzed using a ProteinChip Reader (ProteinChip Biology System II; Bio-Rad Laboratories). TOF spectra were generated with laser shots collected in positive mode. The laser intensity ranged from 190 to 195 with a detector sensitivity of 6. On average, 65 laser shots per spectrum were used. A mixture of standard mass calibrant proteins (All-in-one Peptide Standard; Bio-Rad Laboratories) in 500 nL was used to calibrate the system for mass accuracy. The standards were applied to a single spot of the normal phase chip array (NP20; Bio-Rad Laboratories), after which two 1.0- μ L samples of saturated CHCA were applied. TOF values were compared to the molecular masses of the standard proteins; calibration was performed according to the manufacturer's instructions.⁷

Immunodepletion Assay

Initially, 6 μ L of anti-HNP 1–3 antibody solution (120 ng; Hycult Biotechnology, Netherlands) was bound to 30 μ L of Protein A-agarose (Sigma Chemical, St. Louis, MO) for 15 minutes on ice. The postcentrifugation supernatant was discarded and the pellet was washed twice in buffer containing 20 mM HEPES (pH 7.8), 25 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, and 0.05% NP40. Then 15 μ L of sera from each patient with UC was incubated with a pellet for 45 minutes on ice. As a negative control, samples were incubated with

TABLE 1. Characteristics of Patients with UC or CD

Disease activity ^a	UC		CD	
	Active	Inactive	Active	Inactive
Number	28	20	14	8
Gender (M/F)	19/9	9/11	10/4	6/2
Age (range), yr	41 ± 16 (14-68)	31 ± 16 (12-72)	32 ± 13 (16-57)	28 ± 7 (18-40)
Disease duration (range), yr	5.6 ± 4.8 (1-19)	5.2 ± 4.3 (1-18)	9.4 ± 7.4 (3-22)	6.0 ± 3.8 (1-13)
Treatment ^b				
5-aminosalicylic acid	28	19	14	8
Corticosteroid	28	7	10	2
Leukocytapheresis	23	0	0	0
Type of UC				
Left-side colitis	4	8	—	—
Pancolitis	24	12	—	—
Type of CD				
Ileal	—	—	4	2
Ileocolonic	—	—	9	5
Colonic	—	—	1	1

UC, ulcerative colitis; CD, Crohn's disease. Data are shown as the means ± SD or range.

^aActive UC is defined as an Ulcerative Colitis Disease Activity Index score equal to or greater than 3, and active CD is defined as an International Organization for the Study of Inflammatory Bowel Disease score equal to or greater than 2.

^bIncludes the overlap treatment.

Protein A-agarose in the absence of a specific antibody. After incubation, samples were cleared by centrifugation; 3 μ L of each supernatant was analyzed on NP20 ProteinChip arrays using a PBS II reader.¹¹

ELISA

We determined the HNP 1-3 (P59665, P59666) concentrations in plasma using a human HNP 1-3 ELISA kit (Hycult Biotechnology) according to the manufacturer's instructions. Samples were analyzed in duplicate using a plate reader (Bio-Rad Laboratories) at 450 nm. The concentration of each protein in the plasma was calculated according to a standard curve.

Immunohistochemical Studies

HNP 1-3 expression in colon tissue was evaluated using immunohistochemistry. Abnormal colon tissues were obtained by total colectomy in patients with UC, whereas normal colon tissues were isolated in surgical resections for colon cancer by taking surrounding normal tissue without malignant cells. Colon tissues were fixed in 10% formalin and embedded in paraffin. For histological examination, 5- μ m slices were stained with hematoxylin and eosin (HE). The anti-HNP 1-3 monoclonal antibodies (BMA Biochemicals, Augst, Switzerland) was diluted to a final concentration of 0.5% (w/v) in phosphate-buffered saline (PBS) supplemented with 1% fetal bovine serum (FBS). Immunohisto-

chemical analysis of paraffin-embedded sections using antibodies against HNP 1-3 was performed as described.¹² EnVision plus horseradish peroxidase (Dako, Carpinteria, CA) was applied to samples; chromatin 3',3'-diaminobenzidine was used to detect bound antibody.

Statistical Analysis

Values shown are the means ± SD. Statistical significance, including that for differences in laboratory data and individual peaks in SELDI-TOF/MS, was determined using Mann-Whitney *U*- and paired *t*-tests. *P*-values < 0.05 were considered to be statistically significant. The discriminative power for each putative marker was described via the area under the curve (AUC) from receiver operating characteristic (ROC) analysis. The statistical analyses were performed using StatView 4.5 software (Abacus Concepts, Berkeley, CA), SPSS software (SPSS, Chicago, IL), and Ciphergen ProteinChip Software (Fremont, CA) v. 3.0.2.

RESULTS

Profiling Serum Proteins in Patients with UC

We performed differential profiling of serum proteins in 11 patients with UC and 7 normal healthy controls using the SELDI ProteinChip system. Peaks were automatically detected using Ciphergen ProteinChip Software 3.0.2.¹³ Twenty-seven serum peaks in the 3000-10,000 *m/z* range

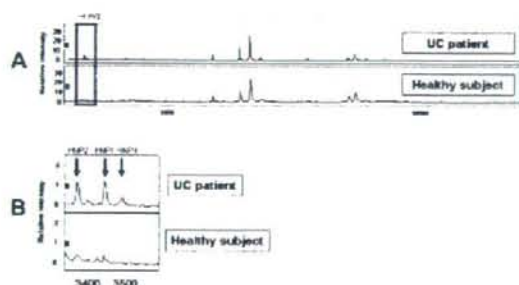


Figure 1. Serum proteomics of UC patients and healthy controls using SELDI-TOF/MS. (A) Spectra representing the serum proteins of a patient with UC and a healthy volunteer. The horizontal axis shows a range from 3000 to 10,000 m/z . Significant differences in peak intensities between patients with UC and healthy volunteers were found for 27 peaks. (B) The intensities of the protein peaks are shown for the range between 3300 and 3600 m/z . Protein peaks with m/z values of 3371, 3443, and 3486 represent HNP 2, HNP 1, and HNP 3, respectively.

were significantly different between the 2 patient groups (Fig. 1). Sixteen peaks resulted in P -values less than 0.01 (Table 2). The most dramatic difference was detected for a 3371 m/z protein, the level of which was increased in the sera of UC patients compared with healthy controls.

Identification of HNP 1–3

A previous study of colon tumor tissue identified a similarly increased signal at 3371 m/z using ProteinChip

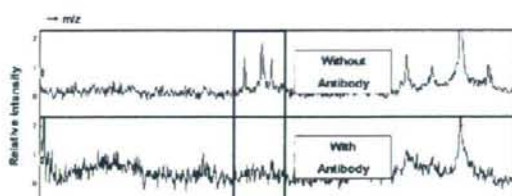


Figure 2. Immunodepletion assay using Protein A beads. Anti-HNP 1–3 antibodies (Hycult Biotechnology) were bound to 30 μ L of Protein A beads. Sera from UC patients were incubated with these beads. After clearing by centrifugation, 3 μ L of each supernatant was analyzed using an NP20 ProteinChip array.

arrays.^{12,14} The peak was confirmed to correspond to HNP 2 with an immunodepletion assay. Peaks at 3443 and 3486 m/z , reported to correspond to HNP 1 and HNP 3 in the previous report, were also found to be significantly increased in analyses of the sera of UC patients compared to results observed for control samples. HNP 1, 2, and 3 have similar structures consisting of 30, 29, and 30 amino acids, respectively; 29 of the amino acids are identical among the peptides.^{12,15} We also subjected the serum samples to immunodepletion assays using monoclonal antibodies against HNP 1–3 and found that the 3371, 3443, and 3486 m/z protein peaks in the SELDI-TOF MS spectra were no longer observed for the sera from patients with UC (Fig. 2). These peaks were clearly observed for negative control samples, which underwent immunodepletion assays in the absence of specific antibodies. These results indicate that the 3371, 3443, and 3486 m/z

TABLE 2. Discriminatory Peaks and Mean Values in Samples from Patients with Ulcerative Colitis and Healthy Volunteers

Mass to Charge (m/z)	Ulcerative Colitis ($n = 11$)	Healthy Subject ($n = 7$)	P -value
3371	1.42 \pm 0.66	0.40 \pm 0.10	4.8 $\times 10^{-4}$
4789	0.51 \pm 0.82	0.05 \pm 0.03	4.8 $\times 10^{-4}$
5421	0.34 \pm 0.24	0.09 \pm 0.02	4.8 $\times 10^{-4}$
8688	0.65 \pm 0.41	1.70 \pm 0.38	6.8 $\times 10^{-4}$
5838	0.79 \pm 0.85	0.21 \pm 0.05	9.4 $\times 10^{-4}$
4351	0.82 \pm 0.62	2.21 \pm 0.56	1.3 $\times 10^{-3}$
5620	0.11 \pm 0.05	0.39 \pm 0.23	1.7 $\times 10^{-3}$
6881	1.00 \pm 0.59	2.24 \pm 0.46	1.7 $\times 10^{-3}$
9358	0.17 \pm 0.06	0.80 \pm 0.52	1.7 $\times 10^{-3}$
7023	0.12 \pm 0.07	0.66 \pm 0.46	2.4 $\times 10^{-3}$
4469	3.31 \pm 2.16	1.02 \pm 0.59	3.2 $\times 10^{-3}$
4542	0.39 \pm 0.17	0.16 \pm 0.02	4.3 $\times 10^{-3}$
4590	0.86 \pm 0.45	1.63 \pm 0.26	4.3 $\times 10^{-3}$
4287	0.68 \pm 0.37	1.26 \pm 0.39	5.7 $\times 10^{-3}$
2900	0.18 \pm 0.12	0.37 \pm 0.14	9.8 $\times 10^{-3}$
2979	1.00 \pm 0.88	0.26 \pm 0.15	9.8 $\times 10^{-3}$

Statistical significance was determined using a Mann-Whitney U -test.