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CASE REPORT

Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report

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Abstract

We present a case of fetal liver failure caused by the activation of lamivudine-resistant hepatitis B virus (HBV) nine months after lamivudine treatment. A 57-year old man visited our hospital for the treatment of decompensated chronic hepatitis B. Lamivudine was started in December 2001. Subsequently, serum HBV was negative for HBV DNA with seroconversion from HBeAg to anti-HBe and improvement of liver function. However, HBV DNA and HBeAg were again detected in September 2002. He was complicated by breakthrough hepatitis and admitted to our hospital in November for severely impaired liver function. Vidarabine treatment was started and serum HBV DNA and alanine aminotransferase (ALT) decreased transiently. However, after the start of α-interferon treatment, HBV DNA level increased and liver function deteriorated. He died 1 mo after admission. An analysis of amino acid sequences in the polymerase region revealed that rtM204I/V with rtL80I/V occurred at the time of viral breakthrough. After the start of antiviral treatment, rtL180M was detected in addition to rtM204I/V and rtL80I/V, and became predominant in the terminal stage of the disease. HBV clone with a high replication capacity may be produced by antiviral treatment leading to the worsening of liver function. Antiviral therapy for patients with breakthrough hepatitis in advanced liver disease should be carefully performed.

Key words: Hepatitis B virus; Lamivudine; Polymerase; Interferon; Tyrosine-methionine-aspartate-aspartate

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INTRODUCTION

Lamivudine is a nucleoside analogue that interrupts the reverse transcription of hepatitis B viral (HBV) pregenomic RNA. Lamivudine is effective for controlling chronic hepatitis B and currently recommended as the first line of treatment for chronic active hepatitis B[1,2]. Even for patients with decompensated liver cirrhosis, lamivudine improves liver function and extends transplantation free intervals[3-10]. Since more than 10% of patients with chronic HBV infection are estimated to develop liver cirrhosis and may eventually suffer from decompensated liver cirrhosis or hepatocellular carcinoma, the role of lamivudine in the treatment of advanced liver disease caused by chronic HBV infection is large[11-14].

The major problems concerning lamivudine treatment are the viral and biochemical breakthroughs caused by drug resistance. Amino acid mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif can occur six months after treatment and often increases alanine aminotransferase (ALT) level. Although the increase is usually mild, a marked increase in ALT level leading to fatal hepatic failure has been reported[15-17]. Factors other than the YMDD motif mutation that are associated with the worsening of liver function remain to be clarified.

Here, we report a case of fatal hepatic failure caused by lamivudine-resistant HBV. A serial analysis of viral amino acid sequences indicated that the acquisition of mutations outside the YMDD motif might be related to the deteriolation of the patient's condition.

CASE REPORT

A 57-year old man visited our hospital in September 2001

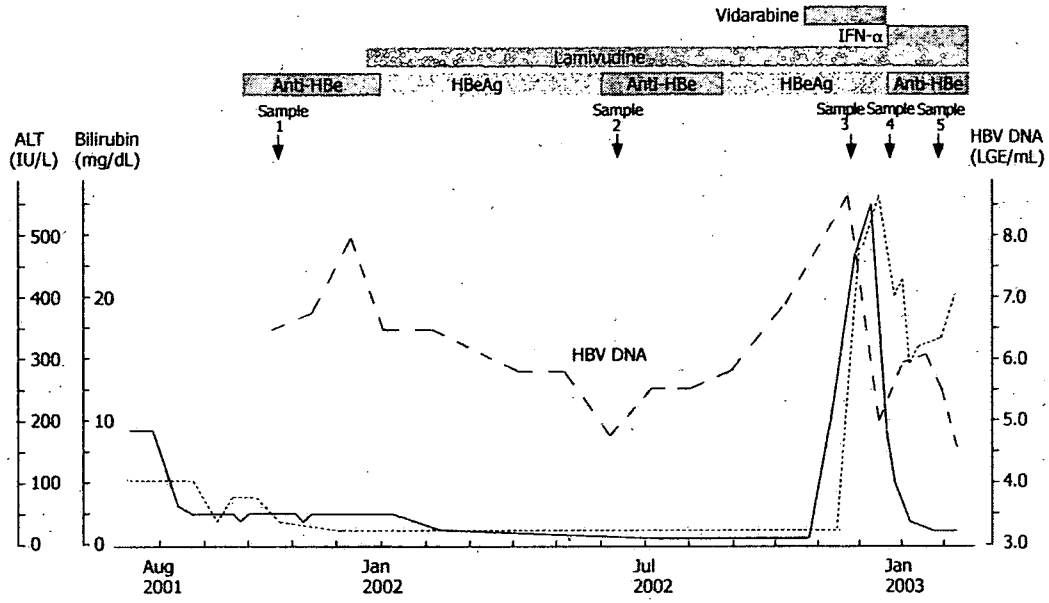

Figure 1 Clinical course of our patient. HBV DNA level was quantified by transcription-mediated amplification assay. The levels of HBV DNA started to increase 8 mo after treatment with reappearance of HBeAg. Breakthrough hepatitis developed 12 mo after treatment. The timing of serum sample analysis for mutations is shown by the arrowhead.

for the treatment of decompensated chronic hepatitis B. In 1978, He was found to be positive for serum HBs antigen (HBsAg). In July 2001, he was admitted to a nearby hospital for ascites where he was diagnosed as having decompensated cirrhosis with exacerbated chronic hepatitis B. The symptomatic control of his ascites improved his general condition. For further treatment, he was referred to our hospital.

On his first visit, he showed no symptoms or signs of worsening hepatic failure or encephalopathy. No ascites or leg edema was observed. His bulbar conjunctiva was slightly jaundiced. Dilated vasculature was observed in his neck and chest. His ALT, total bilirubin and albumin were 50 IU/L, 3.1 mg/dL and 3.7 g/dL, and his prothrombin time was 76%. He was diagnosed as having liver cirrhosis with a Child-Pugh score of 8. He was negative for HBe antigen (HBeAg) and his HBV DNA level measured by transcription-mediated amplification and hybridization protection assay[18] was $10^{6.5}$ genome copies/mL.

In November 2001, he was found to be positive for HBeAg and showed an increase in HBV DNA level. Because he had a history of decompensated chronic hepatitis B, lamivudine treatment (100 mg/d) was started in December. Figure 1 shows the clinical course. The high serum levels of bilirubin and ALT decreased and normalized within 6 mo after lamivudine treatment was started. The patient became negative for HBV DNA and HBeAg.

However, in September 2002, he was found to be positive for HBeAg again and showed an increase in HBV DNA level. In November 2002, he observed jaundice of his bulbar conjunctiva and was admitted to our hospital. Although he was alert, his bulbar conjunctiva and skin were jaundiced. His ALT, total bilirubin, were 474 IU/L, 11.4 mg/dL and 4.3 g/dL. His HBV DNA level was $10^{8.6}$ genome copies/mL. He was diagnosed as having breakthrough hepatitis caused by lamivudine-resistant mutants of HBV. HBV with an amino acid substitution in the YMDD motif in the domain C of polymerase region was detected.

Because interferon is not indicated in patients with decompensated cirrhosis, vidarabine, which is effective for the control of active HBV infection[19-21], was administered together with lamivudine under informed consent. Liver function improved transiently with a decrease in HBV DNA within 2 wk. As prolonged vidarabine administration may induce several complications[22], vidarabine was switched to interferon-α. After the start of interferon-α treatment, HBV DNA level increased and liver function worsened. He died of hepatic failure and rupture of esophageal varices 1 mo after his admission.

The histolopathology of the patient's liver after necropsy showed cirrhosis with zonal necrosis. Hepatocyte regeneration was scarce (Figure 2).

To elucidate the viral factors affecting early viral breakthrough and fatal outcome, amino acid sequences of the upstream polymerase region (aa 1-250) of HBV DNA in serum were examined at 5 points as shown in Figure 1. The methods were as follows.

First, DNA was extracted from 100 μL of a serum sample using the QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). Three fragments spanning the upper polymerase region of HBV DNA were amplified by nested PCR with the primers shown in Table 1. The first stage of amplification was carried out using a thermal cycler for 40 cycles (94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min) in 100 μL of reaction mixture containing 200 mmol/L dNTPs, 1.0 mmol/L each of the primers and 1 × PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$ and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase gold (Perkin Elmer Cetus Corp., CT). Two microliters of the PCR products was subjected to the second stage of amplification under the same conditions as the first stage.

Second, PCR products were purified using Wizard PCR preps DNA purification resin (Promega, WI) and cloned into a plasmid vector using the TA cloning kit (PCR cloning kit Qiagen, CA). Four clones were selected from

Table 1 Primers used for amplification and sequencing of polymerase region of HBV

Region 1		
Outer sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Outer antisense	nt 2490-2509	GGACAGTAGAAGAATAAAG
Inner sense	nt 2222-2241	CTTACTTTTGGAAGAGAAAC
Inner antisense	nt 2478-2497	GAATAAAGCCCAGTAAAGTT
Region 2		
Outer sense	nt 2413-2434	GCGTCGCAGAAGATCTCAATC
Outer antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Inner sense	nt 2434-2452	CTCGGGAATCTCAATGTTAG
Inner antisense	nt 2816-2835	GTTCCCAAGAATATGGTGAC
Region 3		
Outer sense	nt 2490-2509	CTTTATTCTTCTACTGTACC
Outer antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG
Inner sense	nt 2637-2656	ATGCCTGCTAGGTTTTATCC
Inner antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG

each plate, from which recombinant plasmid DNA was purified using a commercially available kit (Plasmid midi kit, Qiagen, Valencia, CA). Nucleotide sequences were determined bidirectionally using the dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, CA) and the PCR primers. Sequencing was performed using an automated DNA sequencer (ABI 377: PE Applied Biosystems).

The determined amino acid sequences in the polymerase region are shown in Figure 3. No amino acid sequence changes were found at the start of lamivudine treatment. At the time of viral breakthrough, rtM204I with rtL80I became dominant. After the start of interferon treatment, rtM204I was replaced by rtM204V and rtL80I by rtL80V. At the final stage of the disease, mutation rtL180M appeared besides rtM204V and rtL80V.

DISCUSSION

Lamivudine monotherapy is effective in suppressing HBV replication and ameliorating liver disease in chronic hepatitis B patients regardless of HBeAg positivity. A one-year study of HBeAg-positive chronic hepatitis B patients showed that 16% of these patients become seroconverted to anti-HBe and 72% of these patients showed normalization of their ALT levels[23]. Furthermore, treatment with lamivudine is associated with histologic improvement not only in terms of necroinflammatory score but also in terms of fibrosis score after long-term treatment[24].

One advantage of lamivudine is that it can be used safely in patients with decompensated cirrhosis[3-10]. In contrast to IFN-α, lamivudine is well tolerated without any significant side effects even in patients with decompensated cirrhosis. Furthermore, lamivudine can improve liver function and survival prognosis.

However, the emergence of a drug-resistant mutant is a big problem in lamivudine treatment. A large-scale Asian study showed that lamivudine resistant HBV infection occurred in 23% of patients in year one and 65% of patients in year five. Hepatitis flares, which occurred more commonly in patients with lamivudine resistant mutations, occurred in 10% of patients in year one, and in 18% to

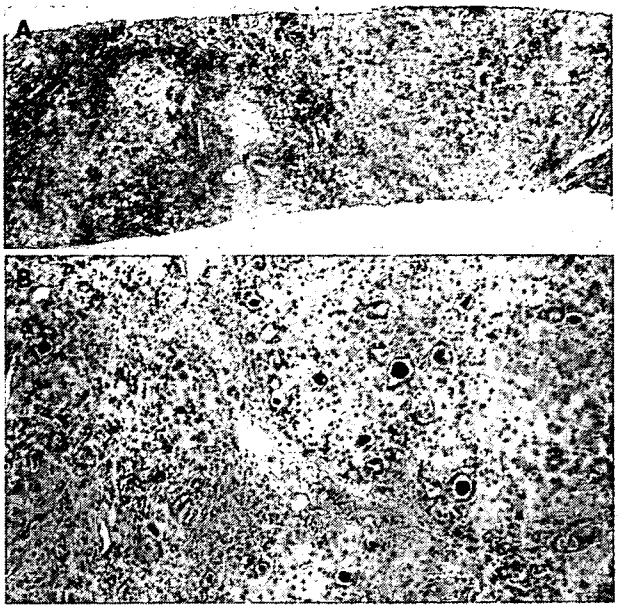

Figure 2 Histopathological findings of liver specimens showing irregularly-shaped parenchymal cells with massive necrosis (A) and scarce hepatocyte regeneration (B) surrounded by extensive fibrosis (A: HE × 20; B: HE × 80).

21% of patients in years two to five. Among patients with lamivudine resistant HBV infection, occurrence of hepatic decompensation increased significantly in patients with lamivudine resistant HBV infection for more than 4 years (from 0% to 6%)[25]. In this large-scale Asian study, liver-disease-related death occurred in two patients.

The prognosis of patients with lamivudine-resistant HBV infection, particularly those with advanced liver disease, may be determined by the timing and severity of breakthrough hepatitis. However, the viral factors that may influence the severity of this hepatitis remain to be clarified. A recent study indicated that patients with a normal ALT level even after the emergence of a YMDD motif mutant are characterized by HBeAg negativity during pretreatment, HBeAg loss during therapy, a longer duration from the commencement of therapy until the emergence of YMDD mutant, and lack of mixed-type YMDD mutants[26]. In contrast, patients with severely exacerbated hepatitis after the emergence of a YMDD mutant tend to have more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of hepatitis exacerbation than those without hepatitis exacerbation[26].

Our patient acquired amino acid mutations in the polymerase region one after the other. Amino acid changes in rtM204/I appeared at the time of viral breakthrough. After the initial treatment with vidarabine, rtM204/V substituted for rtM204/I in one of the four clones. During the interferon treatment, rtM204/V became predominant.

Another mutation observed in our patient was rtL80I/V. Ogata *et al*[27] showed that rtL180M is accompanied with rtM204I in some patients with resistance to lamivudine. Because the mutation at aa position 80 was found at the same time as that at aa position 204 in our patient, it is not clear whether the mutation at aa position 80 affects the clinical course.

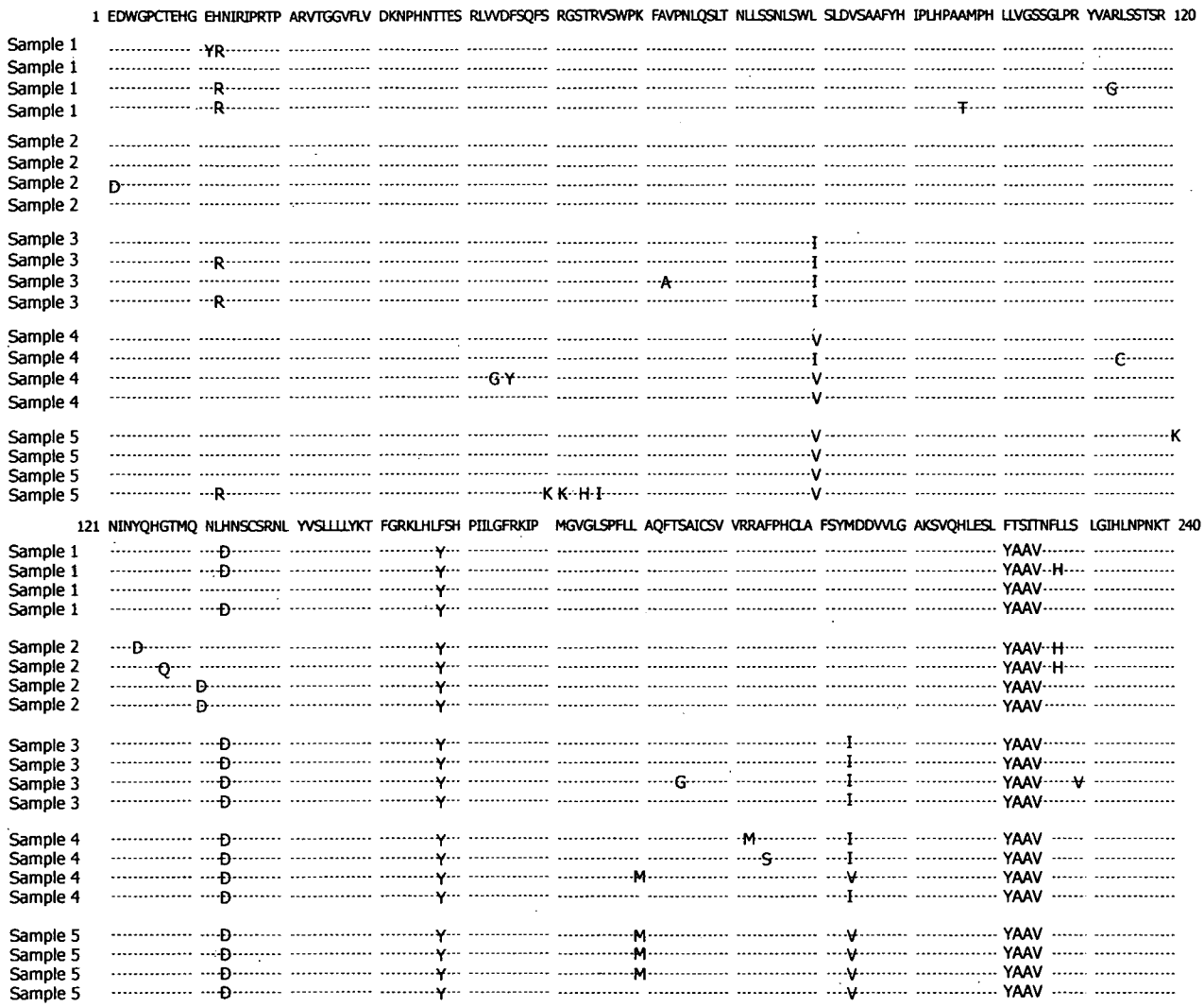

Figure 3 Comparison of amino acid sequences of HBV polymerase gene of isolates before lamivudine treatment (sample 1) and four sequential isolates (samples 2-5) during treatment. A HBV mutant with substitutions of isoleucine for leucine at residue 80 (rtL80I) in combination with isoleucine for methionine at residue 204 (rtM204I) was observed 12 mo after treatment (sample 3). After vidarabine treatment, another HBV mutant with substitutions of valine for leucine at residue 80 (rtL80V) and valine for methionine at residue 204 (rtM204V) was observed (sample 4). These mutations predominated in combination with methionine for leucine at residue 180 (rtL180M) after interferon treatment (sample 5). The published HBV DNA sequence of hepatitis B virus variant (genotype C, AB033550, Okamoto *et al*[38]) was used for comparison.

At the final stage of the disease with deterioration of the condition of the patient, rtL180M, rtM204I/V and rtL80I/V became predominant. Natsuizaka *et al*[28] showed that rtL180M and rtM204V are related to the exacerbation of hepatitis. Interestingly, a patient with a marked elevation in HBV DNA level in Ogata's report had rtL180M in addition to rtL80I and rtM204I. Therefore, the acquisitions of rtL80I/V and rt180M in addition to rtM204I/V may be associated with a severe exacerbation of hepatitis. Large-scale studies are necessary to elucidate this hypothesis.

In our patient, vidarabine decreased HBV DNA levels and improved liver function. Although the long-term use of vidarabine is contraindicated because of its possible side effects including irreversible neurotoxicity[22], its short-term use is effective for controlling active HBV infection and herpes simplex viral infection.

Vidarabine was replaced by interferon-α because adefovir dipivoxil was not available in 2002. Serum HBV DNA and bilirubin levels increased again, which led to a fatal outcome, and HBV clones that have rtL180M became predominant, indicating that withdrawal of vidarabine and administration of interferon may be dangerous for the treatment of severe breakthrough hepatitis. Since interferon may potentially precipitate immunological flares and liver failure[29], nucleotide analogues that are effective for lamivudine-resistant HBV such as adefovir dipivoxil[30-32], entecavir[33-35] and tenofovir[36,37], should be used for the treatment of severe breakthrough hepatitis instead of vidarabine or interferon.

In conclusion, antiviral therapy should be considered in the treatment of patients with hepatic failure after breakthrough hepatitis caused by HBV mutants to lamivudine. The serial acquisition of amino acid mutations outside the YMDD motif in the polymerase region may be associated with severe hepatitis.

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Original Article

Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

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Background: An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

Methods: We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

Results: Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the 26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydropathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydropathy and three-dimensional structure.

Conclusion: Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

Key words: genotype, hepatitis B virus, major hydrophilic region, vaccine

INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.[1–4] To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.[5,6]

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In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.[7]

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsuhashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.[8,9]

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.[8,10] Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.[11] Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.[12,13] Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

METHODS

Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*[14] The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e. $10^{3.7}$ copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ($10^{8.7}$ copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5′-ACCAACCCTCTGGGATTCTTTCC-3′, s2: 5′-ACCAATCCTCTGGGATTCTTCCC-3′, and s3: 5′-AGCAATCCTCTAGGATTCCTTCC-3′ [nt 2902–2924]) and an antisense primer (i.e. as1: 5′-GAGCCTGA GGGCTCCACCC-3′ [nt 3091–3073]) biotinylated at the 5′ end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A-G) immobilized on wells of a 96-well

microplate. Thereafter, hybridization was detected by staining with the streptavidin-horseradish peroxidase (HRP) conjugate.[15]

Amplifying and sequencing the S region of HBV-DNA

The entire aa sequence of MHR in the S region was amplified by two-stage PCR using genotype-specific primers. The outer primers for the amplification of the first fragment were 5′-TTTCCACCAAGCTCTGCAA-3′ (sense: nt 9–28) and 5′-TTCAGGGAATAACCCCATCT-3′ (antisense: nt 872–853) for genotype A, 5′-CTCCA CCACTTTCCA GACT-3′ (sense: nt 1–22) and 5′-CAACTCCCAATTACATATCCC-3′ (antisense: nt 899–879) for genotype B and 5′-TTACAGGCGGGG TTTTTCTT-3′ (sense: nt 70–89) and 5′-TACAGACTT GGCCCCCAATA-3′ (antisense: nt 771–752) for genotype C. The inner primers were 5′-AGAGTCAGGGGCC TGTATTTT-3′ (sense: nt 35–55) and 5′-AGGGAATAA CCCCATCACTTT-3′ (antisense: nt 869–849) for genotype A, 5′-TTCAAGATCCCAGAGTCAGG-3′ (sense: nt 24–43) and 5′-AGGGAATATCCCCACCTTTTT-3′ (antisense: nt 869–849) for genotype B and 5′-CGGGGTT TTCTTGTTGACA-3′ (sense: nt 77–97) and 5′-CCCAAT ACCACATCATCCATA-3′ (antisense: nt 758–738) for genotype C.

The first stage of amplification was carried out in a thermal cycler for 40 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min) in 100 μL reaction mixture containing 200 mM dNTPs, 1.0 mM each of primers and PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 0.001% (wt/vol) gelatin) and 2 U Ampli-Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). PCR products (2 μL) were subjected to the second stage of amplification under the same conditions as those in the first stage. Standard precautions to avoid contamination were taken during PCR, with a negative control serum sample included in each run.

Amplification products were purified on Wizard PCR preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above-mentioned PCR primers. Sequencing was performed in an automated DNA sequencer (ABI 377; PE Applied Biosystems).

The nucleotide sequences of HBV isolates from the patients were compared with those of three reference HBV strains which are used for vaccine production.[16–18]

Phylogenetic trees were constructed with the Mega Program version 2.1 (Center for Evolutionary Functional Genomics, The Biodesign Institute, Tempe, AZ, USA) using the Kimura two-parameter matrix and the neighbor-joining method.[19] To confirm the reliability of phylogenetic tree analysis, boot-strap resampling, and reconstruction were carried out 500 times.

Hydrophobicity and secondary structure analysis

The hydrophobicity profile of the MHR of the S region was predicted by computer-assisted Kyte-Doolittle analysis (an estimate of hydrophobicity based on the bulk phase partitioning of side chain hydrophobicity alone)[20] with GENETYX-MAC software (version 10.1; Software Development, Tokyo, Japan).

The secondary structures of the amino acids in the same region were predicted by computer-assisted Robson[21] and Chou-Fasman analyses[22] with the GENETYX-MAC software.

Statistical analyses

Data were analyzed by the chi-squared test for categorical data and Student's *t*-test or the Mann–Whitney *U*-test for continuous variables. *P*-values less than 0.05 were regarded as statistically significant.

RESULTS

Distribution and clinical characteristics of HBV genotypes

HEPATITIS B VIRUS genotype was determined in the 48 patients with acute hepatitis B. Genotype A was detected in 11 (23%) patients, genotype B in 11 (23%) and genotype C in 26 (54%).

The clinical and demographic backgrounds of the patients with acute hepatitis B who were infected with HBV of different genotypes are shown in Table 1. The mean ages of all the groups were similar. The proportion of male to female patients was higher in genotype A infection than in genotypes B or C infection (100%, 73% and 64%, respectively: A *vs* B, $P = 0.22$; A *vs* C, $P = 0.01$; B *vs* C, $P = 0.16$). The maximum alanine aminotransferase (ALT) levels were lower in patients with genotype A infection than in patients with genotypes B or C infection (1646 ± 1123, 3085 ± 1119 and 2545 ± 981 IU/L, respectively: A *vs* B, $P = 0.01$; A *vs* C, $P = 0.03$; B *vs* C, $P = 0.89$). The maximum HBV-DNA levels were not significantly different between the

Table 1 Demographic and clinical differences among patients with acute hepatitis infected with HBV of distinct genotypes

Features	Genotypes of HBV			Differences (*P*-value)		
	A ($n = 11$)	B ($n = 11$)	C ($n = 26$)	A *vs* B	A *vs* C	B *vs* C
Age (years)	30.6 ± 7.5	28.1 ± 5.1	31.1 ± 9.1	0.41	0.87	0.33
Gender (M:F)	11:0	8:3	15:11	0.22	0.01	0.16
ALT (IU/L)	1646 ± 1123	3085 ± 1119	2545 ± 981	0.01	0.03	0.89
HBV-DNA (LGE/mL)	6.8 ± 1.7	6.6 ± 2.1	5.2 ± 1.2	0.60	0.23	0.06

ALT, alanine aminotransferase; HBV, hepatitis B virus.

genotypes (6.8 ± 1.7, 6.6 ± 2.1 and 5.2 ± 1.2 LGE/mL, respectively: A *vs* B, $P = 0.60$; A *vs* C, $P = 0.23$; B *vs* C, $P = 0.06$).

Amino acid sequence of the S region

The aa sequence of the S region between aa27 and aa203 was determined in the 48 sequences. Figure 1 shows a phylogenetic tree constructed using the 48 sequences and 15 published sequences (four for genotype A, three for genotype B, three for genotype C, one for genotypes D, E, F, G and H). Among the 48 sequences we studied, 11 were classified into genotype A, 11 into genotype B and 26 into genotype C.

The aa sequence of the region between aa101 and aa163 including MHR (aa111-aa156) was compared among 48 sequences and three HBV sequences (X01587, J02205 and huGK-14) currently used for anti-HBV vaccine production. As shown in Figure 2, the aa sequences of X01587 (used for Bimmugen) and J02205 (used for Heptavax) differed in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161). The aa sequence of huGK-14, which is used for the HBV-vaccine Meinyu, differed from that of X01587 in six amino acids and from that of J02205 in two amino acids.

Nine of the 11 isolates classified into genotype A had the same aa sequence as J02205. The remaining two isolates (AB289727 and AB289728) differed from J02205 at aa161 (Fig. 2).

Ten of the 11 isolates classified into genotype B had the same aa sequence as J02205 except for two amino acids (aa114 and aa131). The remaining isolate had another aa substitution at aa112 (Fig. 2).

As shown in Figure 2, 22 of the 26 isolates classified into genotype C had the same sequence as X01587. The remaining four isolates (from patients 10, 24, 30 and 48) had the same sequence as X01587 except for one aa substitution at aa131; the threonine (aa131) of X01587 was substituted with proline for three isolates (AB289714, AB289720 and AB289736) and with alanine for one isolate (AB289701).

Hydrophobicity and secondary structure analysis

As mentioned above, the aa sequences of the MHR from four isolates differed from that of X01587 only at aa131. Furthermore, the aa sequence of the MHR differed between X01587 and J2205 in eight amino acids. We compared the hydropathy and secondary structure of the MHR among J02205, X01587 and two isolates with genotype C (one isolate with proline at aa131 and one with alanine at aa131). The results of Kyte-Doolittle hydropathy analysis based on the hydropathy index are shown in Figure 3. The substitution with alanine-131 was found to alter the patterns on the hydropathy plot, whereas the substitution with proline-131 was found to have little effect. A substitution with alanine-131 could increase the hydrophobicity of the first loop of the MHR, which may affect the antigenicity of HBV.

The secondary structure of our isolate with alanine-131 by Chou-Fasman analysis predicted an α-helix configuration for the region from aa126 to aa135 instead of the β-configuration predicted for the same region of X01587. The predicted secondary structure of our isolate with proline-131 coincided with that of X01587. In contrast, by Robson prediction, the secondary structure of our isolate with alanine-131 coincided with that of X01587; however, that of our isolate with proline-131 was found to have lost a turn structure between aa131 and aa134, which was predicted for X01587.

DISCUSSION

VACCINATION IS THE key to controlling HBV infection. In countries with a high prevalence of HBV infection, universal vaccination is effective not only for controlling viral infections but also for decreasing the incidence of hepatocellular carcinoma.[5,23] Even in

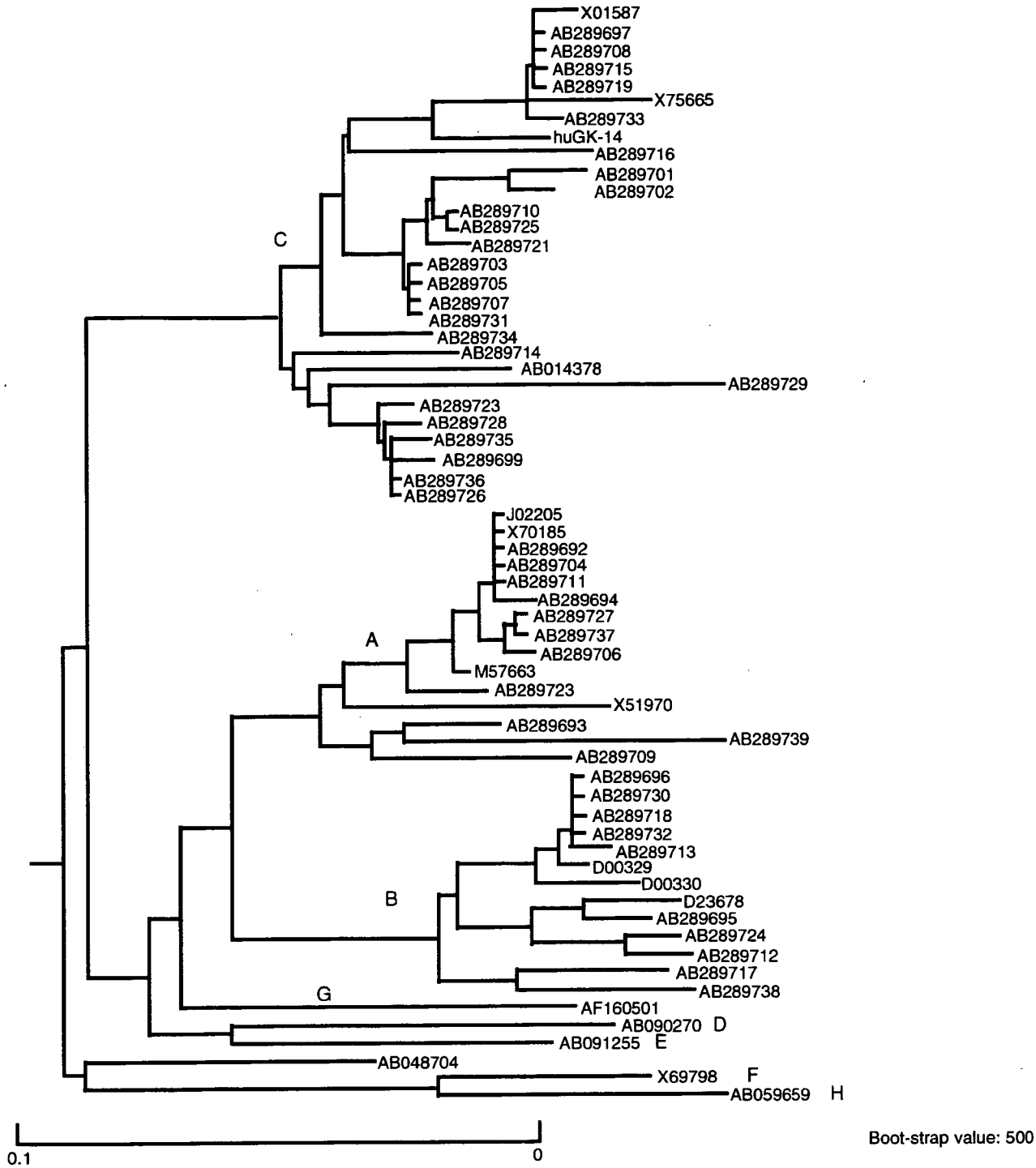

Figure 1 Phylogenetic tree constructed using hepatitis B virus (HBV)-DNA sequences of the *S* gene. The sequences include four with genotype A, four with genotype B, three with genotype C, and those recovered from the serum of 48 patients with acute hepatitis B. J02205 (genotype A) is used for the production of Heptavax and X01587 (genotype C) is used for the production of Bimmugen. The horizontal bar indicates the number of nucleotide substitutions per site. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. The accession numbers for the HBV sequences from the 48 patients are also shown.

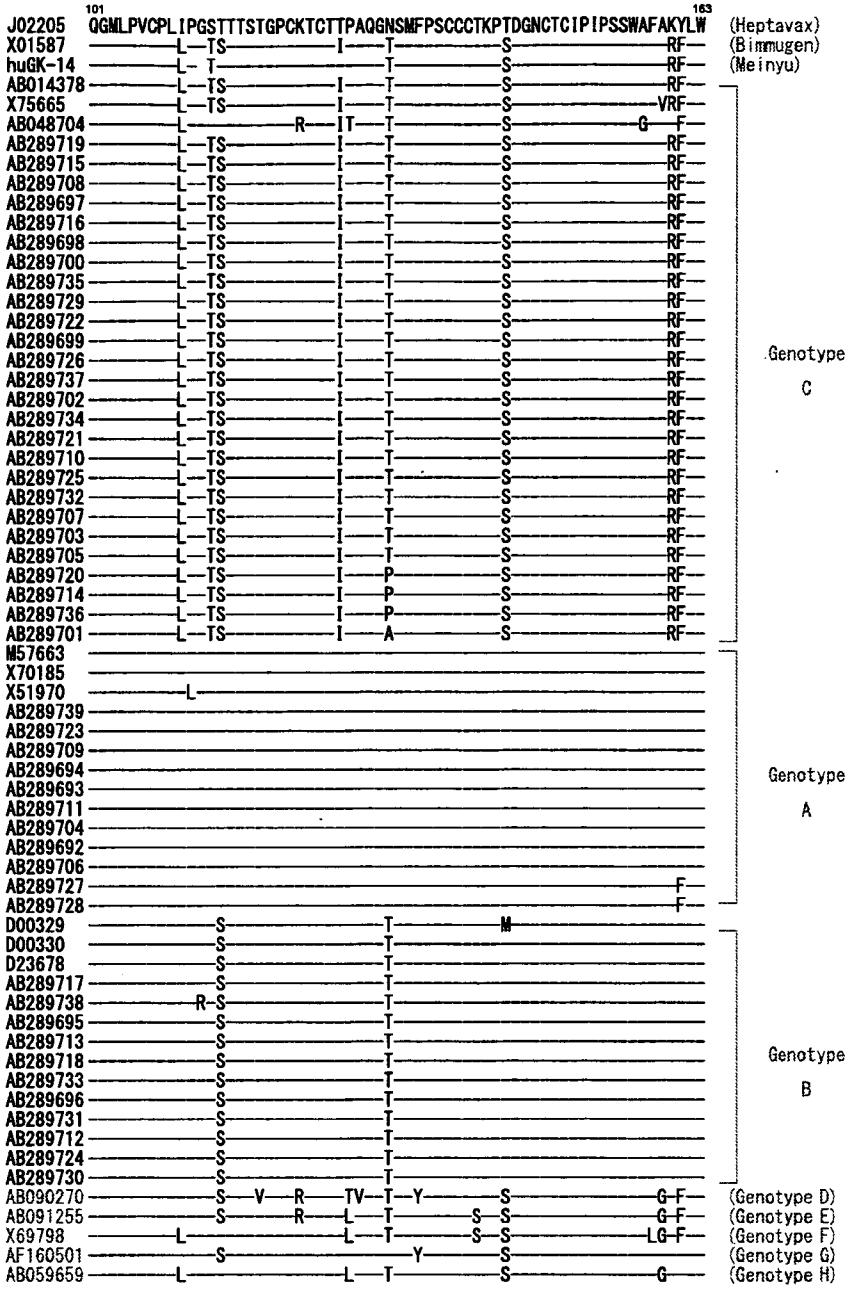

Figure 2 Comparison of amino acid sequences of the major hydrophilic region (MHR) of the *S* gene.

countries with a low prevalence of HBV infection, vaccination is very important for preventing mother-to-child transmission as well as patient-to-staff transmission.

HBV is classified into several genotypes that differ from one another in nucleotide sequence by more than 8% of the entire genome. The aa sequences of their phenotypes also differ among genotypes. The difference in the aa sequence of the 'a' determinant region may alter the three-dimensional structure and antigenicity, and may reduce the protectivity of HBV vaccines.

As mentioned above, the aa sequences of currently available recombinant vaccines differ from each other. J02205 and X01578 differ in eight amino acids (i.e. aa110, aa113, aa114, aa126, aa131, aa143, aa160 and aa161) between aa101 and aa163. Three (i.e. aa126, aa131 and aa143) of them are included in the MHR and may alter the hydropathy and three-dimensional

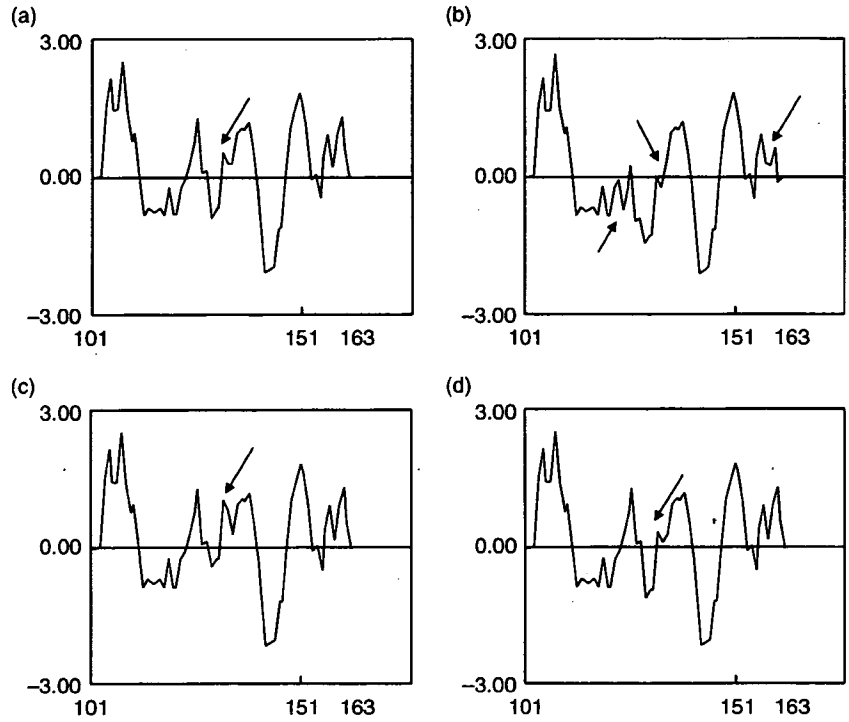

Figure 3 Hydropathy profile of the major hydrophilic region (MHR) of the *S* gene elaborated using the Kyte-Doolittle hydropathy index. Arrows show the positions of amino acids which are different among X01587, J02205, AB289701 (alanine-131) and AB289720 (proline-131). (a) X01587, (b) J02205, (c) AB289701 (alanine-131), (d) AB289720 (proline-131).

structure of the region. Therefore, the antibody produced against J02205 vaccines may not completely neutralize X01578 and vice versa. Indeed, previous studies showed that antibody profiles induced by recombinant vaccines produced from different genotypes are not identical with each other,[12] which suggests that antibodies produced by recombinant vaccines might not protect viral infection with different genotypes.

As shown in Figure 2, the aa sequences of our isolates classified into genotype A are very close to the aa sequence of J02205. Therefore, the transmission of genotype A HBV is prevented by Heptavax which is made from J02205.

The aa sequences of our isolates classified into genotype B are the same as the aa sequence of J02205 except for one substitution at aa131. This aa, which is asparagine and is located in the first stem loop structure of the MHR, was substituted with threonine in our genotype B isolates. Because asparagine and threonine have an uncharged side chain and similar polarity, genotype B HBV infection may be prevented effectively by Heptavax.

The aa sequences of our isolates classified into genotype C were the same as that of X01587 except for four isolates having a substitution at aa131. Bimmugen, which is produced from X01587, may be effective for preventing genotype C HBV infections caused by those four isolates. However, Heptavax may not be effective for preventing genotype C HBV infection because of the difference in eight amino acids as described above.

The four isolates have proline or alanine instead of threonine-131, which has never been reported before. The polarities of threonine and proline/alanine are quite different. The Kyte-Doolittle hydropathy analysis suggests that substituting threonine at aa131 with alanine or proline would increase hydrophobicity, which may then lead to a change in antigenicity. Hou *et al.* reported that some blood donors who were tested negative for serum HBsAg had a substitution of isoleucine for threonine at aa131 in the S region.[24] They suggested that the structure and antigenicity of HBV may be altered by this substitution.

The secondary structure of our isolate with alanine-131 predicted by Chou-Fasman analysis suggested an α-helix configuration instead of a β-configuration in the region from aa126 to aa135. The secondary structure of our isolate with proline-131 predicted by Robson analysis suggested that this change causes the loss of a turn structure between aa131 and aa134. Some changes in the secondary structure can affect the three-dimensional structure of the protein and thus affect antigenicity. These results suggest that the transmission of the four

isolates with an aa substitution at aa131 may not be prevented by either Heptavax or Bimmugen.

However, the protective immunity elicited by HBV vaccines, which is usually polyclonal in nature, may not be totally lost or severely affected *in vivo* by the alteration of only a single amino acid in the 'a' determinant region.[25] Also, antibodies against regions outside the 'a' determinant region may be protective.[26] The protectivity of current vaccines may be elucidated by *in vitro* binding studies using polyclonal antibodies.

It was reported that some individuals immunized with recombinant vaccines are infected with HBV with or without mutations in the 'a' determinant region.[11,27,28] HBV isolates with amino acid substitutions at aa144[29–31] or 145[11,27,28] are known to be transmitted despite vaccination. Indeed, some chronic HBV carriers are reported to have HBV with such amino acid substitutions.[32,33] We were unable to find patients who had these substitutions in the present study. However, large-scale studies are necessary to elucidate the prevalence of 'vaccine-escape mutants' in patients with acute hepatitis B.

In conclusion, we have shown that the aa sequence of the MHR in the *S* gene of HBV is different among isolates from patients with acute HBV infection. Current vaccination may prevent the transmission of these HBV isolates, which should be further investigated.

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A Proteomics Method Revealing Disease-Related Proteins in Livers of Hepatitis-Infected Mouse Model

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In this post-genome era, a sensitive quantitative method is required for differential profiling analyses of clinical proteomes to understand the disease progress. Here, we adopt the FD-LC–MS/MS method, consisting of fluorogenic derivatization (FD), separation by liquid chromatography (LC), and identification by LC-tandem mass spectrometry (MS/MS), to reveal disease-related proteins in livers of hepatocarcinogenesis in transgenic (Tg) and non-transgenic (NTg) mice at three developmental stages. After 6 months, the expression of apoptosis-related proteins is suppressed. After 12 months, proteins related to respiration, the electron-transfer system, and anti-oxidation are significantly up-regulated. After 16 months, proteins related to defense, β-oxidation, and apoptosis are significantly suppressed. This fluctuating expression of proteins could explain the progression of hepatocarcinogenesis. The method would be useful for clinical proteomics analysis because of its high resolution, sensitivity, and reproducibility.

Keywords: DAABD-Cl • FD-LC–MS/MS method • core protein • hepatitis C • liver protein • fluorogenic derivatization

Introduction

Recent work in clinical proteomics has involved quantitative and comparative studies of mixture composition and/or the relative abundance of proteins under differing physiologically relevant conditions and differing experimental approaches, commonly referred to as differential profiling. Numerous approaches have been employed for protein quantification, including a one- or two-dimensional gel electrophoretic and liquid chromatographic (LC) method, followed by mass spectrometry (MS).[1–4] Each of the technical approaches has advantages and limitations. For example, gel-based methods are based on the densitometric quantification of proteins visualized using dyes on gel, followed by in-gel enzymatic digestion of the subject protein spots, with the resulting peptides then subjected to MS analysis. This approach has been widely practiced in proteomics studies because of its high resolution, which enables separating the protein isoforms and post-translational modifications. However, this method suffers from a lack of reproducibility, low sensitivity, low dynamic range, and difficulty in resolving proteins with extreme hydrophobicity or isoelectric points, among other issues.[2,3,5,6] In recent years, the introduction of differential gel electrophoresis (DIGE) using fluorescence reagents such as CyDye DIGE Fluor minimal dye[6–8] and saturation dye[9,10] has somewhat improved the reproducibility, sensitivity, and dynamic range.

LC-based methods offer flexibility of choice over a wide range of stationary and mobile phases to resolve complex biological samples at the protein or peptide level. In these methods, proteins are usually digested into peptides prior to separation by separation columns. The advantage of this approach is that the resolved peptides from the column can be directly introduced into an MS system. To obtain high sensitivity and quantification, the stable-isotope labeling reagents, that is, the isotope-coded affinity tag (ICAT),[11] the cleavable ICAT (cICAT),[3,12,13] and isobaric tags for relative and absolute quantitation (iTRAQ),[3,14] were developed and have gained in popularity. However, a major disadvantage of these strategies is that the obtained peptides cannot be correctly identified as any given protein. Moreover, low-abundance peptides are masked by high-abundance peptides with similar m/z ratios. Thus, for highly complex samples, such as tissue homogenates, these methods are not suitable for the quantification of specific low-abundance proteins unless extensive purification is employed before analysis.[2–4,12,13]

We recently reported a method for proteomics studies called the FD-LC–MS/MS method.[15–18] This method involves fluorogenic derivatization (FD) of proteins using fluorogenic reagents such as 7-chloro-*N*-[2-(dimethylamino)ethyl]-2,1,3-benzoxadiazole-4-sulfonamide (DAABD-Cl), followed by HPLC separation of the derivatized proteins, isolation of the subject proteins, enzymatic digestion of the isolated proteins, and identification of the proteins utilizing HPLC and tandem MS with a database-searching algorithm. The FD-LC–MS/MS method has unique features, differing from other proteome approaches in using a fluorogenic reagent to derivatize proteins

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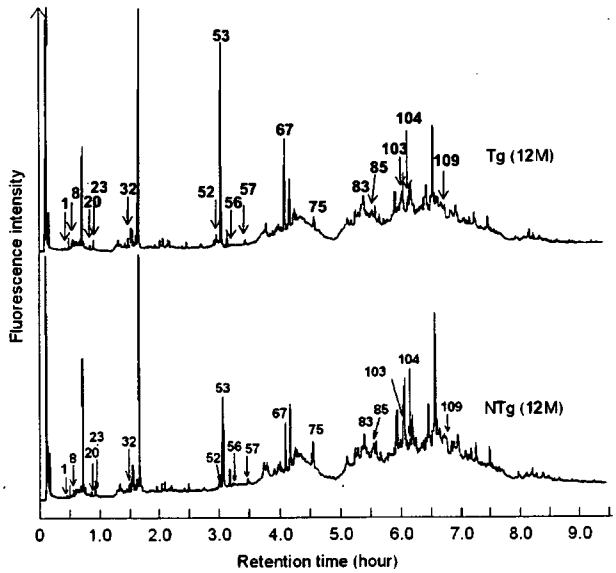

Figure 1. Chromatograms of proteins (8.0 μg protein) in mouse liver derivatized with DAABD-Cl. The chromatograms above and below were obtained from transgenic (12 months) and non-transgenic (12 months) mice, respectively. The altered peaks (106 proteins) between transgenic and non-transgenic mice were numbered, and as representatives, significantly altered peaks (15 proteins) and a peak (no. 53 for accuracy measurement) are described.

and HPLC to separate the derivatized proteins. The fluorogenic reagent is highly reactive and selective to thiols, is nonfluorescent itself, and is water-soluble, so there are few limitations to the complete derivatization of cysteine residues of proteins. The proposed method enables highly sensitive detection of derivatized proteins at the femtomol level,[17,18] whereas other derivatized reagents, such as CyDye DIGE Fluoro minimal dye, would have difficulty forcing the labeling reaction into saturation.[7,8] Separation by HPLC led to highly reproducible quantification. In addition, a protein can be isolated and identified from the corresponding peak fraction without losing any amino acid sequence information, including protein isoforms and post-translational modifications, because the isolated protein itself is digested into peptides following isolation by HPLC. Although this method has already identified more than 100 proteins in a soluble extract of *Caenorhabditis elegans*[15-17] and has identified altered proteins in the islet of Langerhans in dexamethazone-treated rats,[18] there have been no studies involving clinical proteomics analysis utilizing DAABD-Cl as a fluorogenic reagent. Therefore, we attempted to apply it to the quantification and differential profiling analysis of liver proteins taken from hepatitis C virus (HCV) core gene transgenic (Tg) and non-transgenic (NTg) as a model. HCV is the main cause of chronic hepatitis. Chronic hepatitis ultimately results in the progression of hepatocellular carcinoma (HCC). However, the mechanism of hepatocarcinogenesis associated with HCV infection is still unclear. K. Moriya et al. have suggested that the HCV core protein plays a critical role in the progression of HCC and that transgenic mice provide a good animal model for determining the molecular and pathological events in hepatocarcinogenesis with HCV infection.[19-22] Such mice have been investigated previously in terms of morphological and biochemical changes in HCV infection, so far. Therefore, this study investigated the long-term consequences of HCV core gene expression from the viewpoint of proteomics and evaluated the proposed method as the quantification and differential profiling analysis.

Materials and Methods

Transgenic Mice. The production of HCV core gene transgenic mice has been described.[19] Because HCC develops preferentially in male transgenic mice, male mice were used for analysis. Male non-transgenic littermates were utilized as controls. At least three mice were used in each experiment, with the data then subjected to statistical analysis. In the previous studies,[19,20] these transgenic mice developed hepatic steatosis, one of the characteristic histological features of chronic hepatic C, as early as 3 months of age. As the mice grew to 12 months of age, steatosis slowly progressed without neoplastic change. At the age of 16 months, one-fourth of the male mice had experienced hepatic tumors. Moreover, older transgenic mice (>12 months of age) morphologically exhibited an age-dependent increase in oxidative stress. Therefore, in this study, the transgenic and non-transgenic mice used were aged 6, 12, and 16 months, representing the early, medium, and late stages of hepatocarcinogenesis. Also, to exclude the influence of protein variations with advancing age, the amount of protein change due to HCV infection was calculated based on the Tg-to-NTg (Tg/NTg) ratio. All studies were performed according to the Helsinki Declaration and have passed our institutional review board.

Preparation of Sample and Determination of Total Proteins. Liver samples (100 mg) were homogenized in 500 μL of 10 mM CHAPS aq with a pestle on ice. The homogenate was centrifuged at 20 400*g* for 15 min at 4 °C. The supernatant was then collected and stored as a soluble fraction at −20 °C until use. The liver total proteins were determined with the Quick Start Bradford Protein assay kit (Bio-Rad Laboratories, Inc.) by following the written instructions. Bovine serum albumin was used as a protein standard.

FD and HPLC Conditions. The previous method[16] was used for the FD procedure for liver proteins with DAABD-Cl, except that the borate buffer was replaced with a pH 8.7 buffer solution (6.0 M guanidine hydrochloride, Tokyo Chemical Industry). Briefly, homogenized liver tissue was diluted with the CHAPS aq to 4.0 mg/mL, and 10 μL of the sample was mixed with 60 μL of a mixture of 0.83 mM Tris (2-carboxyethyl) phosphine hydrochloride (TCEP), ethylenediamine-*N,N,N',*N'-tetraacetic acid sodium salt (Na_2EDTA), and 16.6 mM CHAPS in the pH 8.7 buffer solution; 25 μL of the buffer solution; and 5.0 μL of 140 mM DAABD-Cl in acetonitrile. After the reaction mixture was placed in a 40 °C water bath for 10 min, 3.0 μL of 20% trifluoroacetic acid (TFA) was added to stop the derivatization reaction. Twenty microliters of the reaction mixture (8.0 μg proteins) was injected into the HPLC system at a flow rate of 0.55 mL/min. The overall system consisted of a Hitachi L-7000 series HPLC system and a fluorescence detector (Jasco FP-2025 plus; λex. 395 nm; λem, 505 nm). Since the derivatives offer adaptable selectivity for the stationary phase, a protein column (Intrada WP-RP, 250 × 4.6 mm i.d., Imtakt Co.) with a column temperature of 60 °C was adopted to further improve the column separation. The mobile phases consisted of 0.15% TFA in acetonitrile/isopropanol/water (A) 9.0/1.0/90 and (B) 69/1.0/30. Mobile phase (C) was the same as (A), except with 0.20% TFA. The gradient condition was established with the following elution: 5.0% B and 1.0% C held for 5.0 min; to 30% B and 35% C in 30 min, and then held for 35 min; to 35% B and 35%

Table 1. Altered Proteins between Tg and NTg Mouse Livers for 6 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		marker		
54	0.55	Major urinary protein (MUP)	20680	gi|295910
56	0.64*	MUP	17549	gi|53271
58	0.58	MUP	17549	gi|53271
55	0.63	Glial fibrillary acidic protein	46498	gi|14193690
		respiration		
52	0.52*	α-globin	15076	gi|49901
		electron-transfer system		
57	0.64	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	18752	gi|16741459
		apoptosis		
1	0.54*	Eukaryotic translation elongation factor 1 α 1 (EF-1 α 1)	50140	gi|13278382
		glycolytic system		
77	0.74	PREDICTED: similar to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	35789	gi|51768209
		other		
34	0.62	ND***		
		Up-Regulated		
		respiration		
27	1.28	α-globin	15076	gi|49900
29	1.28	α-globin	15076	gi|49900
37	1.35	α-globin	15076	gi|49900
		defense		
44	1.14	Cu/Zn-superoxide dismutase (SOD)	15955	gi|201006
75	1.69	Glycine *N*-methyltransferase	32712	gi|15679953
78	1.22	Aldo-keto reductase family 1, member C6	37024	gi|13487925
79	1.32	Glutathione S-transferase, mu 1	25953	gi|61402231
95	1.29	Glutathione S-transferase, α 3	25344	gi|31981724
		fatty acid metabolism (containing β-oxidation)		
35	1.43	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
36	1.24	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
42	1.37	Fatty acid-binding protein, hepatic (fragment)	10173	gi|90485
82	1.31	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
		apoptosis		
3	2.21	EF-1α1	50140	gi|13278382
		glycolytic system		
61	1.40	Fructose-bisphosphate aldolase B	39548	gi|15723269
99	1.36	Enolase 1, α non-neuron	47095	gi|12963491
		metabolism		
68	1.37	Carbonic anhydrase 3	29348	gi|31982861
		other		
26	1.41	Unnamed protein product	58587	gi|12852157
33	1.40	Unnamed protein product	57807, 58587, 57007, 52653	gi|12852157, gi|26345440, gi|2634914, gi|26349459
112	1.42	put. β-Actin (aa 27–375)	39161	gi|49868

[a] Peak numbers correspond to those in Figure 1. Asterisks indicate significant differences (two-tailed Student's *t* test, $*P \leq 0.05$, $** P \leq 0.01$). ***ND, not detected. [b] GI number is simply a series of digits that are assigned consecutively to each sequence record processed by NCBI. The GI system of sequence identifiers runs parallel to the accession.version system, which was implemented by GenBank, EMBL, and DDBJ in February 1999. Therefore, if the protein sequence changes in any way, it will receive a new GI number. (http://www.ncbi.nlm.nih.gov/Sitemap/samplerecord.html#ProteinIDB).

C in 70 min, then to 38% B and 35% C in 130 min; to 44% B and 55% C in 250 min, and then held for 50 min; to 47% B and 53% C in 330 min; to 60% B and 40% C in 480 min; to 70% B and 30% C in 520 min; and then to 90% B and 10% C in 570 min.

Because of the differential profiling of proteins in transgenic and non-transgenic mice, the corresponding peak heights in the different elution profiles were compared for each month age. The correspondence of the peak was judged not only from the specific retention time of the derivatives, but also confirmation of the protein following isolation and identification of the derivatives. The Tg/NTg ratio was also compared between three developmental stages to investigate the consequences of HCV core gene expression during the progression of hepatocarcinogenesis.

Identification of Derivatized Proteins. Each eluate of the subject proteins was concentrated to 5.0 μL under reduced pressure. The residue was diluted with 50 μL of 50 mM ammonium bicarbonate solution (pH 7.8) containing 0.50 U trypsin and 10 mM calcium chloride, and the resultant mixture was incubated for 2.0 h at 37 °C. The peptide mixture (20 μL) was directly subjected to a nanoLC–ESI-tandem MS spectrometer (HCT plus, Bruker Daltonics). Chromatography was performed using an Ultimate/Famos/Switchos suite of instruments (LC Packings, Dionex). The sample was loaded onto a nanoprecolumn (300 μm i.d. × 1.0 mm, C18 PepMap) in the

Table 2. Altered Proteins between Tg and NTg Mouse Livers for 12 Months[a]

peak number	Tg/NTg ratio	protein name	molecular mass (Da)	GI number[b]
		Down-Regulated		
		respiration		
29	0.64	α-globin	15076	gi|49900
		defense		
75	0.56	Glycine *N*-methyltransferase	32712	gi|15679953
76	0.72	Glutathione *S*-transferase, mu 1	25953	gi|61402231
91	0.79	Methionine adenosyltransferase I, α	43481	gi|19526790
		fatty acid metabolism (containing β-oxidation)		
36	0.74	Fatty acid-binding protein, hepatic	10173	gi|90485
40	0.80	Fatty acid-binding protein, hepatic	10173	gi|90485
102	0.77	Peroxisomal acyl-CoA oxidase	74608	gi|2253380
		metabolism		
68	0.75	Carbonic anhydrase 3	29348	gi|31982861
105	0.80	Aldehyde dehydrogenase family 1, subfamily A1	54447	gi|7304881
		amino acid synthesis		
80	0.61	4-Hydroxyphenylpyruvate dioxygenase	45054	gi|849053
		other		
106	0.68	Heat-responsive protein	18462	gi|1255116
		Up-Regulated		
		marker		
55	1.52	Glial fibrillary acidic protein	46498	gi|14193690
56	1.23	MUP	17549	gi|53271
58	1.68	MUP	17549	gi|53271
70	1.51	α-Fetoprotein	47195	gi|191765
		respiration		
4	2.50	Hemoglobin, β adult major chain	15738	gi|31982300
66	1.45	Hemoglobin, β adult major chain	15738	gi|31982300
67	1.98*	Hemoglobin β	15653	gi|229301
27	2.27	α-globin	15076	gi|49900
28	2.43	α-globin	15076	gi|49900
30	1.70	α-globin	15076	gi|49901
31	1.64	α-globin	15076	gi|49900
51	2.12	α-globin	15076	gi|49900
53	2.05	α-globin	15076	gi|49902
		electron-transfer system		
20	1.85*	ATP synthase, H+ transporting, mitochondrial F1 complex, epsilon subunit	5834	gi|13385484
57	1.56*	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	18752	gi|16741459
		protein synthesis		
10	2.43	Ribosomal protein L28	15700	gi|56541228
46	2.33	Ribosomal protein S16	16319	gi|70920
		defense		
9	1.80	SOD	15955	gi|201006
11	2.28	SOD	15752	gi|226471
15	1.24	SOD	15955	gi|201006
18	1.96	SOD	15955	gi|201006
44	1.85	SOD	15955	gi|201006
12	1.42	60S Ribosomal protein	24692	gi|899445
43	1.31	Thioredoxin 1	11668	gi|6755911
50	1.99	Chain C, Crystal Structure Of Macrophage Migration Inhibitory Factor	12365	gi|5542287
69	1.60	D-Dopachrome tautomerase	13069	gi|6753618
71	2.24	Albumin 1	68648	gi|19353306
73	2.11	Albumin 1	68648	gi|19353306
89	1.63	Albumin 1	68648	gi|19353306
83	1.47**	Betaine-homocysteine methyltransferase (BHMT)	44992	gi|62533211
90	2.17	Methionine adenosyltransferase I, α	43481	gi|19526790
100	1.25	Glycine *N*-methyltransferase	32712	gi|15679953
		fatty acid metabolism (containing β-oxidation)		
7	1.95	3-Ketoacyl-CoA thiolase B	43968	gi|18043769
35	1.27	Fatty acid-binding protein, hepatic - (fragment)	10173	gi|90485
41	1.28	Fatty acid-binding protein, hepatic - (fragment)	10173	gi|90485
82	1.32	Acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	41831	gi|20810027
		apoptosis		
1	2.86	EF-1α1	50139	gi|13278381
2	2.79	EF-1α1	50140	gi|13278382
3	1.64	EF-1α1	50140	gi|13278382
8	2.02	Ribosomal protein S29, isoform 1	6672	gi|22267962
24	2.06	Ribosomal protein L14	23549	gi|13385472