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# Pathogenesis of HCV-associated HCC: Dual-pass carcinogenesis through activation of oxidative stress and intracellular signaling

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Overwhelming lines of epidemiological evidence have indicated that persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). It remains controversial, however, in the pathogenesis of HCC associated with HCV, whether the virus plays a direct role or merely an indirect one. The studies using transgenic mouse models by us and others, in which the core protein of HCV has oncogenic potential, indicate that HCV is directly involved in hepatocarcinogenesis, albeit other factors such as continued cell death and regeneration associated with inflammation would play a role, as well. The downstream events of the core protein are segregated into two components. One is the augmented production of oxidative stress along with the activation of scavenging system including catalase and glutathion (GSH) in the putative preneoplastic stage with steatosis in the liver. Thus, oxidative stress production in the absence of inflammation by the core protein would partly contribute to the development of HCC. The generation of oxidative stress is estimated to originate from mitochondrial dysfunction in hepatocytes by HCV infection. The other is the alteration of intracellular signaling cascade of MAPK (JNK), AP-1, cyclin D1, and CDK4. The combination of these pathways, collective with HCV-associated alterations in lipid and glucose metabolism, would lead to the frequent development of HCC in persistent HCV infection. Our results suggest that there would be a mechanism for hepatocarcinogenesis in persistent HCV infection that is distinct from those for other cancers. Similar to the pathogenesis of other cancers, the accumulation of a set of genetic aberrations may also be necessary for multistage development of HCC. However, HCV core protein, to which an oncogenic potential is ascribed, may allow some of the multiple steps to be bypassed in hepatocarcinogenesis. Therefore, unlike other cancers, HCV infection can elicit HCC in the absence of a complete set of genetic aberrations. Such a scenario, "non-Vogelstein-type" carcinogenesis, would explain the unusually high incidence and multicentric nature of HCC development in HCV infection.

**Key words:** hepatitis C virus, hepatocarcinogenesis, intracellular signaling transduction, oxidative stress, transgenic mouse

## INTRODUCTION

WORLDWIDE, HEPATITIS C virus (HCV) infects hundreds of millions of people persistently, and induces a spectrum of chronic liver diseases.[1] Hence, it affects society in a number of domains including medical, sociological, and economic. Hepatocellular carcinoma (HCC) has become the most frequent cause of death in individuals persistently infected with HCV. In particular, HCV has been given increasing attention because of its wide and deep penetration in the community, coupled with a very high incidence of HCC in persistent HCV infection. Once liver cirrhosis is established in hosts infected with HCV, HCC develops at a yearly rate of 5–7%.[2] Knowledge of the mechanism of HCC development in chronic HCV infection, therefore, is imminently required for the prevention of HCC.

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## UNIQUENESS OF HCC DEVELOPMENT IN HCV INFECTION

HOW HCV INDUCES HCC is not yet clear, despite the finding that more than 70% of patients with HCC in Japan are infected with HCV.[1,3,4] HCV infection

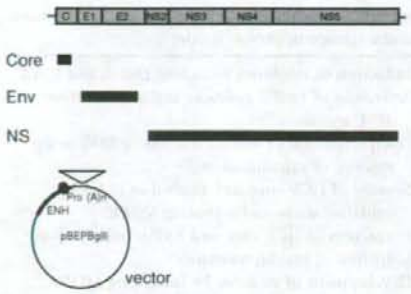


Figure 1 Hepatitis C virus (HCV) transgenic mouse lines. Among the three different transgenic mouse lines established, only the transgenic mice carrying the HCV core gene develop hepatocellular carcinoma (HCC) after an early phase with hepatic steatosis in two independent lineages. The mice transgenic for the envelope genes or non-structural genes do not develop HCC. core, core genes; env, envelope genes; NS, non-structural genes.

is also common in patients with HCC in other countries, albeit to a lesser extent. These lines of evidence obligate hepatologists to a considerable task of determining the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV, manifesting in various forms of hepatitis, should be considered in a study on the carcinogenic capacity of hepatitis viruses. It has been proposed repeatedly that the necrosis of hepatocytes caused by chronic inflammation and ensuing regeneration enhances mutagenesis in host cells, the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC *via* hepatic inflammation. However, this leaves specialists in hepatology with a serious question: can inflammation *per se* result in the development of HCC in such a high incidence or multicentric nature in HCV infection? The secondary role of HCV would have to be weighed against an extremely rare occurrence of HCC in patients with autoimmune hepatitis in whom severe inflammation in the liver persists indefinitely.

This background and reasoning lead to a possible activity of viral proteins for inducing HCC. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. A difficulty in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest. It takes 30–40 years for HCC to develop in individuals infected with HCV. Another constraint common to studies on carcinogenesis is the development of HCC by transformed cells that might have gone out of growth control and escaped surveillance of the host. Should this be the case, the analysis of transformed cells would not be sufficient for solving the mystery of carcinogenesis. On the basis of these viewpoints, we started tackling carcinogenesis in chronic viral hepatitis by transgenic mouse technology.

## CORE PROTEIN OF HCV HAS ONCOGENIC ACTIVITY *IN VIVO*

AS ILLUSTRATED IN Figure 1, transgenic mouse lines with parts of the HCV genome were engineered by introducing the genes excised from the cDNA of the HCV genome of genotype 1b.[5,6] The background of the mouse lines is a C57BL/6 strain, which is known for a rare spontaneous occurrence of HCC.[7] Established are three different transgenic mouse lines, which carry the core gene, envelope genes, or non-structural genes, under the same transcriptional control element. Among these mouse lines, only the transgenic mice carrying the core gene develop HCC in two independent lineages (Fig. 1).[6] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins.[8,9] The transgenic mice carrying the entire non-structural genes have not developed HCC.

The transgenic mice carrying the core gene express the core protein of an expected size, approximately 21 kDa, the level of which in the liver is similar to that in the liver of chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is a histologic characteristic of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[10] Thus, the core gene transgenic mouse model well reproduces this feature of chronic hepatitis C. Of note, significant inflammation is not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Most hepatic nodules disclose a pathology characterized by "nodule-in-nodule", and HCC with a low degree of differentiation develops within adenoma as well as within HCC with a higher degree of differentiation.[6] Although numerous lipid droplets are found in cells forming adenoma, as in non-tumorous cells, they are rarely observed in HCC cells. These histologic features

closely resemble those observed in HCC developing in chronic hepatitis C patients, in which prominent lipid droplets are found in small differentiated HCC and its precursors; poorly differentiated HCC without lipid droplets develops from within differentiated HCC.[8] Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[11] These outcomes indicate that the core protein of HCV has an oncogenic potential when expressed *in vivo*.

## MECHANISM OF HEPATOCARCINOGENESIS IN MOUSE MODEL FOR HCV-ASSOCIATED HCC

IT IS DIFFICULT to sort out the mechanism of carcinogenesis even for our simple model, in which only the core protein is expressed in otherwise normal liver tissue. There is a notable feature in the localization of the core protein in hepatocytes; while the core protein predominantly exists in the cytoplasm associated with lipid droplets, it is also present in the mitochondria and nuclei.[6,12] On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were meticulously analyzed.

One activity of the core protein is an increased production of oxidative stress in the liver. The production of oxidative stress is increased in our transgenic mouse model in the absence of inflammation in the liver (hepatitis). This reflects a state of an overproduction of reactive oxygen species (ROS) in the liver, or predisposition to it, which is staged by the HCV core protein without any intervening inflammation.[13,14] The overproduction of oxidative stress results in the generation of deletions in the mitochondrial DNA, an indicator of genetic damage. Thus, the core protein induces oxidative stress overproduction in the absence of inflammation, and may, at least in part, contribute to hepatocarcinogenesis in HCV infection. If inflammation is induced in the liver with the HCV core protein, the production of oxidative stress is escalated to an extent that can no longer be scavenged by a physiologically antagonistic system. This indicates that the inflammation in chronic HCV infection would have a characteristic different in quality from those of other types of hepatitis, such as autoimmune hepatitis. The basis for the overproduction of oxidative stress may be ascribed to mitochondrial dysfunction.[13,15] The function of the electron transfer system of the mitochondrion is suggested in association with the presence of the HCV core protein.[16] Hepatic steatosis in hepatitis C may work as fuel for oxidative stress overproduction.[14,17,18]

**Table 1** Biomolecular alterations with core protein expression observed in the transgenic mouse model

| | |
|---|---|
| 1. | Induction of cytokines including TNF-α and IL-1β[19] |
| 2. | Activation of MAPK pathway and enhancement of AP-1 activation[19,20] |
| 3. | Overproduction of oxidative stress or ROS in the absence of inflammation[13] |
| 4. | Synergy of HCV core and alcohol in inducing oxidative stress and activating MAPK[13,20] |
| 5. | Interaction of HCV core and RXR-α and PPAR-α[21] |
| 6. | Induction of insulin resistance[17] |
| 7. | Development of steatosis by inhibiting MTP activity[5,14,22] |
| 8. | Interaction of HCV core and proteasome activator PA28γ[23] |
| 9. | Inhibition of SOCS-1[24] |

AP-1, activated protein-1; HCV, hepatitis C virus; IL-1β, interleukin-1β; MAPK, mitogen-activated protein kinase; MTP, microsomal triglyceride transfer protein; PPAR-α, peroxisome proliferator agonist receptor-α; ROS, reactive oxygen species; RXR-α, retinoid X receptor; SOCS-1, suppressor of cytokine signaling; TNF-α, tumor necrosis factor.

Other possible pathways are the alteration of the expression of cellular genes, interacting with cellular proteins, and modulation of intracellular signaling pathways (Table 1). For example, tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β) have been found transcriptionally activated.[19] The core protein has also been found to interact with some cellular proteins, such as retinoid X receptor (RXR)-α, that play pivotal roles in cell proliferation and metabolism.[20] The mitogen-activated protein kinase (MAPK) cascade is also activated in the liver of the core gene transgenic mouse model. The MAPK pathway, which consists of three routes, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (ERK), is involved in numerous cellular events including cell proliferation. In the liver of the core gene transgenic mouse model prior to HCC development, only the JNK route is activated. Downstream in the JNK activation, transcription factor AP-1 activation is markedly enhanced.[19,21] Far downstream, both the mRNA and protein levels of cyclin D1 and CDK4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes.

Such an effect of the core protein on the MAPK pathway, combined with that on oxidative stress, may

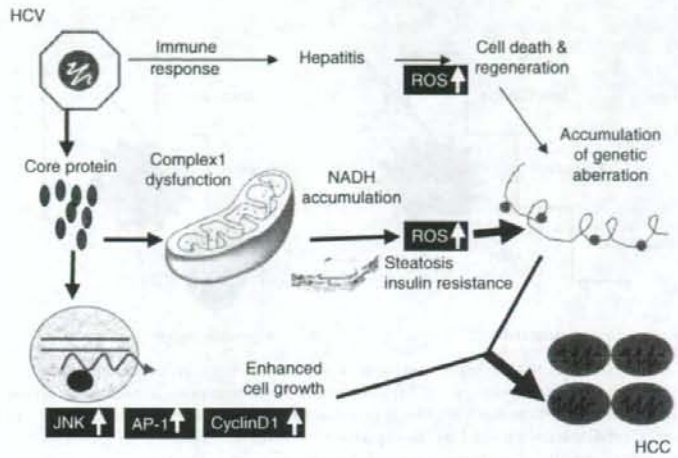


**Figure 2** Mechanism of hepatitis C virus (HCV)-associated hepatocarcinogenesis. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus would contribute to hepatocarcinogenesis via two pathways: (i) the core protein acts on the function of mitochondrial electron transfer system, leading to the overproduction of oxidative stress. Inflammation may act synergistically with the core protein in inducing oxidative stress. The presence of steatosis and insulin resistance would enhance the production of oxidative stress; and (ii) modulation of cellular gene expression and signal transduction, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of hepatocellular carcinoma (HCC) in HCV infection. AP-1, activated protein-1; JNK, Jun N-terminal kinase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species.

explain the extremely high incidence of HCC development in chronic hepatitis C.

## HEPATOCARCINOGENESIS INDUCED BY HCV INFECTION: MECHANISM DISTINCT FROM OTHER CANCERS

THE RESULTS OF our studies on transgenic mice indicated a carcinogenic potential of the HCV core protein *in vivo*; thus, HCV may be directly involved in hepatocarcinogenesis.

In research studies of carcinogenesis, the theory by Kinzler and Vogelstein[25] has gained wide popularity. They proposed that the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations. They deduced that mutations in the *APC* gene for inactivation, those in *K-ras* for activation and those in the *p53* gene for inactivation accumulate, which cooperate toward the development of colorectal cancer.[25] The theory has been extended to the carcinogenesis of other cancers as well, called "Vogelstein-type" carcinogenesis (Fig. 2).

On the basis of results we obtained for the induction of HCC by the HCV core protein, we introduce a mechanism different from that of Kinzler and Vogelstein[25] for hepatocarcinogenesis in HCV infection. We allow multistages in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that many mutations accumulate in hepatocytes. Some of these steps, however, may be skipped in the development of HCC in HCV infection to which the core protein would contribute (Fig. 3). The overall effects achieved by the expression of the viral protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis.

By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events happening in HCV carriers.[26] Now it does not seem so difficult as before to determine why HCC develops in persistent HCV infection at an outstandingly high incidence. Our theory may also give an account of the non-metastatic and multicentric *de novo* occurrence characteristics of HCC, which would be the result of persistent HCV infection.

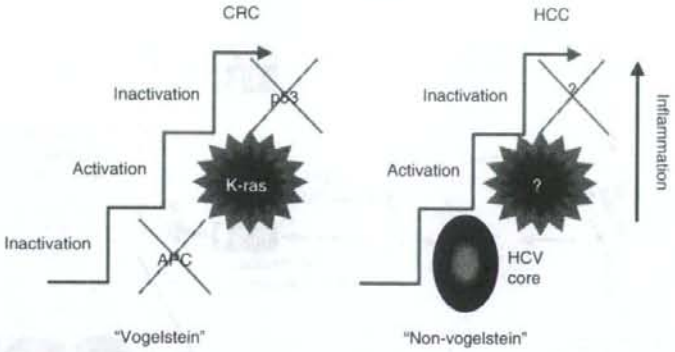


**Figure 3** Hepatitis C virus (HCV)-associated hepatocarcinogenesis is a non-Vogelstein-type. Multiple steps are required in the induction of all cancers; it would be mandatory for hepatocarcinogenesis that genetic mutations accumulate in hepatocytes. However, in HCV infection, some of these steps may be skipped in the development of hepatocellular carcinoma (HCC) in the presence of core protein. Overall effects achieved by the expression of core protein would be the induction of HCC, even in the absence of a complete set of genetic aberrations, required for carcinogenesis. By considering such a "non-Vogelstein-type" process for the induction of HCC, a plausible explanation may be given for many unusual events in HCV carriers. CRC, colorectal cancer.

## CONFLICT OF INTEREST

NO CONFLICT OF interest has been declared by the author.

## REFERENCES

1 Saito I, Miyamura T, Ohbayashi A *et al.* Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci USA* 1990; **87:** 6547–9.
2 Ikeda K, Saitoh S, Suzuki Y *et al.* Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998; **28:** 930–8.
3 Kiyosawa K, Sodeyama T, Tanaka E *et al.* Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12:** 671–5.
4 Yotsuyanagi H, Shintani Y, Moriya K *et al.* Virological analysis of non-B, non-C hepatocellular carcinoma in Japan: frequent involvement of hepatitis B virus. *J Infect Dis* 2000; **181:** 1920–8.
5 Moriya K, Yotsuyanagi H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatic steatosis in transgenic mice. *J Gen Virol* 1997; **78:** 1527–31.
6 Moriya K, Fujie H, Shintani Y *et al.* Hepatitis C virus core protein induces hepatocellular carcinoma in transgenic mice. *Nat Med* 1998; **4:** 1065–8.
7 Smith GS, Walford RL, Mickey MR. Lifespan and incidence of cancer and other diseases in selected long-lived inbred mice and their F 1 hybrids. *J Natl Cancer Inst* 1973; **50:** 1195–213.
8 Koike K, Moriya K, Ishibashi K *et al.* Expression of hepatitis C virus envelope proteins in transgenic mice. *J Gen Virol* 1995; **76:** 3031–8.
9 Koike K, Moriya K, Yotsuyanagi H *et al.* Sialadenitis resembling Sjögren's syndrome in mice transgenic for hepatitis C virus envelope genes. *Proc Natl Acad Sci USA* 1997; **94:** 233–6.
10 Bach N, Thung SN, Schaffner F. The histological features of chronic hepatitis C and autoimmune chronic hepatitis: a comparative analysis. *Hepatology* 1992; **15:** 572–7.
11 Lerat H, Honda M, Beard MR *et al.* Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. *Gastroenterology* 2002; **122:** 352–65.
12 Moriya K, Fujie H, Yotsuyanagi H *et al.* Subcellular localization of hepatitis C virus structural proteins expressed in transgenic liver. *Jpn J Med Sci Biol* 1997; **50:** 169–77.
13 Moriya K, Nakagawa K, Santa T *et al.* Oxidative stress in the absence of inflammation in a mouse model for hepatitis C virus-associated hepatocellular carcinogenesis. *Cancer Res* 2001; **61:** 4365–70.
14 Moriya K, Todoroki T, Tsutsumi T *et al.* Increase in the concentration of carbon 18 monounsaturated fatty acids in the liver with hepatitis C: analysis in transgenic mice and humans. *Biophys Biochem Res Commun* 2001; **281:** 1207–12.

15 Okuda M, Li K, Beard MR *et al.* Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. *Gastroenterology* 2002; **122**: 366–75.
16 Moriya K, Tajima A, Tsutsumi T *et al.* Hepatitis C virus core protein insults mitochondrial function through reducing the ETS complex I activity. In Miyamura T, Shimotohno K, eds. *Proceedings of the 10th International Meeting on Hepatitis C and Related Viruses, 2–6 December 2003, Kyoto, Japan.* Kyoto: Japan Linkage Service, 2003, 73.
17 Shintani Y, Fujie H, Miyoshi H *et al.* Hepatitis C virus and diabetes: direct involvement of the virus in the development of insulin resistance. *Gastroenterology* 2004; **126**: 840–8.
18 Koike K, Moriya K. Metabolic aspects of hepatitis C: steatohepatitis distinct from NASH. *J Gastroenterol* 2005; **40**: 329–36.
19 Tsutsumi T, Suzuki T, Moriya K *et al.* Intrahepatic cytokine expression and AP-1 activation in mice transgenic for hepatitis C virus core protein. *Virology* 2002; **304**: 415–24.
20 Tsutsumi T, Suzuki T, Shimoike T *et al.* Interaction of hepatitis C virus core protein with retinoid X receptor-a modulates its transcriptional activity. *Hepatology* 2002; **35**: 937–46.
21 Tsutsumi T, Suzuki T, Moriya K *et al.* Hepatitis C virus core protein activates ERK and p38 MAPK in cooperation with ethanol in transgenic mice. *Hepatology* 2003; **38**: 820–8.
22 Perlemuter G, Sabile A, Letteron P *et al.* Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. *FASEB J* 2002; **16**: 185–94.
23 Moriishi K, Okabayashi T, Nakai K *et al.* Proteasome activator PA28g-dependent nuclear retention and degradation of hepatitis C virus core protein. *J Virol* 2003; **77**: 10 237–49.
24 Miyoshi H, Fujie H, Shintani Y *et al.* Hepatitis C virus core protein exerts an inhibitory effect on suppressor of cytokine signaling (SOCS)-1 gene expression. *J Hepatol* 2005; **43**: 757–63.
25 Kinzler KW, Vogelstein B. Lessons from hereditary colorectal cancer. *Cell* 1996; **87**: 159–70.
26 Koike K, Tsutsumi T, Fujie H *et al.* Role of hepatitis viruses in hepatocarcinogenesis. *Oncology* 2002; **62**: 29–37.

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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]

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

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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 ($1646 \pm 1123$, $3085 \pm 1119$ and $2545 \pm 981$ 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

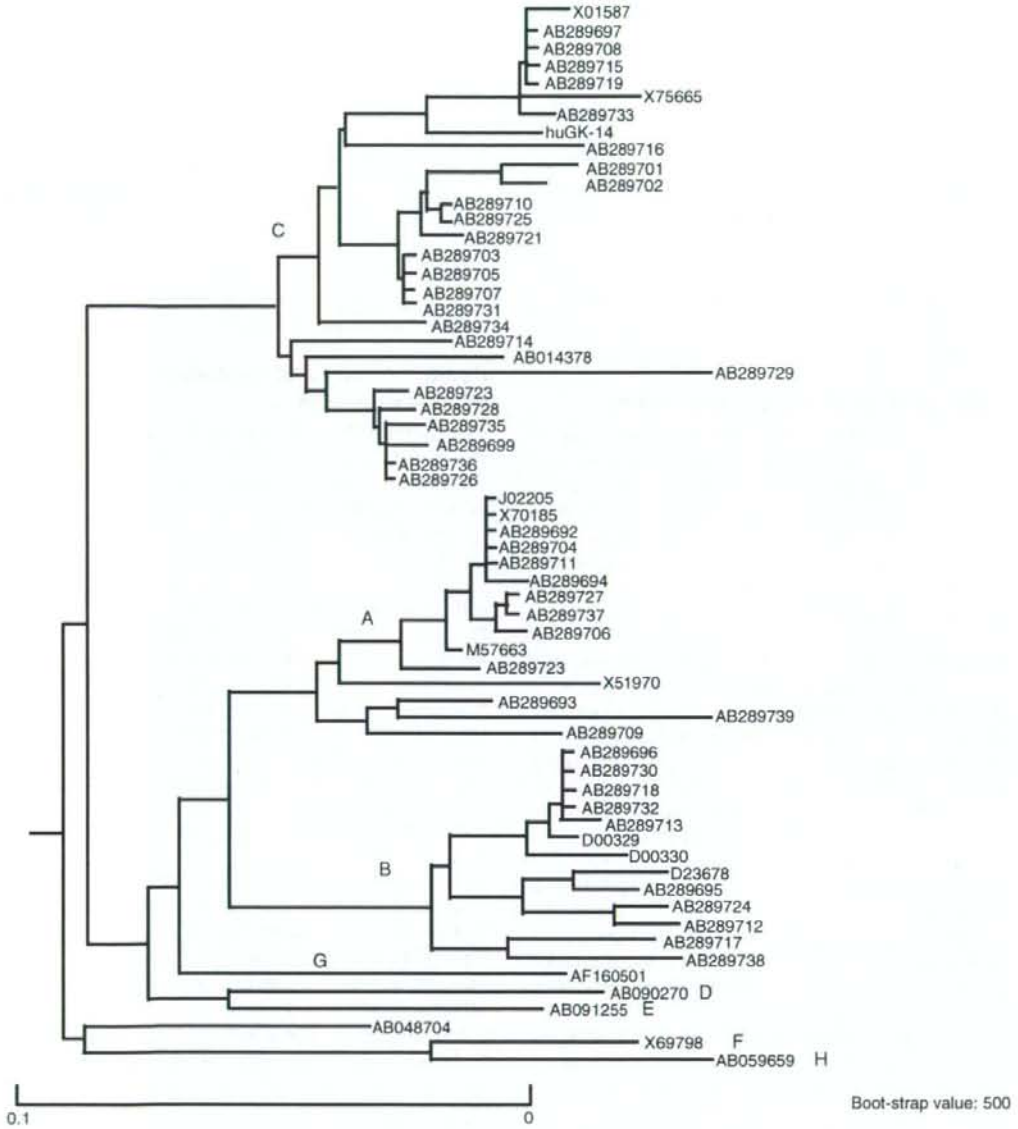


**Figure 1** Phylogenetic tree constructed using hepatitis B virus (HBV)-DNA sequences of the *S* gene. The sequences include four with genotype A, four with genotype B, three with genotype C, and those recovered from the serum of 48 patients with acute hepatitis B. J02205 (genotype A) is used for the 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.

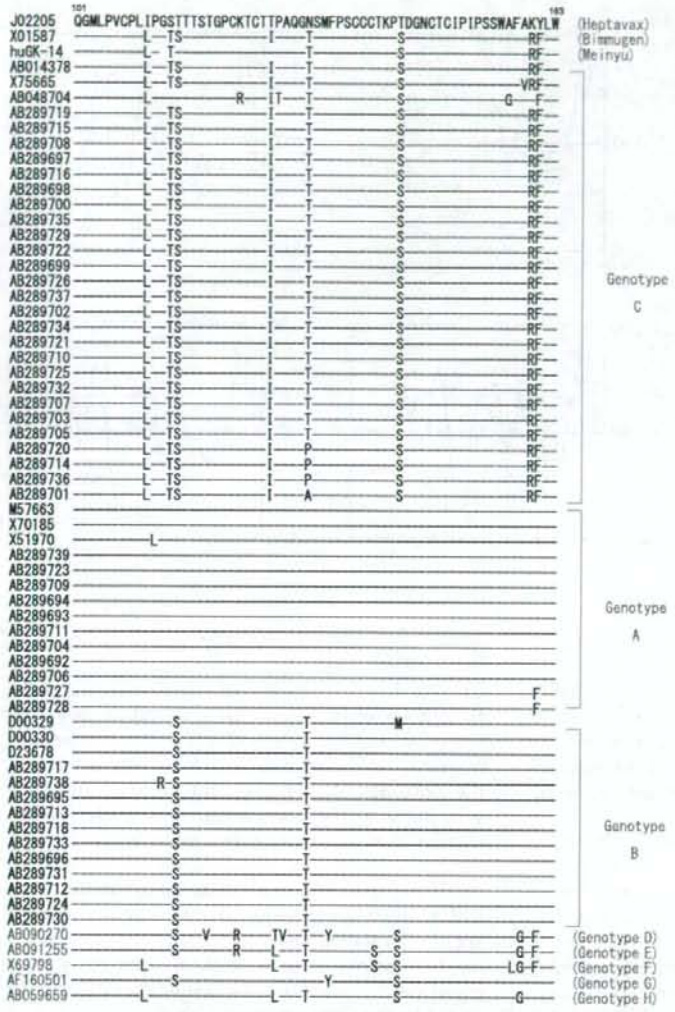


**Figure 2** Comparison of amino acid sequences of the major hydrophilic region (MHR) of the *S* gene.

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

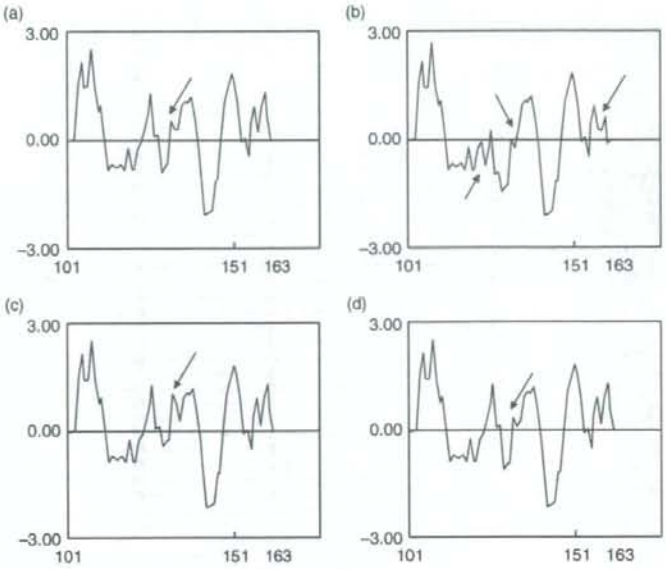


**Figure 3** Hydropathy profile of the major hydrophilic region (MHR) of the *S* gene elaborated using the Kyte-Doolittle hydropathy index. Arrows show the positions of amino acids which are different among X01587, J02205, AB289701 (alanine-131) and AB289720 (proline-131). (a) X01587, (b) J02205, (c)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

1 Manno M, Camma C, Schepis F *et al.* Natural history of chronic HBV carriers in northern Italy: morbidity and mortality after 30 years. *Gastroenterology* 2004; **127**: 756–63.
2 Liaw YF, Lin DY, Chen TJ, Chu CM. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Liver* 1989; **9**: 235–41.
3 Fattovich G, Brollo L, Giustina G *et al.* Natural history and prognostic factors for chronic hepatitis type B. *Gut* 1991; **32**: 294–8.
4 Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Hepatology* 1988; **8**: 493–6.
5 Chang MH, Chen CJ, Lai MS *et al.* Universal hepatitis B vaccination in Taiwan and the incidence of hepatocellular carcinoma in children. Taiwan Childhood Hepatoma Study Group. *N Engl J Med* 1997; **336**: 1855–9.
6 Ni YH, Chang MH, Huang LM *et al.* Hepatitis B virus infection in children and adolescents in a hyperendemic area: 15 years after mass hepatitis B vaccination. *Ann Intern Med* 2001; **135**: 796–800.
7 Tanaka J, Kumagai J, Katayama K *et al.* Sex- and age-specific carriers of hepatitis B and C viruses in Japan estimated by the prevalence in the 3 485 648 first-time blood donors during 1995–2000. *Intervirology* 2004; **47**: 32–40.
8 Kobayashi M, Suzuki F, Arase Y *et al.* Infection with hepatitis B virus genotype A in Tokyo, Japan during 1976 through 2001. *J Gastroenterol* 2004; **39**: 844–50.
9 Yotsuyanagi H, Okuse C, Yasuda K *et al.* Distinct geographic distributions of hepatitis B virus genotypes in patients with acute infection in Japan. *J Med Virol* 2005; **77**: 39–46.
10 Suzuki Y, Kobayashi M, Ikeda K *et al.* Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005; **76**: 33–9.
11 Water JA, Kennedy M, Voet P *et al.* Loss of the common 'A' determinant of hepatitis B surface antigen by a vaccine-induced escape mutant. *J Clin Invest* 1992; **90**: 2543–7.
12 Heijtink RA, Schneeberger PM, Postma B, Crombach W. Anti-HBs levels after hepatitis B immunisation depend on test reagents: routinely determined 10 and 100 IU/L seroprotection levels unreliable. *Vaccine* 2002; **20**: 2899–905.
13 Hieu NT, Kim KH, Janowicz Z, Timmermans I. Comparative efficacy, safety and immunogenicity of Hepavax-Gene and Engerix-B, recombinant hepatitis B vaccines, in infants born to HBsAg and HBeAg positive mothers in Vietnam: an assessment at 2 years. *Vaccine* 2002; **20**: 1803–8.
14 Kamisango K, Kamogawa C, Sumi M *et al.* Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. *J Clin Microbiol* 1999; **37**: 310–14.
15 Kato H, Orito E, Sugauchi F *et al.* Frequent coinfection with hepatitis B virus strains of distinct genotypes detected by hybridization with type-specific probes immobilized on a solid-phase support. *J Virol Methods* 2003; **110**: 29–35.
16 Koike K, Yoshida E, Katagiri K *et al.* Production of hepatitis B virus surface antigen particles by the human hepatoma cell line huGK-14 in a serum-free medium. *Jpn J Cancer Res* 1987; **78**: 1341–6.
17 McAleer WJ, Buynak EB, Maigetter RZ, Wampler DE, Miller WJ, Hilleman MR. Human hepatitis B vaccine from recombinant yeast. *Nature* 1984; **307**: 178–80.
18 Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982; **298**: 347–50.
19 Sugita S, Yoshioka Y, Itamura S *et al.* Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. *J Mol Evol* 1991; **32**: 16–23.

20 Kyte J, Doolittle F. A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 1982; **157**: 105–32.
21 Garnier J, Osguthorpe DJ, Robson B. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J Mol Biol* 1978; **120**: 97–120.
22 Chou PY, Fasman GD. Conformational parameters for amino acids in helical, β-sheet, and random coil regions calculated from proteins. *Biochemistry* 1974; **13**: 211–12.
23 Ni YH, Chang MH, Huang LM *et al.* Hepatitis B virus infection in children and adolescents in a hyperendemic area: 15 years after mass hepatitis B vaccination. *Ann Intern Med* 2001; **135**: 796–80.
24 Hou J, Wang Z, Cheng J *et al.* Prevalence of naturally occurring surface gene variants of hepatitis B virus in nonimmunized surface antigen-negative Chinese carriers. *Hepatology* 2001; **34**: 1027–34.
25 Ogata N, Cote PJ, Zanetti AR *et al.* Licensed recombinant hepatitis B vaccines protect chimpanzees against infection with the prototype surface gene mutant of hepatitis B virus. *Hepatology* 1999; **30**: 779–86.
26 Paulij WP, de Wit PL, Sunnen CM *et al.* Localization of a unique hepatitis B virus epitope sheds new light on the structure of hepatitis B virus surface antigen. *J Gen Virol* 1999; **80**: 2121–6.
27 Carman WF, Zanetti AR, Karayiannis P *et al.* Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990; **336**: 325–9.
28 Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet* 1995; **345**: 1406–7.
29 Lee PI, Chang LY, Lee CY, Huang LM, Chang MH. Detection of hepatitis B surface gene mutation in carrier children with or without immunoprophylaxis at birth. *J Infect Dis* 1997; **176**: 427–30.
30 Protzer-Knolle U, Naumann U, Bartenschlager R *et al.* Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high-dose hepatitis B immune globulin after liver transplantation. *Hepatology* 1998; **27**: 254–63.
31 Shields PL, Owsianka A, Carman WF *et al.* Selection of hepatitis B surface 'escape' mutants during passive immune prophylaxis following liver transplantation: potential impact of genetic changes on polymerase protein function. *Gut* 1999; **45**: 306–9.
32 Yamamoto K, Horikita M, Tsuda F *et al.* Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol* 1994; **68**: 2671–6.
33 Kato J, Hasegawa K, Torii N, Yamauchi K, Hayashi N. A molecular analysis of viral persistence in surface antigen-negative chronic hepatitis B. *Hepatology* 1996; **23**: 389–95.

research articles Journal of proteome research

# 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, $\beta$-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[8,10] 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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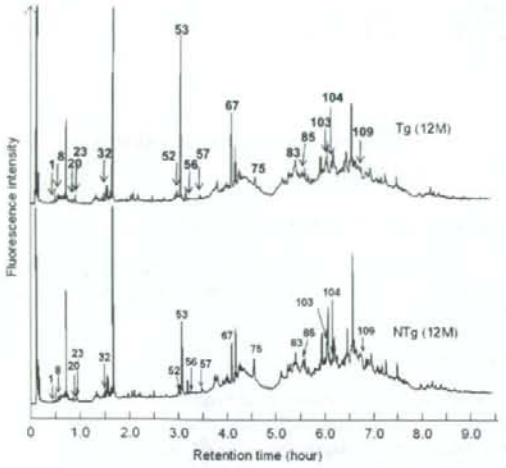


**Figure 1.** Chromatograms of proteins (8.0 $\mu$g protein) in mouse liver derivatized with DAABD-Cl. The chromatograms above and below were obtained from transgenic (12 months) and non-transgenic (12 months) mice, respectively. The altered peaks (106 proteins) between transgenic and non-transgenic 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 $\mu$L 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 $\mu$L of the sample was mixed with 60 $\mu$L 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 $\mu$L of the buffer solution; and 5.0 $\mu$L of 140 mM DAABD-Cl in acetonitrile. After the reaction mixture was placed in a 40 °C water bath for 10 min, 3.0 $\mu$L of 20% trifluoroacetic acid (TFA) was added to stop the derivatization reaction. Twenty microliters of the reaction mixture (8.0 $\mu$g 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; $\lambda$ex. 395 nm; $\lambda$em, 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 |

**Table 2.** (Continued)

| peak number | Tg/NTg ratio | protein name | molecular mass (Da) | GI number[b] |
|---|---|---|---|---|
| | | **glycolytic system** | | |
| 59 | 2.96 | Fructose-bisphosphate aldolase B | 39548 | gi\|15723268 |
| 62 | 1.21 | Fructose-bisphosphate aldolase B | 39548 | gi\|15723268 |
| 77 | 1.39 | PREDICTED: similar to GAPDH | 35789 | gi\|51768209 |
| 112 | 1.64 | Lactate dehydrogenase 1, A chain | 36475 | gi\|6754524 |
| | | **metabolism** | | |
| 21 | 1.79 | TI-225 | 14167 | gi\|1167510 |
| 22 | 2.02 | TI-225 | 14167 | gi\|1167510 |
| 32 | 2.02 | Cystatin B | 11039 | gi\|6681071 |
| 72 | 3.23 | Carbonic anhydrase 3 | 29348 | gi\|31982861 |
| 104 | 1.83* | Acetaldehyde dehydrogenase (ALDH) | 54410 | gi\|9755362 |
| 110 | 1.82 | Aldh2 protein | 56502 | gi\|13529509 |
| 115 | 1.90 | Malate dehydrogenase (EC 1.1.1.37) | 31692 | gi\|164543 |
| 116 | 2.10 | Argininosuccinate lyase | 51707 | gi\|19526986 |
| | | **signal transduction** | | |
| 47 | 1.70 | Phosphatidylethanolamine binding protein | 20847 | gi\|9256572 |
| | | **amino acid synthesis** | | |
| 74 | 2.21 | Glycine-*N*-acyltransferase | 34076 | gi\|22122359 |
| | | **other** | | |
| 5 | 2.02 | ND*** | | |
| 6 | 1.99 | ND*** | | |
| 118 | 1.97 | ND*** | | |
| 19 | 3.56 | γ-actin | 40992 | gi\|809561 |
| 23 | 1.40* | Diazepam binding inhibitor, splice form 1b | 15219 | gi\|67511482 |
| 39 | 2.80 | Saposin | 61353 | gi\|249387 |
| 45 | 2.13 | Unnamed protein product | 58587, 57007, 52653, 49471 | gi\|12852157, gi\|26345440, gi\|26349141, gi\|26349459, |
| 49 | 1.42 | Peptidylprolyl isomerase A | 17960 | gi\|71051228 |
| 103 | 1.53* | Sorbitol dehydrogenase precursor | 40066 | gi\|1009706 |
| 117 | 3.03 | Unnamed protein product | 57614 | gi\|52787 |

[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).

injection loop, and washed using 0.10% TFA in 2.0% acetonitrile at 30 μL/min using the Switchos pump. Peptides were then separated on a nanoflow column (75 μm i.d. × 15 cm, C18 PepMap) at a flow rate of 170 μL/min, employing a gradient from 5.0% to 60% buffer B (0.10% formic acid in 80% acetonitrile) over a period of 35 min (A buffer: 0.10% formic acid in 2.0% acetonitrile). One-second MS/MS scans were performed on each precursor ion. Ions observed with $m/z$ between 350 and 1250 were fragmented with capillary energies from 1300 to 1800 V. The proteins were identified in accord with the previous method.[15,17] There were several candidates with the same score for the unnamed protein products (peak numbers 33 and 45).

**Statistical Analysis.** Results are expressed as the mean ± SD. The significance of the difference in means was determined by a two-tailed Student's *t* test.

## Results and Discussion

**Validation of The FD-LC–MS/MS Method.** With the FD-LC–MS/MS method, more than 500 peaks were obtained from an extract of mouse liver tissue derivatized with DAABD-Cl. Typical chromatograms derived from transgenic and non-transgenic mice are depicted in Figure 1. Only the proteins which expression was estimated to fluctuate between transgenic and non-transgenic mice on the same months were identified after isolation, tryptic digestion, and LC–MS/MS identification of arbitrarily selected peak fractions (113 proteins). As a result, 106 proteins differed between transgenic and non-transgenic mice from 6 to 16 months of age, as summarized in Tables 1–3. The total protein amount required for quantification and identification was only 8.0 μg per injection, and identification of even low-abundance proteins was possible with 40 μg of total protein per injection into an HPLC column. In general, proteome analysis of biological samples labeled with CyDye, ICAT, cICAT, or iTRAQ requires from dozens to hundreds of micrograms of protein samples.[3,7–10,11,13,14,28] The accuracy of the method was acquired based on the reproducibility of the peak heights using peaks 53, 83, and 32 as representatives of the high, medium, and low peaks obtained from each individual mouse. The relative standard deviation (RSD, %) for each between-day peak was less than 16 (high peak), 17 (medium peak), and 23% (low peak) ($n = 3$). The reproducibility of the retention time was also calculated using peak 32. The between-day RSD was 0.41% ($n = 3$). As an additional benefit, the simple apparatus, consisting of a pump, a column, and a fluorescence detector, does not require a complex facility for operation. In this study, we attempted a comprehensive profiling analysis of an 11-h operation to evaluate the utility of the method. After the elution time of a subject protein has been determined, it will be possible to reduce the analysis time for an arbitrary analysis of the subject protein by re-optimizing the separation conditions. It would also be possible to reduce the overall analysis time if we could develop a higher-performance column.