Research Article

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Hepatocarcinogenesis in Hepatitis C: HCV Shrewdly Exacerbates Oxidative Stress by Modulating both Production and Scavenging of Reactive Oxygen Species

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Key Words
Hepatitis C · Hepatocellular carcinoma · Oxidative stress · Transgenic mouse · Core protein

Abstract
Persistent infection with hepatitis C virus (HCV) is a major risk for the development of hepatocellular carcinoma (HCC). One of the characteristics of HCV infection is the unusual augmentation of oxidative stress, which is exacerbated by iron accumulation in the liver, as observed frequently in hepatitis C patients. Using a transgenic mouse model, in which HCC develops late in life after the preneoplastic steatosis stage, the core protein of HCV was shown to induce the overproduction of reactive oxygen species (ROS) in the liver. In excessive generation of ROS, HCV affects the steady-state levels of a mitochondrial protein chaperone, i.e. prohibitin, leading to an impaired function of the mitochondrial respiratory chain with the overproduction of ROS. Insulin resistance and hepatic steatosis, which frequently accompany HCV infection, exacerbate ROS production. On the other hand, HCV compromises some of the antioxidant systems, including heme oxygenase-1 and NADH dehydrogenase quinone 1, resulting in the provocation of oxidative stress, together with ROS overproduction, in the liver with HCV infection. Thus, HCV infection not only induces ROS but also hampers the antioxidant system in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis. Combination with the other activated pathway, including an alteration in the intracellular signaling cascade of MAP kinase, along with HCV-associated disturbances in lipid and glucose metabolism would lead to the unusual mode of hepatocarcinogenesis, i.e. very frequent and multicentric development of HCC, in persistent HCV infection.

Introduction

Approximately 200 million people are infected with hepatitis C virus (HCV) worldwide. More than two thirds of those with acute HCV infection suffer from persistent infection causing active or inactive chronic hepatitis, and approximately 30% of patients with chronic hepatitis are assumed to develop cirrhosis within their lifetime. Once HCV infection develops into cirrhosis, hepatocellular carcinoma (HCC) develops at an annual rate of 7% [1]. The strong association of oxidative stress with HCV infection has been demonstrated and can explain at least part of the clinical progression of the disease. The patho-

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genesis of chronic hepatitis C is not merely ascribed to inflammation caused by viral infection; the role of viral proteins in the pathogenesis has also been reported [2]. Of the proteins constituting HCV, the core protein in particular has various functions with respect to host cells and is closely related to oxidative stress. In this article, the relationship between HCV infection and oxidative stress is analyzed focusing on the pathological effect of the core protein of HCV, and the significance of oxidative stress in the pathogenesis of liver disease is discussed.

HCV Infection and Hepatocarcinogenesis

The mechanism underlying hepatocarcinogenesis in HCV infection is not fully understood yet. Inflammation induced by an immune response to HCV should be considered, of course, in a study on hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations 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 context leaves us with a serious question: can inflammation alone result in the development of HCC in HCV infection with such a high incidence (90% in 15 years) or in a multicentric fashion? The other role of HCV would have to be weighed against a rare occurrence of HCC, even after the development of cirrhosis, in patients with autoimmune hepatitis in which severe inflammation in the liver persists. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C in vivo using transgenic mouse technology.

Transgenic Mouse Model for HCV-Related HCC

One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. For this purpose, several lines of mice have been established which are transgenic for the HCV cDNA. We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b [3, 4]. Four different kinds of transgenic mouse lines are established, and they carry the core gene, envelope genes, the entire nonstructural (NS) genes, or the NS5A gene, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages [4]. The envelope gene transgenic mice did not develop HCC despite high expression levels of both E1 and E2 proteins [5], and the transgenic mice carrying the entire NS or NS5A gene developed no HCC.

Early in life, core gene transgenic mice develop hepatic steatosis, which is one of the histologic characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damages [6]. Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. It is important to note that no significant inflammation is observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the structural genes including the core gene [4, 7, 8]. These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed in vivo.

Augmentation of Oxidative Stress in Hepatitis C

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 [4]. On the basis of this finding, the pathways related to these two organelles, the mitochondria and nuclei, were thoroughly investigated.

One effect of the core protein is an increased production of oxidative stress in the liver. We would like to draw particular attention to the fact that the production of oxidative stress is increased in the core gene transgenic mouse model in the absence of inflammation in the liver [4]. The overproduction of oxidative stress results in the generation of deletions in the mitochondrial and nuclear DNA, an indicator of genetic damage [2].

Augmentation of oxidative stress is implicated in the pathogenesis of liver disease in HCV infection as shown by a number of clinical and basic studies [2, 9]. Reactive

oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism. ROS can induce genetic mutations as well as chromosomal alterations and thus contribute to cancer development in multistep carcinogenesis [10, 11]. Recent studies have shown that oxidative stress is more augmented in hepatitis C than in other types of hepatitis such as hepatitis B [9].

Thus, a major role in the pathogenesis of HCV-associated liver disease has been attributed to oxidative stress augmentation, but little is known regarding the mechanism of increased oxidative stress in HCV infection. Hence, it is important to understand the mechanism of oxidative stress augmentation, in terms of both generation and scavenging of ROS, which may allow us to develop new tools of therapies for chronic hepatitis C.

Oxidative Stress and the Liver

Oxidative Stress and Reactive Oxygen

The main source of ROS in hepatocytes is the mitochondria. Outside of hepatocytes, ROS also originate from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and xanthine oxidase in Kupffer cells and inflammatory cells. A large percentage of consumed oxygen is constantly converted into ROS in the mitochondria accompanied by oxygen consumption in the electron transport system (ETS). Hepatocytes contain many mitochondria and therefore have a high ROS production. Generated ROS are very unstable and highly reactive and attack biomolecules such as DNA, lipids, and proteins. The liver not only produces much ROS but is also the center of the antioxidative effect in the form of protein synthesis. Oxidative stress refers to the oxidation-reaction-dominant state of the living body induced by an imbalance between the oxidation reaction caused by ROS and the antioxidation reaction. Main ROS include superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO$\cdot$). ROS are mainly produced from $\cdot O_2^-$ and converted into stable H_2O_2 through a dismutation reaction. H_2O_2 is converted into highly reactive HO$\cdot$ in the presence of a transition metal.

The Antioxidant System and Oxidative Stress Markers

Antioxidants include glutathione (GSH), thioredoxin (TRX), vitamin E, vitamin C, and β-carotene. Reactive oxygen elimination enzymes include superoxide dismutase (SOD), GSH peroxidase, heme oxygenase (HO)-1, and catalase. SOD is induced by oxidative stress and dismutates $\cdot O_2^-$ to H_2O_2 and oxygen. Catalase in peroxisomes also decomposes H_2O_2 to water and oxygen. TRX is also a protein induced by oxidative stress and is reduced via S-S binding of the substrate protein by two SH groups in TRX and acts on the H_2O_2 elimination system via peroxiredoxins. HO-1 is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin with the release of carbon monoxide. Biliverdin is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities.

ROS cause various forms of cellular damage. 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) are the peroxidation reaction products of lipids, and 8-hydroxydeoxyguanosine (8-OHdG) is the product of DNA base modification. These products serve as oxidative stress markers.

The Origin of ROS Production in HCV Infection

Then, where is the place for oxidative stress overproduction in the liver of hepatitis C patients? The core protein is mostly localized to the endoplasmic reticulum, but we and other groups have shown its localization to the mitochondria in cultured cells and transgenic mice [12]. In addition, the double structure of mitochondrial membranes is disrupted in hepatocytes of core gene transgenic mice. Evidence suggests that the core protein modulates some mitochondrial functions, including fatty acid β-oxidation, the impairment of which may induce lipid abnormalities and hepatic steatosis. In addition, the mitochondrion is an important source of ROS. In livers of transgenic mice harboring the core gene, increased ROS production has been observed [2]. A recent study found, via proteomic profiling of biopsy specimens, that impairment of key mitochondrial processes including fatty acid oxidation and oxidative phosphorylation and of the response to oxidative stress occurs in HCV-infected human liver with advanced fibrosis [13]. Therefore, it is probable that the HCV core protein affects mitochondrial functions since such pathogenesis is observed in both HCV core-transgenic mice and HCV-infected patients.

The recent progress in proteomics has opened new avenues for disease-related biomarker discovery. We performed a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of mitochondria isolated from HepG2 cells stably expressing the HCV core protein and

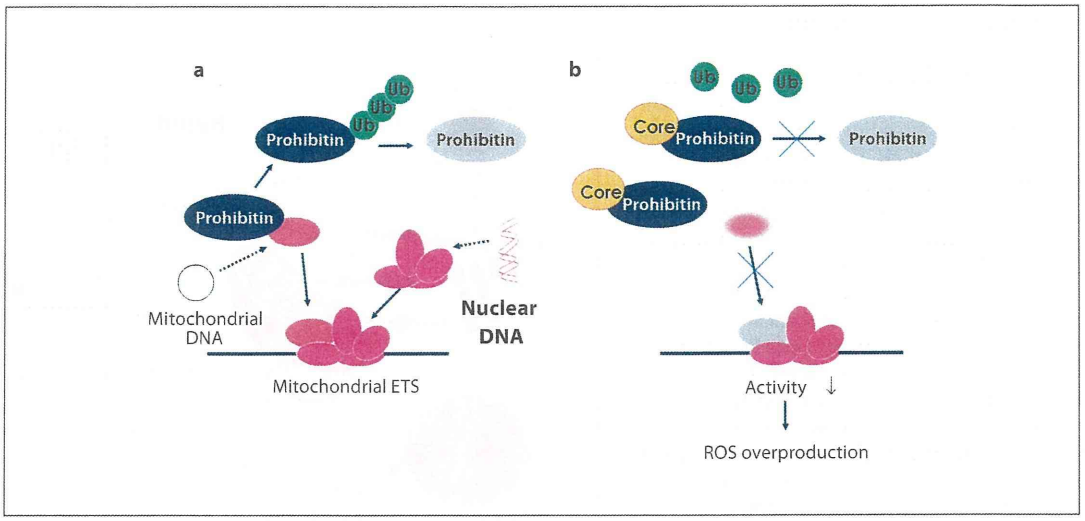

Fig. 1. The HCV core protein binds prohibitin and impairs its chaperone function leading to ROS overproduction. **a** Mitochondrial proteins consist of nuclear DNA-encoded proteins as well as mitochondrial DNA-encoded ones. Prohibitin acts as a protein chaperone for the mitochondrial proteins that are encoded by mitochondrial DNA by stabilizing newly synthesized mitochondrial translation products through direct interaction. **b** The HCV core interacts with prohibitin, disturbing its molecular chaperone function, and leads to a decrease in the activity of ETS complex IV, COX. Subunit II of COX is encoded by the mitochondrial DNA, while other subunits are encoded by the nuclear DNA. This is a new mechanism for oxidative stress overproduction in viral infection in that HCV induces mitochondrial ETS dysfunction by inhibiting chaperone function. Ub = Ubiquitin.

identified several proteins of different expressions when compared with control HepG2 cells. Among upregulated proteins in the core-expressing cells, we focused on prohibitin, which functions as a mitochondrial protein chaperone, and found that the core protein interacts with prohibitin and represses the interaction between prohibitin and subunit proteins of cytochrome c oxidase (COX), which may lead to decreases in the expression level of the proteins and in COX activity.

Prohibitin, a mitochondrial protein chaperone, was identified as an upregulated protein in core-expressing cells. Prohibitin is a ubiquitously expressed and highly conserved protein that was originally determined to play a predominant role in inhibiting cell cycle progression and cellular proliferation by attenuating DNA synthesis [14]. It is present in the nucleus and interacts with transcription factors that are important in cell cycle progression. In core-expressing cells, prohibitin was also detected in the nucleus and its expression level was also higher than that in control Hepswx cells or HepG2 cells. Mitochondrial prohibitin acts as a protein chaperone by stabilizing newly synthesized mitochondrial translation products through direct interaction [15]. We examined the interaction between prohibitin and the mitochondrially encoded subunit II of COX and found a suppressed interaction between these proteins in core-expressing cells. In addition, there are several studies that showed the association of prohibitin with the assembly of mitochondrial respiratory complex I as well as complex IV (COX) [15] (fig. 1). Complex I also consists of both nuclear- and mitochondrial-DNA-encoded subunits; therefore, it is probable that the assembly and function of complex I are impaired by the core protein. In respect to the complex I function, we previously found a decreased complex I activity in core-expressing cells. Other groups have also shown that complex I activity is decreased in cultured cells [16]. Based on these findings, the interaction between prohibitin and the core protein may impair the function of complex I as well as complex IV, leading to an increase in ROS production. In fact, the suppression of prohibitin function has been shown to result in an increased production of ROS [17], a phenomenon observed in the core-expressing cells used in this study as well as in the liver of core-gene transgenic mice [2]. Interestingly, Shelly Lu et al. [18] recently reported that the liver-specific deletion of prohibitin resulted in morphological abnormality and HCC.

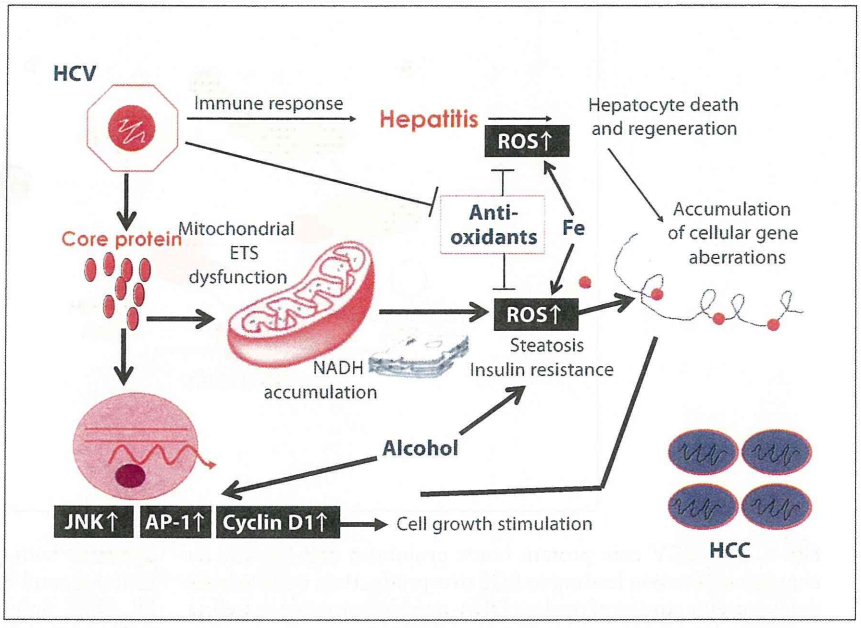

Fig. 2. Molecular pathogenesis of HCC development in HCV infection. Inflammation should contribute to hepatocarcinogenesis by producing genetic aberrations via continual cell death and regeneration. In the case of HCV infection, the virus itself contributes to hepatocarcinogenesis via two pathways. In one pathway, the core protein acts on the function of the mitochondrial ETS, leading to the overproduction of oxidative stress. The core protein also compromises some antioxidants and exacerbates ROS generation. Fe accumulation is an aggravating factor. The presence of steatosis and insulin resistance augments oxidative stress production. The other pathway is the modulation of cellular gene expression and signal transduction including the JNK pathway, which would give a growth advantage to hepatocytes. The combination of these alterations would escalate the development of HCC in HCV infection.

This is a new mechanism for ROS overproduction in viral infection in that HCV induces mitochondrial dysfunction through the inhibition of chaperone function in the mitochondria [19].

HCV Compromises the Antioxidant System

As discussed above, chronic hepatitis C is characterized by its prominent augmentation of oxidative stress. Related to this, iron accumulation in the liver has been shown to aggravate the oxidative stress as shown by the increase in the amount of DNA adducts in the liver [2, 9]. Iron is accumulated in the liver of HCV core gene transgenic mice [20]. The accumulation of iron observed in the liver of the core gene transgenic mice fed with normal chow corroborates the observation in chronic hepatitis C patients [9, 10]. Then, the impact of iron overloading on the oxidant/antioxidant system was examined using this mouse model and cultured cells. Iron overloading caused the induction of ROS as well as antioxidants. However, some of the key antioxidant enzymes, including HO-1 and NADH dehydrogenase quinone 1 (NDQ-1), were not augmented sufficiently by iron overloading, while other antioxidant enzymes such as catalase and GST were augmented more strongly in the iron-overloaded core gene transgenic mice than in the iron-overloaded control or non-iron-overloaded core gene transgenic mice. The attenuation of iron-induced augmentation of HO-1 was also confirmed in HepG2 cells expressing the core protein. HO-1 catalyzes the initial and rate-limiting reaction in heme catabolism and cleaves prooxidant heme to form biliverdin, which is converted into bilirubin in mammals; both of these have been known to have very strong antioxidant activities [21]. In addition, HO-1 has been also suggested to be a central antioxidant in conditions of GSH depletion [22]. Thus, HO-1 is an essential protective endogenous mechanism against oxidative stress, particularly in the case of iron overload. Therefore, it is probable that the attenuation of HO-1 and NQO-1 would hamper the antioxidant system and lead to a robust production of oxidative stress in HCV infection.

Thus, HCV infection not only induces ROS but also hampers antioxidant activation in the liver, thereby exacerbating oxidative stress that would facilitate hepatocarcinogenesis.

Conclusion

Pathways other than oxidative stress provocation in HCV-related hepatocarcinogenesis are alteration of the expression of cellular genes and modulation of intracellular signaling pathways. For example, tumor necrosis factor (TNF)-α and interleukin-1β have been found transcriptionally activated [23]. The mitogen-activated pro-

tein kinase (MAPK) cascade, which is involved in numerous cellular events including cell proliferation, is also activated in the liver of the core gene transgenic mouse model. In the liver prior to HCC development, only the c-Jun N-terminal kinase (JNK) route is activated. Downstream of the JNK activation, transcription factor activating protein (AP)-1 activation is markedly enhanced [23, 24]. Far downstream, both the mRNA and protein levels of cyclin D1 and cyclin-dependent kinase (CDK)4 are increased. Thus, the HCV core protein modulates the intracellular signaling pathways and gives advantage for cell proliferation to hepatocytes. The combination of these pathways that are activated in HCV infection, i.e. ROS overproduction, attenuation of antioxidants, cell growth stimulation via MAPK activation, metabolic disturbances such as hepatic steatosis, and insulin resistance [25], which are all induced by HCV itself, would contribute to hepatocarcinogenesis, together with moderate but long-lasting inflammation in chronic hepatitis C (fig. 2).

The results of our studies on transgenic mice have indicated a carcinogenic potential of the HCV core protein in vivo; thus, HCV would be directly involved in hepatocarcinogenesis. In research studies of carcinogenesis, the development of colorectal cancer is induced by the accumulation of a complete set of cellular gene mutations [26]. Their theory has been extended to the carcinogenesis of other cancers as well, called 'Vogelstein-type' carcinogenesis. On the basis of the results we obtained for the induction of HCC by the HCV core protein, we would like to introduce a different mechanism for hepatocarcinogenesis in HCV infection. We do 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. The overall effect achieved by 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 which occur in HCV carriers. It no longer seem so difficult to determine why HCC develops in persistent HCV infection with an outstandingly high incidence. Our theory may also give an account of the multicentric de novo occurrence characteristics of HCC, which would be the result of persistent HCV infection.

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Disclosure Statement

The authors have nothing to disclose.

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Hepatitis B surface gene 145 mutant as a minor population in hepatitis B virus carriers

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Abstract

Background: Hepatitis B virus (HBV) can have mutations that include the *a* determinant, which causes breakthrough infection. In particular, a single mutation at amino acid 145 of the surface protein (G145) is frequently reported in the failure of prophylactic treatment. The aim of this study was to evaluate the frequency of the *a* determinant mutants, especially the G145 variant, in Japan, where universal vaccination has not been adopted.

Methods: The present study was a retrospective study. The study cohorts were defined as follows: group 1, children with failure to prevent mother-to-child transmission despite immunoprophylaxis (n = 18, male/female = 8/10, age 1-14 years; median 6 years); group 2, HBV carriers who had not received vaccination or hepatitis B immunoglobulin (n = 107, male/female = 107, age 1-52 years; median 16 years). To detect the G145R and G145A mutants in patients, we designed 3 probes for real-time PCR. We also performed direct sequencing and cloning of PCR products.

Results: By mutant-specific real-time PCR, one subject (5.6%) was positive for the G145R mutant in group 1, while the G145 mutant was undetectable in group 2. The *a* determinant mutants were detected in one (5.6%) of the group 1 subjects and 10 (9.3%) of the group 2 subjects using direct sequencing, but direct sequencing did not reveal the G145 mutant as a predominant strain in the two groups. However, the subject who was positive according to the mutant-specific real-time PCR in group 1 had overlapped peaks at nt 587 in the electropherogram. In group 2, 11 patients had overlapped peaks at nt 587 in the electropherogram. Cloning of PCR products allowed detection of the G145R mutant as a minor strain in 7 (group 1: 1 subject, group 2: 6 subjects) of 12 subjects who had overlapped peaks at nt 587 in the electropherogram.

Conclusions: The frequency of the *a* determinant mutants was not high in Japan. However, the G145R mutant was often present as a minor population in children and adults. HBV carriers might have the *a* determinant mutants as a minor form.

Background

Hepatitis B virus (HBV) variants with mutations in the *a* determinant frequently emerge under immunological pressure induced by the HB vaccine or HB immunoglobulin (HBIG)[1-4]. Although the mechanism of the emergence of *a* determinant mutants remains unclear, preexisting mutants as a minor population or as the predominant population could survive, replicate, and cause a breakthrough infection after the host receives the HB vaccine or HBIG. Japan continues to implement an HBV immunization strategy that targets high-risk groups rather than instituting a universal vaccination program. There is no immune pressure induced by universal vaccination, and thus knowing the prevalence of mutants in Japan will be useful for clarifying the mechanism of the emergence of mutants.

Of various mutants with the *a* determinant, the mutant with a single mutation at the 145th amino acid of the hepatitis B surface antigen (HBsAg) has been frequently reported to cause the failure of prophylaxis in mother-to-child transmission [5-10]. Thus, it is indispensable to clarify the frequency of the *a* determinant mutants when developing the future vaccine strategy in Japan. The aim of this study was to evaluate the frequency of the *a* determinant mutants in HBV carriers.

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In particular, we focused on the G145 variant as a minor strain. Mutant-specific real-time polymerase chain reaction (PCR) and direct sequencing were performed. Moreover, cloning of PCR products was used to investigate the presence of the G145variant as a minor strain.

Methods

Patients

The present study was a retrospective study. The study cohorts consisted of the following two groups: group 1, children with failure to prevent mother-to-child transmission despite prophylaxis; group 2, HBV carriers who had not received the HB vaccination or HBIG. A total of 18 children (male/female = 8/10, age: 1-14 years, median 6, genotype A/B/C = 0/0/18) were referred to our institute to failure of prophylactic treatment with the HB vaccine or HBIG in mother-to-child transmission. They belonged to the failure of prophylactic treatment group (group 1). A total of 107 chronically infected patients (male/female = 47/60, age: 1-9 years; n = 19, age 10-19 years; n = 54, age: 20-52 years; n = 34, genotype A/B/C = 1/14/92) were followed in our institute. They had no history of receiving the HB vaccine or HBIG. These subjects belonged to group 2. The patients' characteristics by group are shown in Table 1. Informed written consent for study participation was obtained from all patients or their parents. The study protocol was approved by the ethics committees of Yokohama Eastern Hospital.

HBV DNA extraction and quantification of HBV DNA in serum

HBV DNA was extracted from 200 μL of serum using QIAamp DNA Blood Mini kit (QIAGEN, Hilden, Germany). The real-time PCR was performed for quantification using the genotype-independent real-time PCR method described previously [11]. The PCR assay was performed in a MX3000P (Stratagene), and the results were analyzed with MxPro software (version 3.0). The lower detection limit was > 100 copies/mL. All assays were carried out in duplicate with negative control samples.

Real-time PCR for the G145R and G145A mutants

To detect the G145R and G145A mutants as minor strains in patients, we designed 3 probes based on the sequences of the G145R and G145A mutants [12]. The primers used were 5'-GAT TCC TGC TCA AGG AAC CTC-3' (forward; nt 529-549) and 5'-CGA AAG CCC AGG ATG ATG-3' (reverse; nt 612-629). The point mutation at nt 587 (G145R, mutant 1: G to A, mutant 2: G to C) and nt 588 (G145A, mutant 3: G to C) were applied to the sequence of the probes. The following probes (nt 571-591) were used for mutant-specific real-time PCR: wild type: 5'-FAM-TACAAAACCTTCG-GACGGAAACTGC-TAMRA-3'; mutant 1: 5'-FAM-TACAAAACCTTCGGACAGAAACTGC-TAMRA-3'; mutant 2: 5'-FAM-TACAAAACCTTCGGACC-GAAACTGC-TAMRA-3'; mutant 3: 5'-FAM-TACAAAACCTTCGGACGCAAACTGC-TAMRA-3'. Nucleotide positions were designated on the basis of nucleotide sequences from genotype C (GenBank/EMBL accession number AB300361). PCR was performed in a 50-μL reaction mixture containing 25 μL TaqMan Universal PCR master mix (Applied Biosystems) with 0.2 μM primers, 0.1 μM probes, and 10 μL extracted DNA. The PCR program consisted of an initial pre-cycle incubation at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. All assays were carried out in duplicate with negative control samples.

Sequencing and cloning

All HBV DNA samples were amplified by nested PCR using 2 primer pairs with sequences corresponding to the surface region of the HBV genome, which encompassed *a* determinant region. We used the following primers: outer sense: ACAGAGTCTAGACTCGTGGT (nt 241-260); outer antisense: AAAGCCCTACGAAC-CACTGA (nt 694-713); inner sense: GGACTTCTCT-CAATTTTCTAGGG (nt 261-283); inner antisense:

Table 1 Patient characteristics by group

	Failure of prophylactic treatment for mother-to-child transmission	Chronic HBV infection without HBIG or HB vaccine
	n = 18	n = 107
Gender, male/female	8/10	47/60
Age	1-14 yr. (median, 6)	1-52 yr. (median, 16)
Genotype A/B/C	0/0/18	1/14/92
HBeAg (mother)	16 (15, 3 unknown)	60
HBV DNA levels in blood, log copies/mL	5.6 - > 8.8 (median, 8.6)	2.1 - > 8.8 (median, 4.9)

CAAATGGCACTAGTAAACTGAGC (nt 670-692). Amplification was performed by nested PCR in a 50-μl reaction mixture containing 25 pmol of each primer and 2.5 U of Taq DNA polymerase (TaKaRa Ex Taq, Takara Bio, Shiga, Japan). The first round of amplification was performed for 35 cycles (denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min) with the external primers. In the second round of PCR, 1 μM of the first-round PCR products was submitted to a second round of PCR using the internal primers. The second-round PCR program was the same as that for the first round. PCR products were cloned and sequenced using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). DNA sequences were determined using the Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Results

Sensitivity of mutant-specific real-time PCR

We evaluated the sensitivity and specificity of mutant-specific real-time PCR (Figure 1). HBV DNA from clinical serum samples in which we had confirmed a mutation with G145R (at nt 587 G to A, genotype C, the levels of HBV DNA: 5.7 log copies/mL) was amplified using the mutant-specific real-time PCR in duplicate. The amplification signals were detected for HBV DNA from serum with mutant G145R in the wild probe and the mutant probe. However, cycle threshold (Ct) values in the mutant probe (mutant 1, Ct values = 26.42 and 26.57) were lower than those in the wild probe (Ct values = 27.76 and 27.99), as shown Figure 1A. In addition, the amplification curves in the mutant 1 probe showed a steeper upward slope. Although the mutant primer detected a weak signal of wild-type HBV DNA, these findings suggested that the mutant primer could clearly identify the mutant with a point mutation at nt 587 G to A if the mutant was the predominant strain.

Next, we assessed whether the specific probe could distinguish the G145R mutant as a minor strain from the predominant wild-type strain (Figure 1B-C). Constructed plasmid mutant-type (a point mutation at nt 587 G to A) DNA and wild-type DNA were mixed. The results of real-time PCR using 4 probes for mixed DNA at mutant:wild ratios of 1:1, 1:10, and 1:100 are shown in Figures 1B-C, and 1D, respectively. At the 1:1 ratio, the amplification curve of the mutant 1 probe showed the same Ct values and similar steep slopes (wild-type: Ct values = 24.53 and 24.71; mutant 1: Ct values = 24.86 and 24.27) (Figure 1B). At the 1:10 ratio, however, the amplification curve of the mutant probe showed a less steep upward slope compared to that of the wild-type probe, and the Ct values were greater (wild-type: Ct values = 24.52 and 24.76; mutant 1: Ct values = 26.60 and 27.27; mutant 2: Ct values = 27.61 and 27.89; mutant 3: Ct values = 27.58 and 28.36) (Figure 1C). At the 1:100 ratio, there was no difference in the Ct values or in the shape of the amplification curves among these mutant-specific probes, and it was impossible to identify the mutant with a point mutation at nt 587 G to A (wild-type: Ct values = 24.74 and 24.91; mutant 1: Ct values = 27.65 and 27.75; mutant 2: Ct values = 28.04 and 28.12; mutant 3: Ct values = 26.95 and 27.17) (Figure 1D). These findings suggested that the mutant probe could detect the G145R mutant representing as little as 10% of the wild-type population. Therefore, if the Ct value in a mutant-type probe was the same as or lower than that in the wild-type probe, the sample was considered to be positive for the mutant-specific real-time PCR.

Positive rate of mutant-specific real-time PCR

Chronically infected children despite receiving immunoprophylaxis

The emergence of mutants is a well-known cause of the failure of immunoprophylactic treatment for mother-to-child transmission of HBV. Of the 18 children chronically infected despite receiving immunoprophylaxis, one (5.6%), a 10-year-old girl, was positive for the mutant-specific real-time PCR using the mutant 1 probe (Table 2). This child was also positive for the wild-type probe (Figure 2A).

HBV carriers without HBIG or the HB vaccine

We examined 107 HBV carriers who had never received HBIG or the HB vaccine and found that none of them was positive for the mutant-specific real-time PCR (Table 2). The primers of the mutant-specific real-time PCR did not work in a patient infected with genotype A. Genotype from D to H were not available in this study.

Direct sequencing

To confirm the results of the mutant-specific real-time PCR, we performed direct sequencing. Although the girl with failure of immunoprophylaxis was positive for the mutant 1 probe, the G145R mutant was not detected as a predominant strain in any patients (Table 2). In addition to the G145R mutant, the *a* determinant (aa 124-147) was evaluated using the direct sequencing. As a predominant strain, I/T126S mutant was detected in one (5.6%) of the 18 children with failure of prophylaxis. In addition, I/T126S (n = 4), I/T126V (n = 1), Q129L (n = 1), T131P (n = 2), M133T + T140I (n = 1), and S136Y (n = 1) mutants were detected as a predominant strain in 10 (9.3%) of the 107 HBV carriers who had not received the HB vaccine or HBIG.

Detection of G145R mutant by cloning of PCR products

The mutant-specific real-time PCR (mutant 1 probe) could detect the G145R mutant in a child with failure of

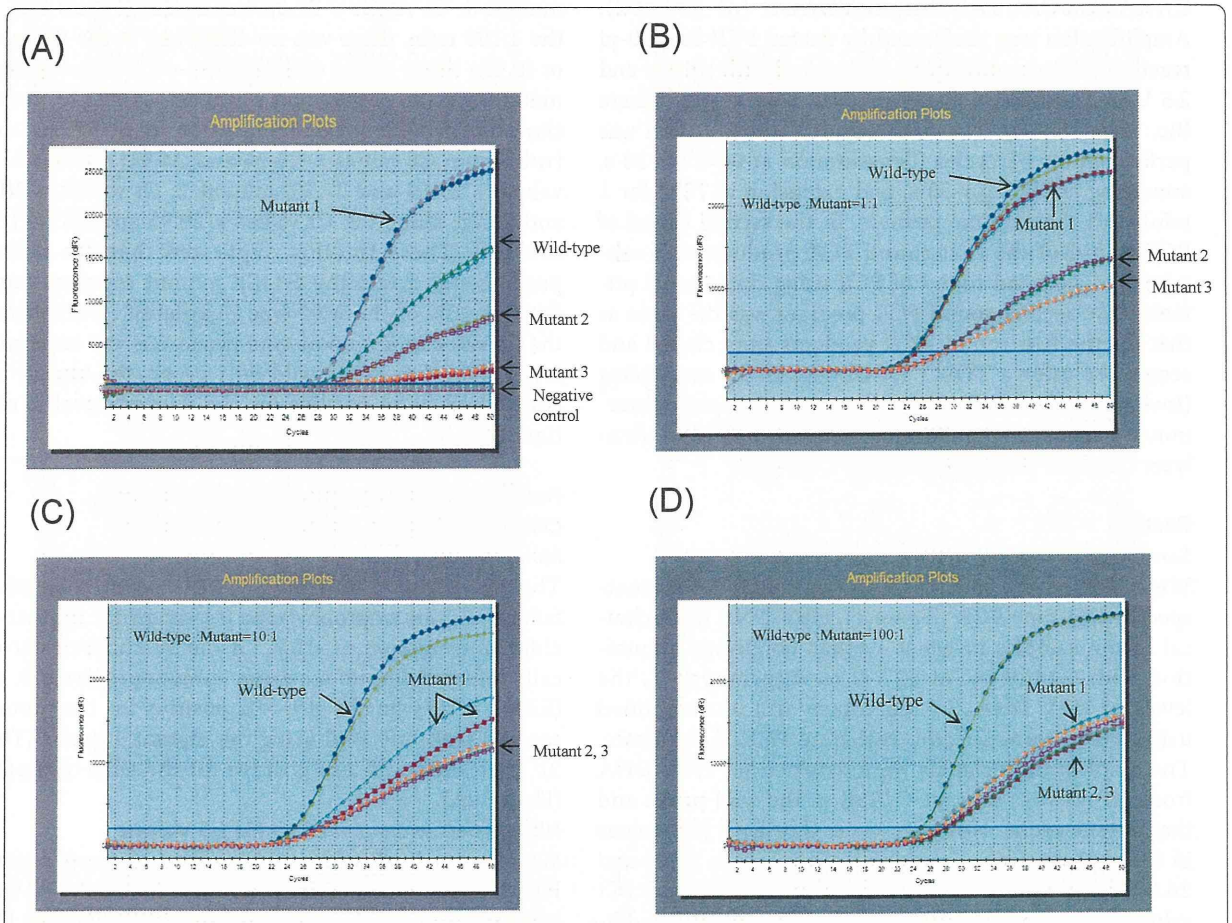

Figure 1 Mutant-specific real-time PCR was performed in duplicate for each probe. (**A**) HBV DNA from serum with an escape mutant (single mutation at nt 587, A to G) was amplified using the wild-type and mutant 1, 2, and 3 probes. (**B**) Constructed plasmid mutant-type (single mutation at nt 587 G to A) DNA and wild-type DNA were mixed. The amount of the mutant-type plasmid DNA was equal to that of the wild-type DNA. The curves of the amplification plots are indicated by arrows. (**C**) Constructed plasmid mutant-type (single mutation at nt 587 G to A) DNA and wild-type DNA were mixed. The mutant:wild ratio in plasmid DNA was 1:10. (**D**) Constructed plasmid mutant-type (single mutation at nt 587 G to A) DNA and wild-type DNA were mixed. The mutant:wild ratio in plasmid DNA was 1:100.

immunoprophylaxis. The amplification curves of this girl indicated that the G145R mutant strain and wild-type strain were coexisting (Figure 2A, upper, the serum sample was taken in May 2007). Consistent with the results of the mutant-specific real-time PCR, her electropherogram indicated that the wild-type HBV was the predominant strain and the G145R mutant was a minor strain. Of the 20 clones, 5 (25.0%) were G145R mutants (Figure 2A, lower) by PCR cloning technique. This finding suggested that the mutant-specific real-time PCR had the ability to detect 25% of minor strains in HBV populations. The girl was positive for HBeAg. Her serum ALT values had been elevated to twice the normal values for 6 years, and the levels of HBV DNA in her blood were more than 7 log copies/ml. She had received antiviral therapy (entecavir, 0.5 mg/day) for 48

Table 2 Frequency of nt 587 G to A (aa 145 Gly to Arg) mutants

	no./total no.	
	Failure of prophylactic treatment for mother-to-child transmission	**Chronic HBV infection without HBIG or HB vaccine**
Mutant specific real time PCR	1/18 (5.6)	0/107
Direct sequencing (major clone)	0/18	0/107

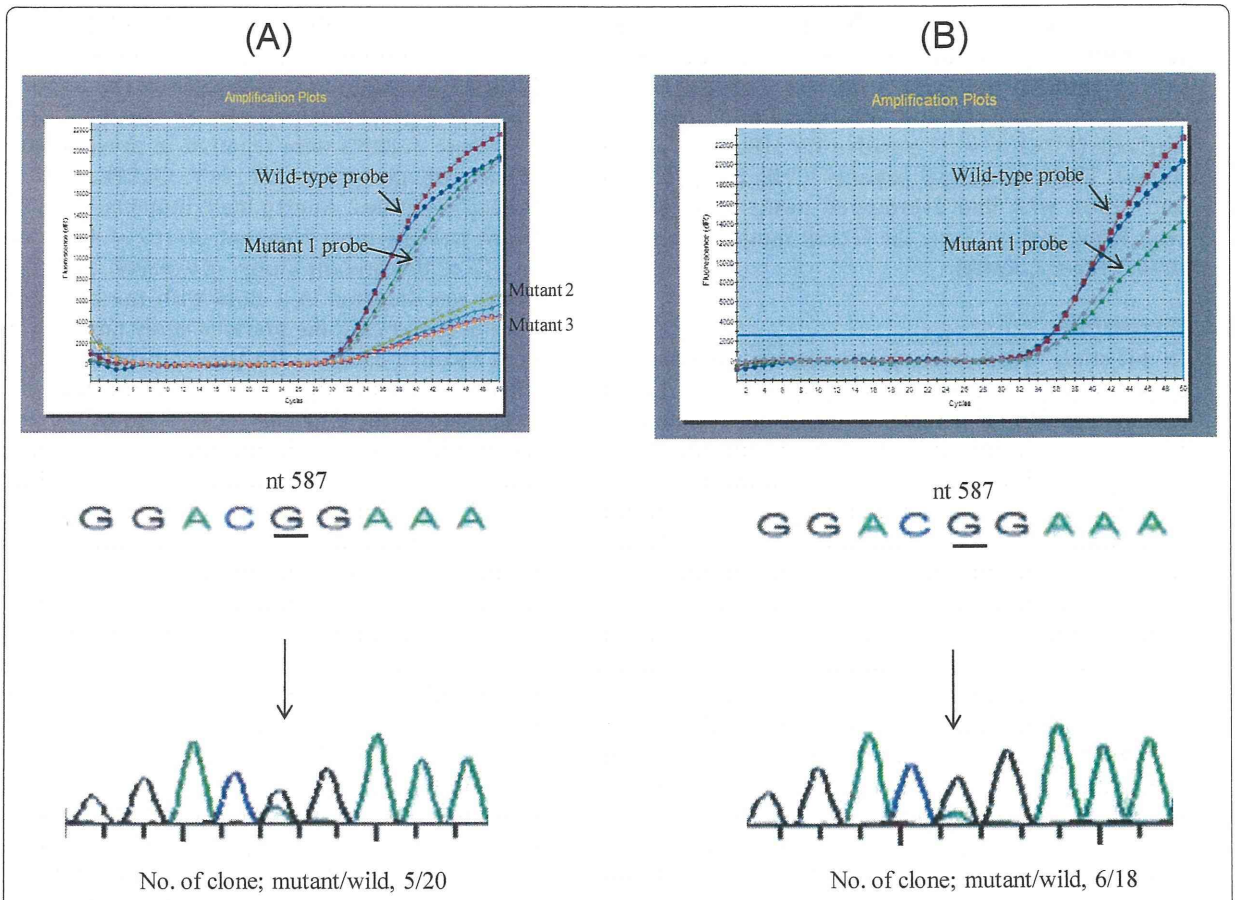

Figure 2 Serum HBV DNA from a girl who became positive for HBsAg and negative for anti-HBs despite immunoprophylaxis was amplified by mutant-specific real-time PCR in duplicate. (**A**) Before antiviral therapy (May 2007), the Ct values of the amplification plots in the mutant 1 probe were the same as those for the wild-type probe. In addition, the amplification plots of both probes were similar in the upward slopes. Although the signals of the mutant 2 and 3 probes were detectable, the curves of the amplification plots were not steep. There was an overlapped peak at nt 587 in the electropherogram. The larger peak was G, and the smaller peak was A. Of the 20 clones, 5 had a mutation at nt 587 from G to A. (**B**) At the cessation of antiviral therapy (May 2009), the girl was negative for serum HBV DNA by real-time PCR. 4 months after the completion of antiviral therapy, however, she became positive again for serum HBV DNA. The signals of the mutant 1 probe as well as the wild-type probe were detected in the amplification plots (the mutant 2 and 3 probes were not used). The curves of the amplification plots were steep. There was an overlapped peak at nt 587 in the electropherogram. Of the 18 clones, six had a mutation at nt 587 from G to A.

weeks (between July 2007 and June 2008) and had become negative for serum HBV DNA by the completion of the therapy. 4 months after the completion of the therapy, however, serum HBV DNA became detectable. The mutant-specific real-time PCR and PCR cloning were performed again (Figure 2B, upper, the serum sample was taken in May 2009), and she was found to be positive for serum HBV DNA. PCR cloning revealed that 6 (33.3%) of 18 clones were G145R mutants (Figure 2B, lower).

The result of the PCR cloning for the G145R mutant in the girl with failure of immunoprophylaxis raised a question. In her sequence electropherogram, a larger peak and a smaller peak overlapped at nt 587 of HBsAg (Figure 2). The larger peak and the smaller peak were considered to represent the predominant strain and the minor strain, respectively. This finding suggested that the electropherogram could be useful for the detection of minor populations, so we investigated whether cloning of PCR products was more sensitive than mutant-specific real-time PCR for the detection of minor populations. Of all the patients in groups 1 and 2, 12 (group 1: F-16, group 2: CHB-2, -5, -26, -30, -42, -62, -67, -73, -94, -100, and -104) had overlapped peaks at nt 587 in the electropherogram. We thus performed cloning of PCR products in all of these patients except for F-16. Ten or more clones were sequenced. Of the 11 patients, 6 (CHB-26, -62, -67, -73, -94, and -104) had the G145R

mutant as the minor strain (Table 3). All 6 of these subjects were HBV carriers with no history of HBIG or the HB vaccine. Of the 7 HBV carriers with the G145R mutant, including the girl with failure of immunoprophylaxis (F-16), 6 were positive for HBeAg (Table 3, upper). The remaining 5 patients (CHB-2, -5, -30, -42, and -100), who had overlapped peaks at nt 587 by sequencing, did not have the G145R mutant as a minor strain (Table 3, lower).

Discussion

In this study, the *a* determinant mutants were detected as predominant strains by direct sequencing in 9.3% of chronic hepatitis B patients who had not received the HB vaccine or HBIG.. This figure was comparable with that reported in a previous study (7.8%), which was conducted in Taiwan before the introduction of universal vaccination [13,14]. This finding indicates that the presence of the *a* determinant mutants was not common in Japanese children. However, previous studies showed a higher prevalence rate of the *a* determinant mutants in Japan. Ogura et al. reported that the *a* determinant mutants were detected in 24% (10/42) of unselected Japanese HBV carriers [15]. In addition, Takahashi et al. reported that 48% (19/40) of HBs-positive hepatocelluar carcinoma patients had the *a* determinant mutants in Japan [16]. Apart from Japan, Avellon et al. reported that the *a* determinant mutants were detected in 39% (106/272) of unselected carriers in Spain [17]. that HBV carriers. Presumably, the age (duration of infection), the degree of chronic hepatitis, and immunological selection by vaccine might have influenced the emergence of mutants. For instance, after the introduction of universal vaccination in Taiwan, the prevalence of the *a* determinant mutants was slightly increased and remained approximately 20% between 1989 and 2004 [13,14].

Direct sequencing showed that I/T126S [9,18,19], I/T126V [16], Q129L [20,21], T131P [22], M133T + T140I [13,14,21,23], and S136Y [24,25] mutants were present as a predominant strain in 107 HBV carriers. All of these mutants were reported in previous studies, but the pathogenicity of I/T126V, Q129L, T131P has not been confirmed. Among these mutants, the I/T126S mutant was the most frequent (4/107 = 4.7%) in the group 2 of the present study. Moreover, the I/T126S mutant was detected in a child with the prophylactic failure in group 1. In the previous study targeting unselected Spanish carriers, the prevalence rate of the I/T126S mutant was 0.4% (1/272)[17]. Compared with the previous study, the frequency of I/T126S of the present study is high. The I/T126S mutant has been frequently observed in the studies from Japan, in 12% (5/42) [15] and 13% (5/40)[16] of adult HBV carriers. In contrast, a mutation at the 127th amino acid of the hepatitis B surface antigen (genotype A:5.9%, genotype D: 8.8%) was the most frequently reported mutation in a Spanish study [17]. Genotypes A and E are predominant in Europe, whereas genotypes B and C prevail in Southeast Asia, including Japan. There is a possibility that the difference among HBV genotype could be associated with the frequency of the I/T126S mutant.

Before the present experiment was performed, we predicted that the mutant-specific real-time PCR technique would be able to more frequently detect the G145 mutant compared with sequencing and cloning. Using the constructed plasmid, the mutant-specific probe could detect 10% of mutants among wild-type virus in this study. Similarly, Zhang et al. reported that the real-time PCR method could detect 5% of mutants among wild-type virus [12]. The present study showed that the mutant-specific real-time PCR identified a child in whom the G145R mutant and wild-type virus were

Table 3 Results of PCR cloning in patients who had overlapped peaks (G and A) at nt 587 in the electropherogram

Patint ID.	age (yr.)	HBsAg	HBeAg	HBV DNA in blood, log copies/mL	no./total no.	
					Wild-type	Mutant-type (%)
F-16	10	positive	positive	5.2	15/20	5/20 (25)
CHB-26	12	positive	positive	6.9	12/14	2/14 (14)
CHB-62	15	positive	negative	2.3	13/15	2/15 (13)
CHB-67	13	positive	positive	4.1	24/25	1/25 (4)
CHB-73	43	positive	positive	5.2	14/16	2/16 (13)
CHB-94	10	positive	positive	7.4	14/15	1/15 (7)
CHB-104	40	positive	positive	> 8.8	15/16	1/16 (6)
CHB-2	7	positive	negative	2.1	16/16	
CHB-5	14	positive	positive	4.9	30/30	
CHB-30	43	positive	positive	5.3	14/14	
CHB-42	24	positive	positive	8.7	28/28	
CHB-100	42	positive	positive	6.3	30/30	

mixed. In this case, the number of the G145R mutant clones was 5 (25%) out of 20 clones. These findings proved that the mutant-specific probe could detect a minor mutant-type virus among viral populations in a clinical sample. However, we found that sequencing and cloning were more sensitive than the mutant-specific real-time PCR, although performance of these techniques required more time. Sequencing and cloning could detect much smaller populations of mutant-type virus, ranging from 6% to 14% of subpopulations in this study. If cloning of PCR products is carefully performed in every nucleotide of the *a* determinant, the prevalence of finding the *a* determinant mutant will be increased. Therefore, the *a* determinant mutant is present as a minor population in a high proportion of HBV carriers. This notion is consistent with the fact that selection immunological pressure such as the HB vaccine and HBIG allows the minor *a* determent mutant to become the predominant strain of the virus.

The virulence of the G145R mutant is indeterminate. The results of previous studies suggested that the G145R mutation reduced the ability of viral assembly and secretion [26,27]. In this study, however, the levels of serum transaminases had been elevated and the levels of HBV DNA had remained high for several years in the girl infected with the G145R mutant, which was detected by the mutant-specific real-time PCR. The levels of HBV DNA in the blood were not significantly lower in patients with the G145R mutant than in patients without the G145R mutant (data not shown). Although the G145R mutant was mixed with the predominant wild-type virus and viral replication was influenced by pre-core and basal core promoter mutation, these findings suggest that infection with the G145R mutant does not always promise a good prognosis. After treatment with entecavir, the G145R mutant as well as the wild-type virus appeared again in the girl.

Of the 18 the children with prophylactic failure in the present study, one had an *a* determinant mutant (I/T126S) as a predominant strain and one had an *a* determinant mutant (G145R) as a minor strain. This finding was consistent with previous studies [5,28,29]. The detection rate of *a* determinant mutation varied from 12% to 26% in children with prophylactic failure, indicating that the main cause of failure to prevent mother-to-child transmission was not the emergence of *a* determinant mutants. Recent studies suggest that high viral load in blood is closely related to the failure of prophylaxis with vaccine and HBIG [30-32].

Conclusions

The *a* determinant mutants were not the predominant mutations in Japan. However, the G145R mutant was present as a minor population in children and adults. Numerous *a* determinant mutants could be lost in the mass of the wild-type virus.

Abbreviations

HBV: Hepatitis B virus; HB vaccine: Hepatitis B vaccine; HBIG: Hepatitis B immunoglobulin; HBsAg: Hepatitis B surface antigen; HBeAg: Hepatitis B e antigen; PCR: Polymerase chain reaction; Anti-HBs: Antibodies against HBsAg

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Authors' contributions

HK contributed to the design of this study and drafted this manuscript. AI, TS, YK, AT, and TF participated in data collection and critical revision of the manuscript. All the authors concurred with the submission and will take responsibility for the manuscript.

Competing interests

The authors declare that they have no competing interests.

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MAJOR ARTICLE

Tears From Children With Chronic Hepatitis B Virus (HBV) Infection Are Infectious Vehicles of HBV Transmission: Experimental Transmission of HBV by Tears, Using Mice With Chimeric Human Livers

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Background. Body fluids such as saliva, urine, sweat, and tears from hepatitis B virus (HBV) carriers are potential sources of HBV transmission.

Methods. Thirty-nine children and 8 adults who were chronically infected with HBV were enrolled. Real-time polymerase chain reaction was used for the quantification of HBV DNA.

Results. HBV DNA was detected in 73.7% of urine samples (14 of 19), 86.8% of saliva samples (33 of 38), 100% of tear samples (11 of 11), and 100% of sweat samples (9 of 9). Mean HBV DNA levels (±SD) in urine, saliva, tears, and sweat were 4.3 ± 1.1 log copies/mL, 5.9 ± 1.2 log copies/mL, 6.2 ± 0.7 log copies/mL, and 5.2 ± 0.6 log copies/mL, respectively. A statistically significant correlation was observed between the HBV DNA level in serum specimens and HBV DNA levels in saliva and tear specimens ($r = 0.88$; $P < .001$). Tear specimens from a child were injected intravenously into 2 human hepatocyte-transplanted chimeric mice. One week after inoculation, both chimeric mice had serum positive for HBV DNA.

Conclusions. The levels of HBV DNA in tear specimens from young children were high. Tears were confirmed to be infectious, using chimeric mice. Strict precautions should be taken against direct contact with body fluids from HBV carriers with high-level viremia.

Hepatitis B virus (HBV) infection causes acute and chronic liver diseases. Fortunately, HBV infection is a vaccine-preventable disease, and as of 2008, 177 countries (92%) have integrated HBV vaccine into routine infant immunization programs. However, Japan and northern European countries, where the endemicity of HBV is low, continue to implement an HBV immunization strategy that targets high-risk groups, rather than a universal vaccination program [1]. Nonetheless, HBV infection by sexual contact and household contact does occur in Japan [2–5]. Children with chronic HBV infection are usually asymptomatic and have high-level viremia. Therefore, it is believed that children with chronic HBV infection may be a major reservoir for spreading HBV to other close susceptible individuals [6–8]. This scenario would especially threaten the countries that adopt an "at-risk" immunization strategy [6, 9–13].

Body fluids such as saliva, semen, urine, sweat, and tears are also potential sources of HBV transmission. Several studies have reported that HBV DNA in these body fluids can be detected by polymerase chain reaction (PCR) [9–18]. Of these body fluids, however, only serum, saliva, and semen have been demonstrated to be infectious in humans or experimental animal models [19–21].

In this study, HBV DNA levels in urine, saliva, tears, and sweat were quantified by real-time PCR.

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Body fluid samples were collected from HBV-carrier children and HBV-carrier mothers. After quantification of HBV DNA levels for each specimen type, we evaluated the infectivity of tears from HBV carriers. Mice with severe combined immunodeficiency, carrying a urokinase-type plasminogen activator transgene controlled by an albumin promoter (uPA/SCID), and with transplanted human hepatocytes have recently been used as an appropriate animal model for studying viral hepatitis due to HBV and hepatitis C virus [22–24]. Using these mice, we evaluated whether tears from HBV-carrier children were infectious.

MATERIALS AND METHODS

Patients and Materials

Eligible patients were chronic HBV carriers who attended our outpatient clinic. Their chronic HBV infection status was routinely evaluated by blood examination. All of the patients were asymptomatic. Serum, urine, saliva, tears, and sweat samples were collected when possible from each patient.

Serum samples were collected in preparation tubes. Each urine sample was collected in a sterile plastic tube. Saliva, tear, and sweat samples were collected using an indicating FTA Micro Card (Whatman, GE Healthcare, Tokyo, Japan) and sterile foam-tipped applicators (Whatman). When children shed tears spontaneously, we collected tear samples using the FTA cards. Serum, urine, saliva, tear, and sweat specimens were collected on the same day. Informed consent was obtained from all patients or all patients' parents. This study was approved by the Research Ethics Committee of Eastern Yokohama Hospital.

HBV DNA Extraction and Real-Time PCR

HBV DNA in serum was measured by COBAS TaqMan HBV DNA test, version 2.0 (Roche Diagnostics, Tokyo, Japan). HBV DNA was extracted from 200 μL of urine, using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). HBV DNA was extracted from saliva, tear, and sweat specimens that were spotted on FTA cards, using QIAamp DNA Mini kit (QIAGEN). Three circles were punched from the FTA card by use of a single-hole paper puncher (Harris Micro Punch 3.00 mm, GE Healthcare) and were used for HBV DNA extraction. The extracted DNA was dissolved in 100 μL of elution buffer.

Quantification of HBV DNA in urine, saliva, tear, and sweat samples was performed using an in-house TaqMan real-time assay. The real-time PCR was performed using a genotype-independent method described previously [25]. PCR was performed in an MX3000P (Stratagene), and the results were analyzed with MxPro software (version 3.0). The lower limit of detection was >100 copies/mL. All assays were performed in duplicate with negative control samples. This assay was standardized using HBV DNA samples of known concentrations measured by the COBAS TaqMan HBV DNA test and recombinant plasmid controls. In this study, the standard of qualification is based on the result of COBAS TaqMan HBV DNA test. Therefore, the conversion factor between HBV copies/mL and HBV IU/mL is considered to be 5.82 copies/IU. Genotyping of HBV was determined by the PCR-Invader assay [26].

Tear Specimen for Experimental Transmission

For experimental transmission, a tear specimen was collected from a 10-month-old girl with chronic HBV infection. The source of her HBV infection was mother-to-child transmission due to the failure of prophylactic treatment. A total of 200 μL of tears were gently collected from her face when she cried, using a 1.0-mL syringe. The 200-μL tear specimen was diluted with 1300 μL of sterile saline, yielding a total volume of 1500 μL. The specimen underwent filter sterilization with a 0.2-μm filter.

Inoculation of Chimeric Mice With Livers Repopulated by Human Hepatocytes

Three male chimeric mice were purchased from PhoenixBio (Hiroshima, Japan). Human hepatocytes were imported from BD Bioscience (Woburn, MA). Of the 3 mice, 2 (mouse 101 and mouse 102) were inoculated once intravenously with 100 μL of the sterilized tear sample. The remaining mouse (mouse 103) was orally inoculated with 100 μL of the sterilized tear sample every 4 weeks. After inoculation, blood samples for real-time PCR assay were collected from the chimeric mouse every week.

HBV DNA Extraction From Mice Samples and Real-Time PCR

A total of 50 μL of whole blood samples were collected from the mice every week after inoculation, and serum was separated. Saliva and tear specimens were collected from chimeric mice, using FTA cards. HBV DNA was extracted from 20 μL of mouse serum, using SMI-TEST EX-R&D (Medical Biological Laboratories, Aichi, Japan). The extracted DNA was dissolved in 20 μL of nuclease-free water. HBV DNA was quantitatively measured using real-time PCR with the TaqMan PCR Core Reagent kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed in a 25-μL reaction mixture containing 0.125 μL Ampli Taq Gold with 0.2 μM primers (forward primer: 5′-CACATCAGGATTCCTAGGAC C-3′ [nucleotides 166-186]; reverse primer: 5′-AGGTTGGTG AGTGATTGGAG-3′ [nucleotides 325-344]), 0.3 μM probe (5′-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-TAMRA-3′ [nucleotides 242-267]), and 5 μL extracted DNA. The nucleotide position was based on GenBank accession number AB300361 (genotype C). After incubation for 2 min at 50°C and for 10 min at 95°C, the PCR cycling program underwent 53 2-step cycles, one at 95°C for 20 seconds and the other at 60°C for 1 minute. TaqMan PCR was performed with an ABI

Prism 7500 (Applied Biosystems). In this study, the volume of serum collected from each mouse was 20 μL, which is a very small amount compared with that used in human studies. Therefore, we considered the upper limit of detection of real-time PCR for a small-volume sample to be >10 000 copies/mL, which provided us with more reliable results. This assay was standardized using mouse HBV DNA samples of known concentrations and the recombinant plasmid controls, as previously described [27].

Immunostaining for HBV Surface Antigen (HBsAg) and HBV Core Antigen (HBcAg)

Immunostaining for HBsAg and HBcAg was performed on frozen sections, using the Ventana i VIEW DAB detection kit (Ventana Medical Systems, Tucson, AZ) and the Dako Envision kit (Dako, Tokyo, Japan), respectively. Primary monoclonal antibodies to HBsAg (Santa Cruz Biotechnology, CA), at a 1:100 dilution, and polyclonal antibodies to HBcAg (Dako), at a 1:500 dilution, were used. Liver tissue was taken from mice after they were euthanized, and the tissue was stored at −80°C.

Statistical Analysis

Categorical variables were compared between groups, using the Yates corrected χ^2 test or the Fisher exact test. Noncategorical variables were compared between groups by the Mann–Whitney *U* test. For analysis of the correlation between log HBV DNA level in serum and in saliva and tears, we used the Pearson correlation coefficient. All tests were 2-sided, and a *P* value of ≤.05 was considered to indicate statistical significance. All statistical analyses were performed with StatMate IV for Windows (Advanced Technology for Medicine & Science, Tokyo, Japan) and Microsoft Office Excel 2007.

RESULTS

Patients and Materials

Between August 2009 and September 2010, 39 children and 8 adults who were chronically infected with HBV were randomly enrolled in this study. Twenty-six subjects were male, and 21 were female; the mean age (±SD) was 12.4 ± 12.0 years, and the median age was 9 years (range, 0–47 years. The 47 HBV carriers fell into the following age groups: 0–5 years, n = 18 (16 were HBV e antigen [HBVeAg] positive); 6–10 years, n = 11 (9 were HBeAg positive); 11–19 years, n = 9 (7 were HBeAg positive); and 20–27 years: n = 9 (7 were HBeAg positive). Of the 47 patients with chronic HBV infection, 39 were positive for HBeAg. In addition, 39 patients had serum HBV DNA levels of ≥6 log copies/mL. One, 6, and 40 patients were infected with genotype A, genotype B, and genotype C, respectively. Serum samples were collected from all patients. From the 47 patients, we collected 19 urine samples, 38 saliva samples, 11 tear samples, and 9 sweat samples. One subject provided urine, saliva, and tears only; 3 provided urine, saliva, and sweat only; 10 provided urine and saliva only; 10 provided saliva and tears only; 1 provided urine and sweat only; 1 provided saliva and sweat only; 4 provided urine only; 13 provided saliva only; and 4 provided sweat only. Samples were collected individually at the same time. The characteristics of body fluid samples are shown in Table 1. There were no significant differences in sex, the number of patients with a serum HBV DNA level of >6 log copies/mL, and the prevalence of genotype C among patients supplying different types of samples. However, there was a significant difference in the age of patients supplying the different kinds of samples.

HBV DNA Detection in Body Fluids

All patients were positive for HBV DNA in serum by the COBAS TaqMan HBV DNA test. The levels of serum HBV DNA ranged from 2.1 log copies/mL to >9 log copies/mL. The median HBV DNA level in serum was >9 log copies/mL. HBV DNA was detected in 73.7% of urine specimens (14 of 19), 86.8% of saliva specimens (33 of 38), 100% of tear specimens (11 of 11), and 100% of sweat specimens (9 of 9) (*P* = .07). In patients with a high viral load (ie, >6 log copies/mL), HBV DNA was detected in 85.7% of urine samples (12 of 14), 100% of saliva samples (32 of 32), 100% of tear samples (11 of 11), and 100% of sweat samples (9 of 9) (*P* = .24). Although the frequency of HBV DNA detection in urine was slightly lower than that in other body fluids, there were no significant differences in the frequency of HBV DNA detection among body fluids.

Quantification of HBV DNA From Body Fluids

Figure 1 shows the levels of HBV DNA in body fluids. Mean levels (±SD) of HBV DNA in urine, saliva, tears, and sweat specimens were 4.3 ± 1.1 log copies/mL, 5.9 ± 1.2 log copies/mL, 6.2 ± 0.7 log copies/mL, and 5.2 ± 0.6 log copies/mL,

Table 1. Characteristics of Body Fluid Samples

Characteristic	Body Fluid				*P*
	Urine (n = 19)	Saliva (n = 38)	Tears (n = 11)	Sweat (n = 9)	
Male sex, no. (%)	10 (52.6)	23 (60.5)	8 (72.7)	4 (44.4)	.29
Age, years, median (range)	11 (1–40)	7 (1–38)	1 (0–3)	16 (8–40)	<.05[a]
HBV DNA in serum, no. (%)					
>6 log copies/mL	14 (73.7)	32 (84.2)	11 (100)	9 (100)	.13
Genotype C	14 (73.7)	33 (86.8)	9 (81.8)	9 (100)	.31

[a] Significant difference between urine and saliva, between urine and tears, between saliva and sweat, and between tears and sweat.

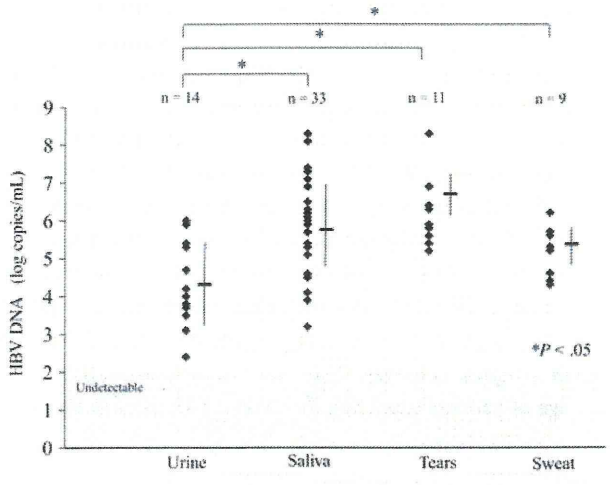

Figure 1. Hepatitis B virus (HBV) DNA levels in urine, saliva, tear, and sweat specimens from 47 patients. The levels of HBV DNA in urine samples were significantly lower than those in saliva, tear, and sweat samples ($P < .05$). The bar indicates the mean of the levels of HBV DNA. SDs are indicated by vertical bars.

respectively. Levels of HBV DNA in urine were significantly lower than those in other body fluids. Levels of HBV DNA in body fluids from patients who had a high viral load (ie, >9 log copies/mL) in serum are shown in Figure 2. Mean levels (±SD) of HBV DNA in urine (n = 10 specimens), saliva (n = 23), tears (n = 8), and sweat (n = 8) were 4.4 ± 0.9 log copies/mL, 6.4 ± 0.9 log copies/mL, 6.4 ± 0.9 log copies/mL, and 5.3 ± 0.6 log copies/mL, respectively. Even after the HBV load in serum was well matched, the HBV DNA levels in urine specimens were significantly lower than those in saliva and tear specimens.

Although there was no significant difference in HBV DNA levels between saliva, tears, and sweat specimens from patients with high viral load in serum, the quantification of HBV DNA in saliva and tear specimens showed almost the same levels (Figure 2). Levels of HBV DNA in the 11 pairs of saliva and tear specimens are shown in Figure 3. Mean HBV DNA levels (±SD) in saliva and tear specimens were 6.1 ± 1.0 log copies/mL and 6.2 ± 0.8 log copies/mL, respectively. The levels of HBV DNA in tear specimens were as high as those in saliva specimens.

The association between the levels of HBV DNA in serum specimens and in saliva and tear specimens was evaluated. Because the upper detection limit of the COBAS TaqMan HBV DNA test was >9 log copies/mL, we used data from patients in whom the levels of HBV DNA in serum ranged from 2.9 to 8.8 log copies/mL. Data from 15 patients (15 serum samples, 15 saliva samples, and 3 tears samples) were available for the correlation analysis. A significant correlation was observed in the levels of HBV DNA between serum specimens and saliva and tear specimens ($r = 0.88$; $P < .001$) (Figure 4*A*). The relationship between HBV DNA in serum specimens and HBV DNA in saliva and tear specimens was described as follows: [log HBV DNA load in saliva and tear specimens] = −3.23 + 1.06 × [log HBV DNA load in serum specimens]. On the other hand, there was no significant

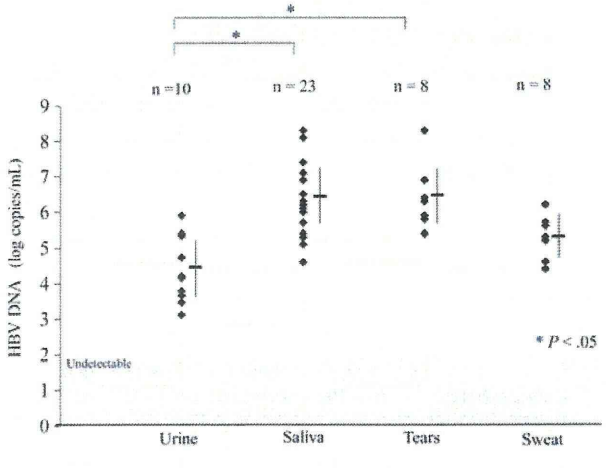

Figure 2. To adjust serum hepatitis B virus (HBV) DNA levels among groups, we show the HBV DNA levels in urine, saliva, tear, and sweat samples from patients whose levels of HBV DNA in serum were ≥9 log copies/mL. Although a significant difference in HBV DNA levels between urine and sweat specimens was not present, HBV DNA levels in urine specimens were significantly lower than those in saliva and tear specimens ($P < .05$). The bar indicates the mean of the levels of HBV DNA. SDs are indicated by vertical bars.

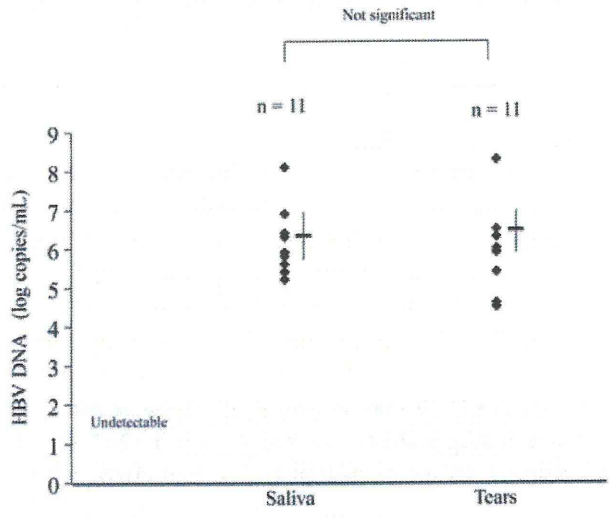

Figure 3. Hepatitis B virus (HBV) DNA levels in saliva and tear samples that were paired. Both groups showed the same HBV DNA levels. The bar indicates the mean of the levels of HBV DNA. SDs are indicated by vertical bars.