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PRECORE REGION MUTATION IN HEPATITIS B VIRUS GENOME IN "HEALTHY" ASYMPTOMATIC ANTI-HBE POSITIVE CARRIERS

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The precore region mutation of hepatitis B virus (HBV) genome characterized from G to A at nucleotide 83 resulting a stop codon in codon 28 has been identified in severe chronic or fulminant hepatitis suggesting that the mutant might be more pathogenic. Since little is known about the mutant in asymptomatic carriers, we investigated the mutant HBV in 19 "healthy" asymptomatic anti-HBe positive carriers by direct sequencing of viral DNA amplified by polymerase chain reaction from serum. Among 18 cases in whom viral DNA could be sequence, wild type HBV was found in only one case. Seventeen cases including three with co-infection of wild type showed a point mutation resulting in a stop codon from TGG to TAG in codon 28 of precore region. This mutation is identical as that found in severe liver disease by other investigators. HBeAg positive patients used as positive control showed only wild type HBV. In conclusion, the precore mutation was found also in the "healthy" asymptomatic carriers. Although this mutation was tightly associated with the lack of HBeAg in serum, it dose not seem to have pathogenic potential compared with wild type HBV.

Recent observations that some hepatitis B (HBV) carrier have HBV DNA detected by dot-blot hybridization in their serum but negative for HBeAg led to the discovery of a variant virus that is unable to synthesize precore/core protein from which HBeAg is derived (1,2). This variant form of HBV has a mutation from guanosine (G) to adenosine (A) at nucleotide (nt) 83 in 28th codon in precore region of the genome, resulting a stop codon (TGG to TAG) which abolishes the production of HBeAg. After discovery, this HBV mutant has been reported to be associated with fulminant hepatitis (3-5) and severe chronic hepatitis (1,2,6). Although infection with HBV can lead to a variety of outcome, majority of persons (60 to 70%) infected with HBV do not manifest an overt illness (7,8). Rather, they have a subclinical infection producing antibody and seemingly permanent immunity. Therefore, association of this HBV variant with severe liver disease may suggest that not only host but also viral factors influence the development of the disease. HBV mutant might have more pathogenic potential.

Since the majority of HBV carrier are anti-HBe positive asymptomatic carriers without liver disease, it would be important to investigate this mutation in the group so called "healthy" aymptomatic HBV carriers (6,9) to investigate the pathogenic role of precore mutant. However, most studies have concentrated mainly on identifying mutant sequence in sera of patients with either fulminant acute hepatitis or chronic active hepatitis (1-6). Although some reports have dealt patients with anti-HBe, they did not refer to the liver histology which seems most important to know the stage of liver disease. In the present study, we could investigate the precore mutation in patients whose liver histology was normal (healthy carrier). In addition, a long term follow-up was possible in these patients to deny the episode of elevation of serum alanine aminotransferase level. By investigating the precore mutation in these patients, we intended to clarify whether this precore mutation is also associated with asymptomatic carrier state or additional factors may influence the hepatitis activity rather than precore mutation itself.

PATIENTS AND METHODS

Patient

Serum samples were taken from 19 HBeAg negative but anti-HBe positive "healthy" asymptomatic carriers (12 male and 7 female, mean age 47 yr, range 23 to 66 yr). All patients were followed-upon regular basis of clinical, biochemical and viral markers in duration of mean time of 6 years (range 3 to 9 yr). Liver biopsy was performed in all cases for histological evaluation before the study. Liver histology showed non specific reaction in 18 cases and minimal fibrosis in one case. In parallel, sera from 5 HBeAg positive patients were also taken and analyzed as positive controls.

Viral markers

HBsAg, HBeAg and antibodies to HBs, HBe, HBc were determined with commercially available RIA kits (Abbott Laboratories North Chicago, IL). Anti-HCVAb was tested by enzyme immunoassay (Ortho Diagnostic Tokyo).

DNA analysis

HBV DNA was determined and quantified by dot-blot hybridization with a probe of cloned HBV DNA labeled with [α^{32}P]dCTP (Amersham Japan, Tokyo) by the multiprime labeling system in comparison with known standards(10).

DNA amplification

HBV DNA was extracted from 0.5 ml HBeAg-positive sera or 1.0 ml anti-HBe sera by using proteinase K (133 μg/ml) digestion according to the method described by Okamoto *et al.* (11). Enzymatic

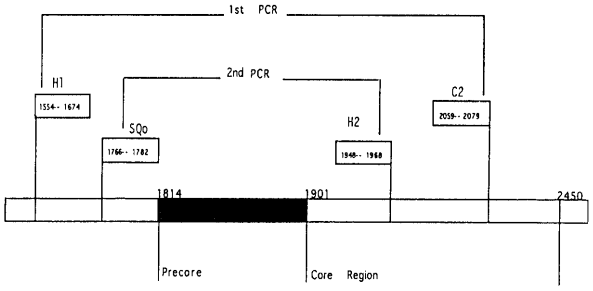


Figure 1 : The locations of the primers for PCR of precore region. H1 and C2 primer set for the first PCR, SQ_0 and H2 for the second PCR and also used for sequencing. Map position of precore was derived the report by Ono *et al*. (13).

amplification of the precore/core region was performed by 2 stage-polymerase chain reaction (PCR). The first-stage PCR was performed using the outer primers comprising H1 (5'-CATAAGAGGACTCTTGGACT-3'; nt 1654 to 1674 of plus-strand HBV DNA) and C2 (5'-AGTGCCTACTTCTTCTTCTGTAT-3';nt 2059 to 2079) capable of multiplying 427 base pair by 35 cycles under the condition;95° C 1 min, 55° C 1 min, 72° C 2 min in the presence of 2.5 unit of Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) (12). One ml of the first-stage PCR product was subjected for second-stage PCR of 30 cycles with inner primers comprising SQ0 (5'-CTTTGTACTAGGC-3';nt 1766 to 1768) and H2 (5'-CCTGACTCCTTACCCCCCTC-3';nt 1948 to 1968) covering precore region nt 1814 to 1900, depicted in Figure 1, numbered by Ono *et al*. (13). As negative controls, one serum from a HBV DNA negative vaccinated staff member and a substitution of water were used as template for PCR. All the proper precautions were taken to minimize the contamination (14).

Direct sequence of the PCR products

The amplified DNA was purified using Gene Clean II kit (Bio 101, La Jolla, CA). Sequencing was performed according to dideoxy-chain termination method (15), using Sequenase version 2.0 (USB, Cleveland, OH). The primers SQ0 and H2 were also used as sequencing primer. In addition, amplified DNA was sub-cloned from patients who showed the mixture of wild type and mutant type virus to test the possibility of coinfection by blunt-end insertion into the Sma I site of pUC19 DNA and sequenced.

RESULTS

Serological markers

The serological data, the results of HBV DNA detection and sequencing results are summarized in Table.1. Anti-HBeAb and anti-HBcAb were positive in all patients. Along with the positive HBsAg profile, four "healthy" carriers developed anti-HBsAb. Anti-HCVAb was negative in all cases.

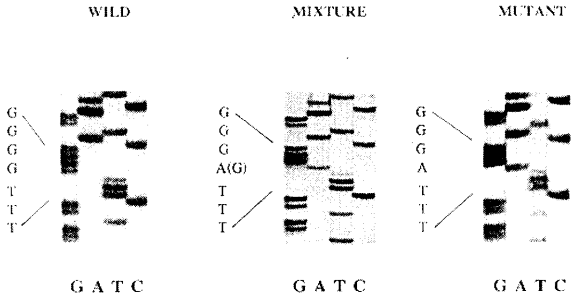
HBV DNA analysis

The viral DNA was negative by dot-blot hybridization in all 19 asymptomatic cases, but was found positive by PCR technique. Two of them were positive by the first-stage PCR, remaining 17 patients were

Table 1 :Serological, Histological Data of 19 Asymptomatic Carriers at the time of study and the Results of Precore Sequenes.

Case No	Age(yr) /Sex	ALT (IU/L)	HBsAg/ anti-HBs		HBeAg/ anti-HBe		anti-HBc	HBV-DNA 1st	HBV-DNA 2nd PCR	Histology	Virus Type
1	38 M	36	+	-	-	+	+	+	+	NSR	Wild
2	29 M	29	+	-	-	+	+	-	+	NSR	Mutant
3	31 M	20	+	-	-	+	+	-	+	MF	Mutant
4	30 F	14	+	-	-	+	+	-	+	NSR	Mutant
5	38 F	17	+	+	-	+	+	-	+	NSR	Mutant
6	23 M	24	+	-	-	+	+	-	+	NSR	Mutant(*)
7	45 M	12	+	+	-	+	+	-	+	NSR	Mutant
8	46 M	16	+	-	-	+	+	-	+	NSR	Mutant
9	51 F	16	+	-	-	+	+	-	+	NSR	Mutant(*)
10	52 F	14	+	-	-	+	+	-	+	NSR	Mutant
11	53 F	20	+	-	-	+	+	-	+	NSR	Mutant
12	54 M	27	+	+	-	+	+	-	+	NSR	Mutant
13	54 M	14	+	-	-	+	+	-	+	NSR	Mutant
14	56 M	16	+	-	-	+	+	-	+	NSR	Mutant(*)
15	57 M	22	+	-	-	+	+	-	+	NSR	Mutant
16	60 M	18	+	-	-	+	+	-	+	NSR	ND
17	63 F	17	+	+	-	+	+	-	+	NSR	Mutant
18	66 F	06	+	-	-	+	+	+	+	NSR	Mutant
19	66 M	11	+	-	-	+	+	-	+	NSR	Mutant(#)

NSR : Non Specific Reaction
MF : Minimal Fibrosis
(*) : Mixture of Predominant Mutant and Wild Types
(#) : The Additional Mutation in 29th Codon included
ND : Not Detectable



Figuer 2 : Wild : Sequence of wild type.
Mixture : Predominant mutant mixed with wild type.
Mutant : Complete mutation from G to A at nt 83 resulting a stop cordon.

positive only by the second-stage PCR, thereby indicating the low level of HBV DNA in all symptomatic carriers. On the contrary HBV DNA was detected by the dot-blot hybridization and was positive by the first-stage PCR. PCR using negative controls gave no positive result.

Sequencing results

Except one patient (No 16) whose HBV DNA amount was insufficient for DNA sequencing, HBV DNA sequence was determined for the remaining 18 of 19 anti-HBe positive "healthy" carriers and also for 5 cases of HBeAg positive controls. HBV variants with a point mutation from G to A in codon 28 of precore region resulting in a stop codon were detected in 94% (17/19) of detectable patients (mutant in Figure 2) including 3 patients (No 6,9,14) co-infected with the mixture of wild and mutant type viruses. In the cases with mixture, the mutant strain was predominant as estimated from the intensity of A band compared with G band on autoradiography (Mixture in Figure 2). An additional G to A mutation was identified in the

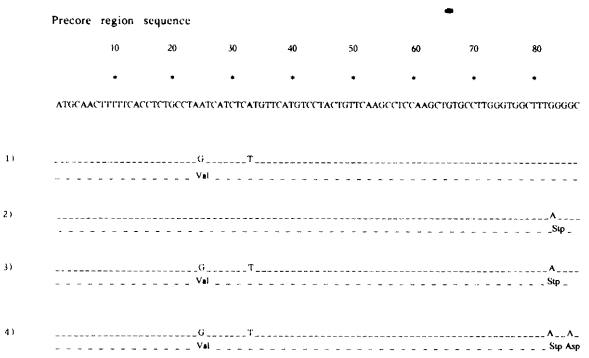


Figure 3 : Four patterns of precore sequence observed in "healthy" carriers were compared with the wild type sequence depicted on the top.

1) Precore sequence from patient 1 (wild type) but had a point mutation in codon 9 creating a substitution of Isoleucine to Valine(Val).
2) The pattern of precore in patients 2-12,14,17,18 with mutation from G to A in codon 28 resulted a stop codon(Stp).
3) The stop codon mutation included mutation in codon 9 was detected in patients 13,15.
4) An additional G to A mutation in codon 29 creating a substitution of Glycine to Asparagine(Asp) in patient 19. Numbers are corresponded to the patients in table 1.

terminal codon of precore region from GGC to GAC creating a substitution of Glycine to Asparagine. Only one case (6%) (No 1) in whom HBV DNA was detectable by the first-stage PCR, was infected with wild type showed a point mutation in codon 9 from ATC to GTC creating a substitution of Isoleucine to Valine which was also found in patients 13, 15 and 19 along with a stop codon (Figure.3). Wild type precore region sequence was found for HBV in all HBeAg positive controls (data not shown).

DISCUSSION

Whether the precore mutant is more pathogenic compared with wild type HBV has been the most interesting and important question. Evidences of the association of the precore mutant with fulminant hepatitis (3,5,16,17) suggest that the precore mutant is more pathogenic. The "ab initio" infection of the mutant in fulminant hepatitis has been suggested by Carman (17). Terazawa *et al.* (18) has reported a baby with fulminant hepatitis vertically infected with the mutant virus from an anti-HBe positive asymptomatic carrier mother. Fulminant hepatitis in a spouse of asymptomatic carrier infected with the precore mutant was also noted by Yotsumoto *et al.* (16). Although these results suggest that HBV carriers bearing this mutant are more likely to have severe hepatitis, Tur-Kaspa et al has reported the precore mutations in patients with various activity of the liver disease, regardless of the carrier's ethnic origin (19). In the present study, we had the advantage of selection of patients without liver disease confirmed by liver biopsy. These so-called healthy asymptomatic carriers also harbored HBV with precore mutation and the mode of mutation was identical as that previously reported in anti-HBe positive patients regardless of hepatitis activity (1-6,9,12), indicating that the precore mutant itself is not pathogenic.

Since this precore mutation is unable to produce HBeAg, it seems probable that decrease of wild type HBV leads to seroconversion of HBeAg as Okamoto *et al.* has reported (11). Our HBeAg-positive patients also showed wild type sequence in the precore region. Hepatitis has been considered as immunological destruction of HBV-infected hepatocytes by cytotoxic T cells (CTL). In CH(B), amino acids in the core region have appeared to be the target antigen for CTL (20,21). Accordingly, result by Okamoto et al indicates that population being eliminated during hepatitis is wild type HBV, but not precore mutant. In this meaning, the precore mutant may escape host immune response (22). However, chronic active hepatitis has been also reported in patients infected with the precore mutant showing high HBV DNA in serum (23). This clearly indicate that precore region mutation does not concern the pathogenic potential. Instead, the small amount of HBV, which is common feature of anti-HBe positive state, seems to be more likely associated with the absence of hepatitis. Small amount of HBV correlates to the small amount of target antigen in the liver for CTL. Akahane et al had suggested the existence of two kinds of precore mutant; One is associated with high level of HBV DNA as observed in active hepatitis, and the other with low level of HBV DNA accompanied by asymptomatic carriers who had seroconverted to anti-HBe positive state (24). Although the reason of difference in viral replication level had not been clear, we have recently shown that HBV infecting in anti-HBe positive healthy carriers had mutations in X region resulting in stop codons in X region, whereas this mutation was not seen in anti-HBe positive chronic active hepatitis(25). Since the X region contains many important sequences for viral replication in vivo, mutations in this region may responsible for low level of viral replication (26,27). These results indicate that the activity of hepatitis depends on the amount of HBV, but not on the precore mutation. Although some additional mutation in the precore region have been reported in relation to the severity of hepatitis (1,2,8,16), these mutations may also have no pathogenic roles. Since inflammation in the liver causes precore mutation in avian hepatitis B virus (28), these additional precore mutations may be the result from continuos inflammation in the liver.

Similarly, it may be difficult to correlate the precore mutant with fulminant hepatitis. Recently, apoptosis by means of Fas antigen has been suggested as the cause of fulminant hepatitis where precore mutation has no role on the pathogenesis (29). However, we can not deny a possibility that precore mutant is not pathogenic in persistently infected host, but may elicit unexpected reaction such as apoptosis of the hepatocyte if transmitted to different host.

Our data provide an evidence of the high prevalence of precore mutant in the group so called "healthy" asymptomatic carriers with anti-HBe suggesting the non-significant role of this mutation on the severity of the disease. However, it is still not clear whether precore mutation have some purposeful meaning in the strategy of HBV infection. Further studies are needed

to clarify this problem and the relationship between precore and X region mutation.

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