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Quantitative Analysis of Hepatitis B Virus Precore Mutant in Hepatitis Type B

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ICHIKAWA, T., TAKAGI, H., KINOSHITA, M., SHIMODA, R., NAGAMINE, T. and MORI, M. *Quantitative Analysis of Hepatitis B Virus Precore Mutant in Hepatitis Type B.* Tohoku J. Exp. Med., 1998, **186** (4), 323-333 —— Active liver disease has been detected in chronic hepatitis B after seroconversion from positive HBe antigen to positive anti-HBe antibody. Active replication of HB virus (HBV) containing a precore stop-codon mutation has been implicated in this condition. The usual methods, such as direct sequencing, to characterize the responsible mutant of HBV are not suitable for routine clinical use. Here we employed the competitive mutation site specific assay (CMSSA) to detect precore mutant HBV-DNA in patients with positive HB surface antigen. In patients with HBe antigen, precore mutant HBV-DNA was significantly higher than in patients with HBe antibody. The level of precore mutant HBV-DNA in patients with elevated serum ALT was significantly higher than in patients with normal serum ALT. Sex, age and the level of serum HBV-associated DNA polymerase levels were not correlated with levels of precore mutant HBV-DNA. Ten of 11 negative patients for the precore mutant by polymerase chain reaction followed by restriction fragment length polymorphism assay (PCR-RFLP) were positive for the precore mutant by CMSSA. These results suggest that the precore mutant has already emerged in the HBeAg-positive phase as determined by CMSSA, which is more sensitive than PCR-RFLP and is useful for evaluating the clinical course of patients with chronic hepatitis B. ———— hepatitis B virus; precore mutant; competitive mutation site specific assay

In patients with chronic hepatitis B virus (HBV) infection, the appearance of a variant HBV that cannot express hepatitis B e-antigen (HBeAg) due to a point mutation in the precore region (precore mutant) has been reported to result in the seroconversion from HBeAg-positive to hepatitis B e-antibody (anti-HBe)-

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positive (Ou et al. 1986; Carman et al. 1989; Okamoto et al. 1990). Seroconversion has been characterized by minimal virus replication and was once accepted as the quiescent phase in the progression of hepatic liver damage (Realdi et al. 1980). However, recent studies have suggested that an HBV variant with a precore stop-codon mutation is associated with fluctuating levels of serum transaminase (Matsuyama et al. 1995) or with severe liver damage in patients with fulminant hepatitis B (Carman et al. 1991; Yotsumoto et al. 1992) or chronic hepatitis B with anti-HBe (Bonino et al. 1986; Carman et al. 1989; Akahane et al. 1990; Kosaka et al. 1991; Liang et al. 1991; Omata et al. 1991). Active viral replication was detected in these patients. The replicating virus contained a G-to-A point mutation at nucleotide 83 in the precore region (Brunetto et al. 1989; Carman et al. 1989). This mutation converted codon 28 from tryptophan to a stop codon.

Direct sequencing was employed in the above studies to detect the site of the mutation. However, this method is not suitable for routine clinical use. For further investigation of the HBV-precore mutant in the pathological sequelae of hepatitis B, it is necessary to quantify the contribution of wild-type and mutant-type HBV in vivo because mutant-type HBV is associated with severe liver disease (Omata et al. 1991). Previously, semi-quantitative studies have suggested that the precore mutant HBV predominates over the wild-type in severe chronic liver disease (Naoumov et al. 1992; Lai et al. 1994). The present study analyzed precore mutant HBV-DNA quantitatively by means of a competitive mutation-site-specific-assay (CMSSA) (Shin and Kinishita 1994), a method that has recently become available for clinical use as a simple method to detect the precore mutation. We evaluated the relationship between the levels of precore mutant detected by CMSSA and the clinical or pathological status of patients who were positive for HBsAg.

Methods

Patients

We studied 55 Japanese patients with chronic HBV infection, 34 males and 21 females, with a mean age of 43 years. The diagnosis of chronic inactive hepatitis (CIH), chronic active hepatitis (CAH), CAH with lobular disorganization (LD), or liver cirrhosis (LC) was based on histological evaluation of liver biopsy. LC and hepatocellular carcinoma (HCC) were clinically diagnosed by blood chemistry, ultrasonography and computed tomography. The asymptomatic HBsAg carrier (ASC) state was defined as the absence of clinical symptoms and normal serum alanine aminotransferase (ALT) levels for at least 3 years without histological analysis. Chronic hepatitis (CH) was defined by the presence of elevated liver function values for more than 6 months and chronic liver damage revealed by ultrasonography and computed tomography without histological analysis. Patients included 21 with CAH, 7 with liver cirrhosis, 6

TABLE 1. *Classification of liver disease type B and the state of HBeAg and anti-HBe*

	HCC	LC	LD	CAH	CIH	ASC	CH	Total
HBe(+) anti-HBe(−)	1	3	1	13	0	3	8	29
HBe(+) anti-HBe(+)	0	0	0	1	0	0	0	1
HBe(−) anti-HBe(+)	2	4	0	5	2	3	6	22
HBe(−) anti-HBe(−)	0	0	0	2	1	0	0	3
Total	3	7	1	21	3	6	14	55

HCC, hepatocellular carcinoma; LC, liver cirrhosis; LD (CAH with LD), chronic active hepatitis with lobular disorganization; CAH, chronic active hepatitis; CIH, chronic inactive hepatitis; ASC, asymptomatic carrier; CH, chronic hepatitis.

asymptomatic HBsAg carriers, 3 with HCC, 1 with CAH with LD and 14 with CH (Table 1).

Assays of HBV marker

HBsAg, HBeAg, and anti-HBe were detected by enzyme immunoassay (EIA) using commercial assay kits (Dinabot, Tokyo). The activity of HBsAg-associated DNA polymerase (HBV-DNA-p) was measured by a method (Moritsugi et al. 1975) modified from Kaplan et al. (1973). Total HBV-DNA in serum was assayed by competitive PCR with the primer as described previously (Kinoshita et al. 1994).

Competitive mutation site specific assay (CMSSA)

Detection of HBV mutant was performed by mutation-site specific assay (MSSA) using the principle of mutant-allele-specific amplification (Takeda et al. 1993) as shown in Fig. 1. Serum samples from patients with hepatitis B were each denatured with an equal volume of 0.2 M NaOH and incubated for 15 minutes at 17°C. Then the mixture was neutralized with 0.2 M HCl. A 5.0 μl aliquot of the resulting mixture was subjected to PCR. PCR was performed with 1 μM each of primer "a", "b" and "c" primers in Fig. 1, using a Gene Amp kit (Takara, Tokyo) with a Robocycler PCR machine (Stratagene, La Jolla, CA, USA). Each PCR reaction cycle included denaturation at 94°C for 1 minute, primer annealing at 56°C for 1.5 minutes and then primer extension at 72°C for 1.5 minutes. Then 10 μl of the above reaction mixture was electrophoresed on a 3% agarose gel. Bands were identified following ethidium bromide staining. The identification of a 224 base-pair (bp) band was regarded as demonstraing the presence of HBV with a mutation in the precore region of the HBV-DNA genome. This band was compared with a 133 bp and 91 bp bands originated from HBV standard and the concentration was calculated by video-digitizer (ACI Japan, Tokyo) as 10^2 to 10^9 copies/ml (Fig. 2).

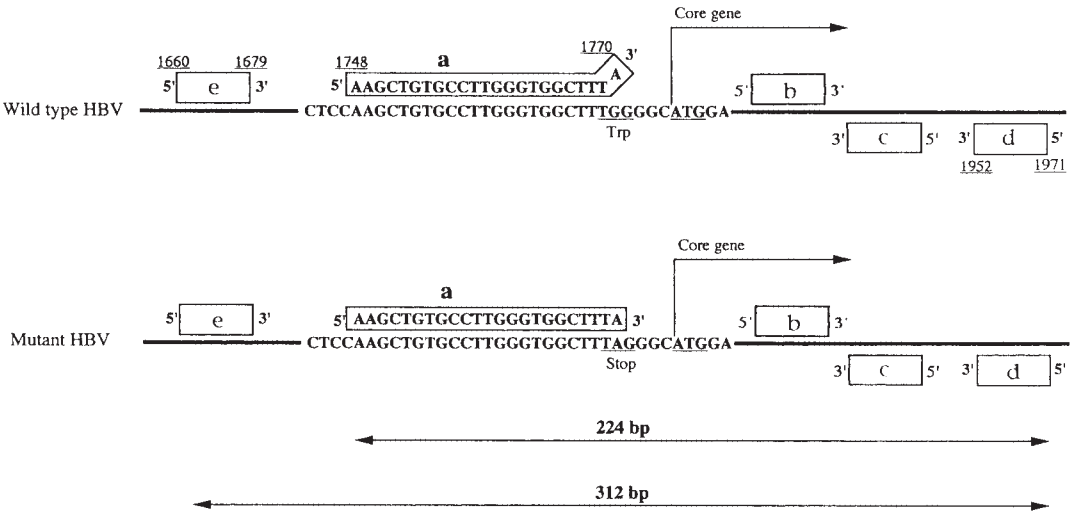

Fig. 1. Schematic representation of principle used in CMSSA. Primers are shown as open boxes; (a) OAL41 as mutation-trapped oligonucleotide (MTO) primer,
(b) OAL45: dATTCGAGATCTCCTCGACACCG,
(c) OAL40: dATAGCTTGCCTGAGTGCTGTA,
(d) OAL46: dATCAACTCACCCCAACACAG,
(e) OAL47: dGGCATAAATTGGTCTFTTCA.
The underlined numbers indicate the nucleotide positions in the HBV-DNA strain (Kinoshita et al. 1993).

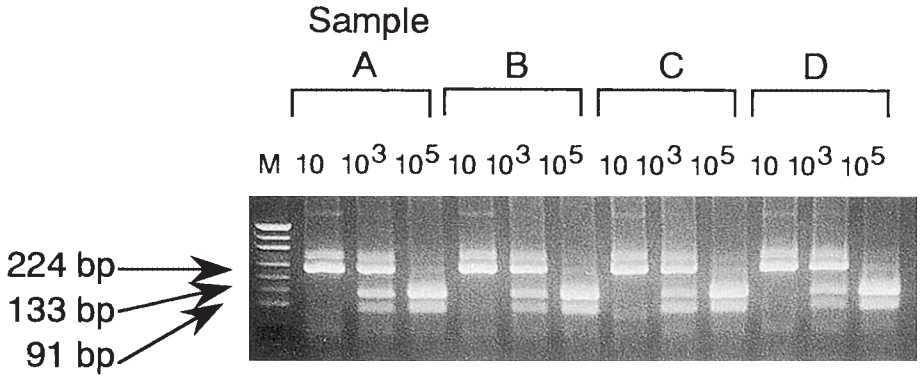

Fig. 2. Quantification of precore mutant HBV-DNA by CMSSA. HBV-DNA standard (10^1, 10^3 and 10^5 copies in this case) were added. Bands of 224 base-pair (bp) derived from precore mutant HBV-DNA and bands of 133 bp and 91 bp derived from HBV-DNA standard were identified and the HBV-DNA concentration was determined by the density of the band with a video digitizer. Each sample A, B, C and D was determined to have the precore mutant as 1×10^8, 9×10^7, 1×10^8, 8×10^7 copies/ml respectively. The top bands were derived from the first PCR and the other three bands (224, 133 and 91 bp) were produced from the second PCR.

PCR-RFLP (restriction fragment length polymorphism assay)

To confirm the presence of the HBV precore mutant, precore mutant HBV-DNA from 21 patients was detected by PCR in combination with a restriction fragment length polymorphism assay (PCR-RFLP: Special Immunology Laboratory, Tokyo) (Fig. 3). PCR was performed using a mismatched oligonucleotide

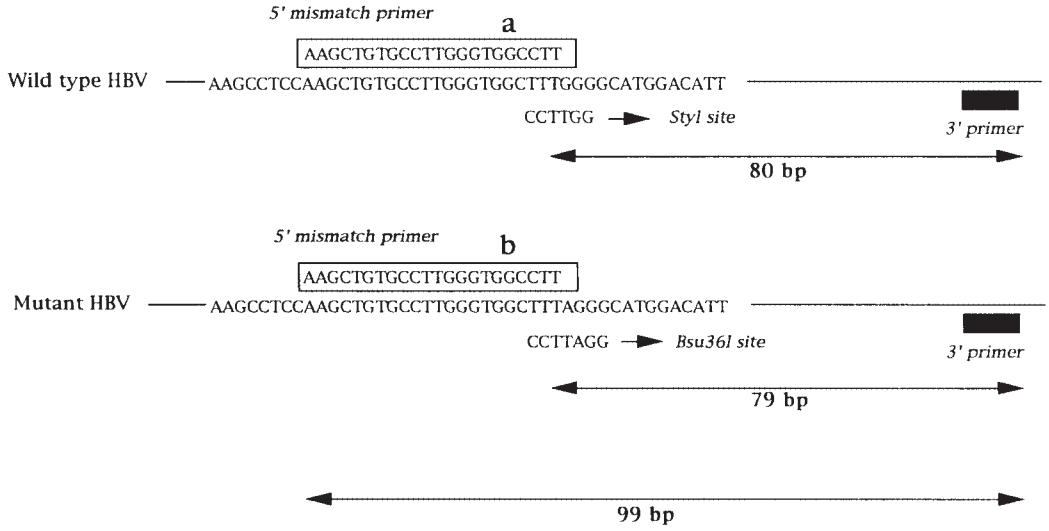

Fig. 3. Schematic representation of the principle of PCR-RFLP.

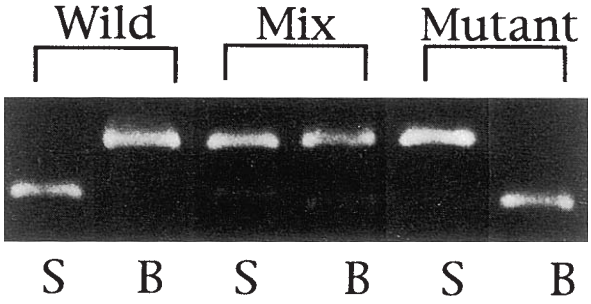

Fig. 4. Detection of precore mutant HBV-DNA by PCR-RFLP. Wildtype HBV-DNA was identified as 80 bp band digested with Sty I and as 99 base pair band by Bsu 36I. Mutant type HBV-DNA was identified as 99 and 79 bp band digested by Sty I and Bsu 36I, respectively. Mixed type had two bands of different size digested by Sty I and Bsu 36I, respectively. Wild, Wild type; Mix, Mix type; Mutant, Mutant type; S, Sty I digestion; B, Bsu 36I digestion.

primer as the 5' primer, as indicated by open box "a" for the wild type and open box "b" for the mutant type in Fig. 3. The PCR product of HBV-DNA amplified with these primers was digested with the appropriate restriction enzyme (Sty I and Bsu 36I) and was analyzed by electrophoresis (Fig. 4) as with CMSSA.

Statistical analysis

The Mann-Whitney U-test and Wilcoxon's single-rank test were used for statistical analysis of unpaired and paired samples. The relationships between precore mutant HBV-DNA levels and other laboratory data were analyzed using Spearman's correlation coefficient. A level of $p<0.05$ was accepted as statistically significant.

Results

Correlation of serological markers with precore mutant HBV-DNA levels The serum level of precore mutant HBV-DNA was significantly higher in patients with HBeAg than in those with anti-HBeAg (Fig. 5a, $p<0.05$). Serum precore mutant HBV-DNA was detected in 44 of 55 (80%) HBsAg-positive patients, 28 of 29 (97%) HBeAg-positive patients, and in 13 of 22 (59%) anti-HBe-positive patients. The serum level of precore mutant HBV-DNA was significantly higher

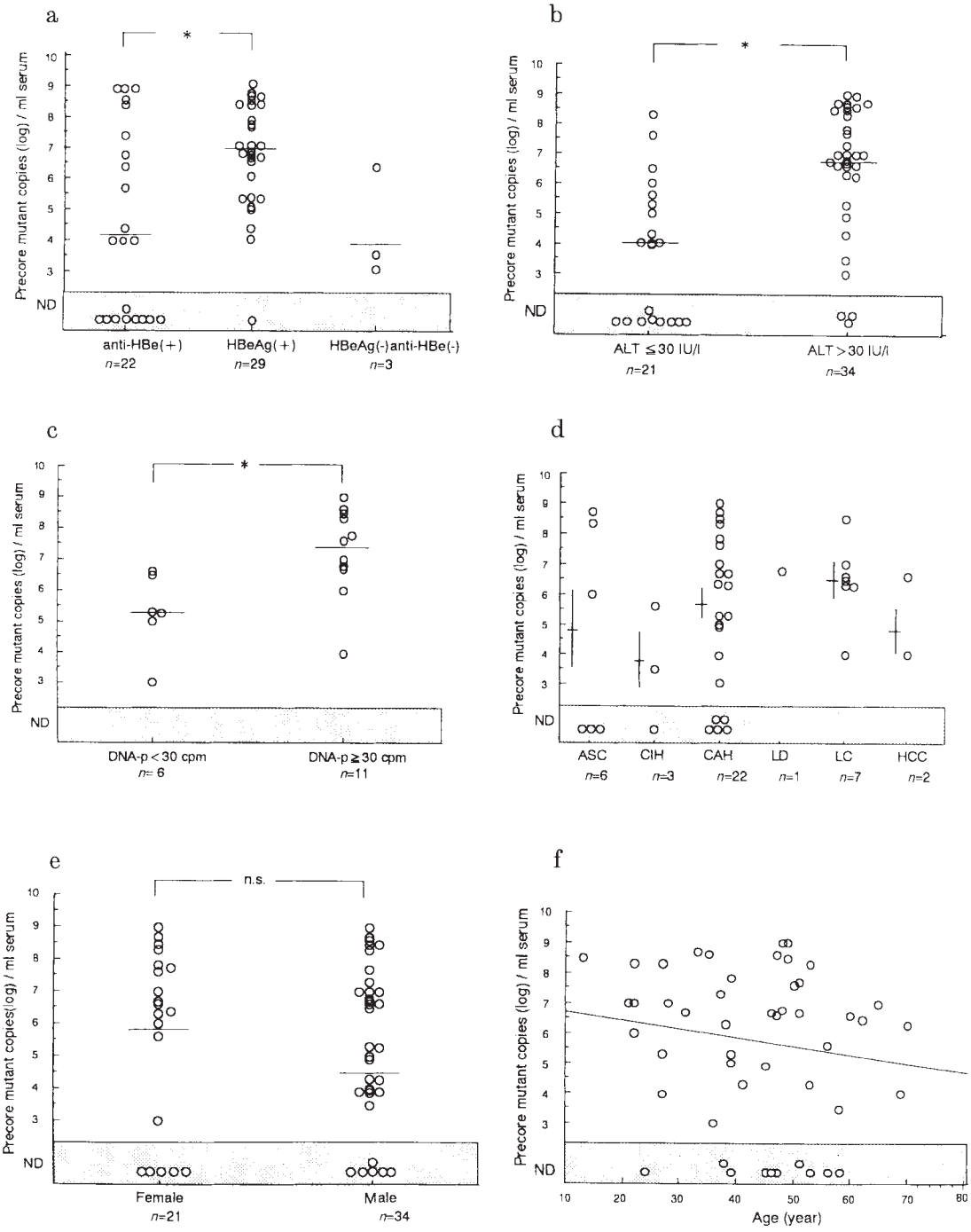

Fig. 5

in patients with elevated ALT (Fig. 5b) compared with patients with normal ALT levels ($p<0.05$). The level of precore mutant HBV-DNA differed significantly between patients with elevated HBV-DNA-p and patients with normal DNA-p levels in those without anti-HBeAg (Fig. 5c, $p<0.05$), but if those with anti-HBeAg would be added, the level of precore mutant HBV-DNA did not differ significantly. Precore mutant HBV-DNA levels in serum did not differ significantly among serum samples from patients in various disease states (CIH, CAH, CAH with LD, LC, HCC or ASC) (Fig. 5d). No correlation was observed between the precore mutant HBV-DNA level and sex (Fig. 5e, $p=0.57$) or age (Fig. 5f, $p=0.23$).

Detection of HBV-DNA type by PCR-RFLP. All 10 HBeAg-positive patients had the wild-type HBV-DNA (100%), whereas mutant DNA was also found in only 1 of these 10 patients (10%). Wild-type DNA was detected in 6 of 11 anti-HBe positive patients (54.5%) and 9 of the 11 patients had mutant DNA (81.8%) by PCR-RFLP (Table 2).

Comparison of clinical features with precore mutant levels by comparison of CMSSA and RFLP in patients with HBsAg. Ten of 11 patients negative for the

TABLE 2. *The distribution of wild type and mutant of precore region according to the state of HBeAg and anti-HBe*

	W(+)M(−)	W(+)M(+)	W(−)M(+)	W(−)M(−)	Total
HBeAg(+) anti-HBe(−)	9(90%)	1(10%)	0(0%)	0(0%)	10
HBeAg(−) anti-HBe(+)	0(0%)	6(54.5%)	3(27.3%)	2(18.2%)	11
Total	9	7	3	2	21

W, Wild type; M, Mutant.

Fig. 5. a) Comparison of serum precore mutant HBV-DNA with immune markers. anti-HBe(+): patients with anti-HBe. HBeAg(+): patients with HBeAg. HBeAg(−): patients without HBeAg. anti-HBeAb(−): patients without anti-HBe. Fifty five patients were analyzed. One patient who was positive for both HBeAg and anti-HBe, had 1×10^7 copies/ml precore mutant HBV-DNA and was excluded from this Fig. ND, not determined; $^*p<0.05$. b) Comparison of serum precore mutant HBV-DNA with serum ALT levels. ALT below 30 IU/liter is considered normal. $^*p<0.05$. c) Comparison of serum precore mutant HBV-DNA with activity of HBsAg-associated DNA polymerase (DNA-p). Patients without anti-HBe were analyzed. $^*p<0.05$. d) Comparison of serum precore mutant HBV-DNA levels with various indicators of liver disease. No significant differences were detected. Vertical bars represent the mean+S.E. ASC: asymptomatic HBV carrier. CIH: chronic inactive hepatitis. CAH: chronic active hepatitis. LD: CAH with lobular disorganization. LC: liver cirrhosis. HCC: hepatocellular carcinoma. e) Comparison of serum precore mutant HBV-DNA between males and females. No significant difference was observed. f) Correlation between precore mutant HBV-DNA level and age of patients. $r=0.176$ ($p=0.23$), $y=7.041-0.031x$.

TABLE 3. *Clinical features and precore mutant detected by CMSSA and PCR-RFLP in patients with positive HBsAg*

Patients[a]	Age	Sex	Histology[b]	HBV-DNA[c]	HBe[d]	Mutant by CMSSA (copies/ml)[e]	PCR-RFLP[f]	ALT (IU/liter)
Group A								
1	45	F	ASC	(−)	Ab(+)	100>	W(−)M(−)	9
Group B								
2	13	F	CAH	1+	Ag(+)	300000000	W(+)M(−)	163
3	39	F	CAH	2+	Ag(+)	70000000	W(+)M(−)	123
4	22	M	CAH	2+	Ag(+)	10000000	W(+)M(−)	119
5	65	F	LC	2+	Ag(+)	10000000	W(+)M(−)	47
6	31	M	CAH	2+	Ag(+)	5000000	W(+)M(−)	44
7	33	F	ASC	2+	Ag(+)	500000000	W(+)M(−)	39
8	60	F	HCC	2+	Ag(+)	4000000	W(+)M(−)	37
9	47	M	LC	2+	Ag(+)	4000000	W(+)M(−)	36
10	69	M	HCC	1+	Ab(+)	10000	W(−)M(−)	17
11	27	M	ASC	2+	Ag(+)	200000000	W(+)M(−)	15
Group C								
12	38	M	CAH	2+	Ab(+)	*100000>	W(+)M(+)	126
13	24	F	ASC	1+	Ab(+)	100>	W(−)M(+)	25
14	47	M	ASC	(−)	Ab(+)	100>	W(−)M(+)	22
15	58	F	CIH	(−)	Ab(+)	100>	W(+)M(+)	15
Group D								
16	22	F	CAH	2+	Ag(+)	200000000	W(+)M(+)	175
17	51	M	CAH	2+	Ab(+)	500000000	W(+)M(+)	167
18	49	M	LC	2+	Ab(+)	300000000	W(+)M(+)	61
19	58	M	CIH	1+	Ag(−)Ab(−)	3000	W(−)M(+)	48
20	70	F	LC	2+	Ab(+)	2000000	W(+)M(+)	31
21	56	F	CIH	1+	Ab(+)	400000	W(+)M(+)	13

[a]Patients are divided by the state of precore mutant as follows;
Group A: Not detected by both of CMSSA and PCR-RFLP. Group B: Detected only CMSSA.
Group C: Detected only by PCR-RFLP. Group D: Detected by CMSSA and PCR-RFLP.
[b]See in the legends of Table 1.
[c]HBV related DNA assayed by the method described in method in the text.
[d]HBeAg and HBeAb.
[e]The titer of precore mutant quantitated by CMSSA. *when hemolysis (+).
[f]PCR in combination with a restriction fragment length polymorphism assay. W, wild type; M, mutant type.

precore mutant by PCR-RFLP were positive for the precore mutant by CMSSA (Groups A and B in Table 3). In group B, in which only CMSSA detected the precore mutant, the amount of precore mutant decreased as the liver damage revealed by histological evaluation progressed and ALT decreased except in the case of ASC. In the anti-HBe-positive phase, ALT levels were usually normal when the mutant was not detected by CMSSA (Patients 13, 14, and 15 of group C in Table 3). Group D patients, in which both CMSSA and PCR-RFLP detected the precore mutant, tended to have high levels of precore mutant as revealed by CMSSA, but no definitive characteristics could be deduced from Group D.

Discussion

To detect the G^{83} to A^{83} transition in HBV, mutation-trapped oligonucleotide was used as a primer of PCR in CMSSA (Fig. 1). Only mutated HBV-DNA was amplified by the PCR because the mutation-trapped oligonucleotide primer was synthesized to detect the precore mutation. The mutated virus was quantitated and compared with the standard HBV-DNA cloned into the plasmid on the same gel (Shin and Kinoshita 1994). Although CMSSA was usually more sensitive than RFLP and was suitable for the titration of the precore mutant, precore mutant in some cases was not detected by CMSSA but by PCR-RFLP (Table 3). Basically one copy of precore mutant among 10^5 copies of wild type (0.001%) was detectable by CMSSA (Shin and Kinoshita 1994) but the minimum requisite for CMSSA is 10^2 copies/ml. On the other hand, precore mutant is detected by PCR-RFLP when the precore mutant exsisted more than 10% of wild type even if the absolute amount of precore mutant was lower than 10^2 copies ml (unpublished data). According to these data, precore mutant was detected by PCR-RFLP alone in the part of cases below 10^2 copies/ml.

The other method, PCR-coupled ligase chain reaction, could detect the ratios of precore mutant to total viruses and estimate the progression to fulminant hepatitis in patients with acute heatitis B (Minamitani et al. 1997). On the other hand, selective amplification method could detect the precore mutant in the majority of HBV carriers but this method was not able to quantify precore mutant and estimate the liver function (Nakahori et al. 1995). We showed the imporatance of quantification of precore mutant HBV-DNA in understanding of liver dysfunction. In this study, the precore mutant was detected by CMSSA in 28 of 29 cases in patients with positive HBeAg, but was detected in only 1 of 10 cases by PCR-RFLP.

In our study, the progression of hepatitis as evaluated by histological studies was not correlated with the level of precore mutant HBV-DNA in the serum. The titer of the precore mutant decreased after seroconversion, but the mutant was detected by CMSSA in most cases with positive HBeAg. The level of serum precore mutant HBV-DNA was compared with several markers of hepatitis. Levels of the precore mutant were higher in patients with elevated ALT compared

with those with normal ALT levels. Those were also higher in the HBeAg-positive phase than in the anti-HBe-positive phase. Because a high titer of precore mutant HBV-DNA is usually accompanied by total HBV-DNA (Table 3), it cannot be concluded that the precore mutant is directly responsible for liver injury. In the anti-HBe-positive phase, when the mutant is not detected by CMSSA, ALT levels are usually normal. These results suggest that detection of the precore mutant by CMSSA can be a useful marker of progression of liver injury in patients with positive HBeAb.

In conclusion, precore mutant HBV-DNA was detected by CMSSA in the phase of positive HBe-Ag and the precore mutant HBV-DNA levels were correlated with ALT levels. Thus precore mutant was useful for the evaluation of the clinical course of chronic hepatitis B.

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