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Original Article

Mutations in the Hepatitis B Virus PreS2 Region and Abrogated Receptor Activity for Polymerized Human Albumin

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The preS2 region of the hepatitis B virus (HBV) has been reported to have human polymerized albumin receptor (PAR) activity, which correlates with viral replication. Here, we studied the genomic sequence of the preS region from rare patients lacking PAR activity, despite active viral replication. PAR and DNA polymerase activity was identified in 178 HBe antigen-positive HBV carriers, and a significant correlation between 2 markers was shown, except in 2 hepatitis patients lacking PAR activity. Nucleotide sequences of the preS region of HBV from both patients were examined by direct sequencing of PCR products. In one patient, a 45-base deletion was found to overlap half of the putative polymerized human albumin binding site in the preS2 region. In the other patient, a point mutation at the first nucleotide of the start codon of the preS2 region of HBV was found. There was no such genomic change in the 3 control HBV sequences. These results indicate that the preS2 region is necessary for binding of polymerized human albumin, and this is the first report of naturally existing mutant virus with no or low PAR activity.

Key words: hepatitis B virus, preS region, polymerized albumin receptor, genetic mutation, genetic deletion

Hepatitis B virus (HBV) has an envelope consisting of 3 glycoproteins coded by parts of the HBV genome, all sharing the C-terminal sequence, which contains the major surface antigen (HBsAg). Of these, the N-terminal sequence of the middle surface protein is encoded by the preS2 region located just upstream of the S region of the HBV genome and is functionally associated with binding activity of HBV surface proteins to polymerized human albumin through putative polymerized albumin receptors (PAR) both on HBV and hepatocytes [1]. It has therefore been suggested that HBV may enter into hepatocytes through this species- and organ-specific binding machinery. Also the preS1 region has been reported as a principal binding site for hepatocytes [2]. Bruss [3] has reported that hepatitis B virus particles contain 2 forms of the L protein: an external (e-preS) and internal (i-preS) pre-S domain. It is thus indicated that the external preS domain is involved in binding to virus receptors. As such, the preS region of the HBV genome appears to be important in the first phase of HBV's entry pathway into the hepatocyte.

We have previously demonstrated that PAR activity in serum of the HBV carrier is closely correlated with serum HBV DNA polymerase [4] levels, which reflect

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viral replication. Here, we have defined the amount of polymerized human albumin bound to HBs antigen as PAR activity [4]. While PAR activity is a clinically useful tool in evaluating the viral replicative state in HBV carriers [5], we found 2 patients with active viral replication but less PAR activity in 178 HBe antigen-positive carriers. To clarify this dissociation, we analyzed in the present study the genomic sequences of the pre-S region of HBV from these patients, finding that genomic changes in this region may be directly linked to the biological function of this viral protein.

Materials and Methods

Patients. All patients, who visited our hospital between September 1987 and December 1994 and were positive for HBs antigen and HBe antigen were enrolled in this study. During this period, 178 patients were found to be HBe antigen-positive out of 256 patients positive for the HBs antigen. Their sera of these patients was collected and stored at − 20 °C until use.

HBV markers. HBsAg was determined by a reverse passive hemagglutination test (SERODIA-HBs; Fujirebio Inc., Tokyo, Japan). HBeAg was determined by radioimmunoassay (HBe RIAKIT II; Dainabot, Tokyo, Japan). HBV DNA-polymerase was determined by Kaplan's method [6]. PAR activity was determined by the method reported by Tsuji [4], with the optical density being measured at 492 nm in Photo-ELISA I (Organon, Oss, the Netherlands) and expressed as arbitrary unit. Negative controls showed a range of 0.04-0.23.

Sequencing of the preS1 and preS2 regions of HBV. Nucleotide sequences of the preS1 and preS2 regions of HBV were determined from patient serum by direct sequencing of PCR products. In brief, DNA was extracted from 100 μl of serum by the SDS-proteinase K method [7]. PreS1 and preS2 regions of HBV DNA were amplified by 45 cycles of polymerase chain reaction (PCR) using a sense primer (5'-GGGTCACCATATTCTTGGGA-3', 2814-2833) and an anti-sense primer (5'-GTCCTAGGAATCCTGATG-3', 185-168) [8]. PCR products were purified using a SUPREC-2 column (Takara, Ohtsu, Japan) and sequenced with an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA, USA) in a 373A DNA Sequencing System (Applied Biosystems Japan, Chiba, Japan) [9].

Results

Correlation between DNA-P and PAR. DNA-polymerase (DNA-P) and PAR activity was determined in one serum specimen from each of the 178 HBe antigen-positive patients and is plotted in Fig. 1. These 2 parameters were found to be correlated ($r^2 = 0.533$, $P < 0.001$).

In this population, 2 patients (patients 1 and 2 in Fig. 1) were found to be outside of this correlation. We then examined the genomic sequences of the preS regions of HBV from these 2 patients, with another 3 patients (patients 3, 4, and 5 in Fig. 1) as a control. The clinical backgrounds of studied patients are given in Table 1. Although alanine aminotransferase (ALT) and DNA-P levels fluctuated in patients 1 and 2, as seen in control patients, the PAR activity of patients 1 and 2 remained low. Subtypes of HBV were adr, except for the adwr subtype in patient 2. These 5 patients had chronic hepatitis histologically diagnosed by perioneoscopic liver biopsy performed under patients' written informed consent.

Sequence of the HBV preS region. Nucleotide sequences of the preS regions of HBV from

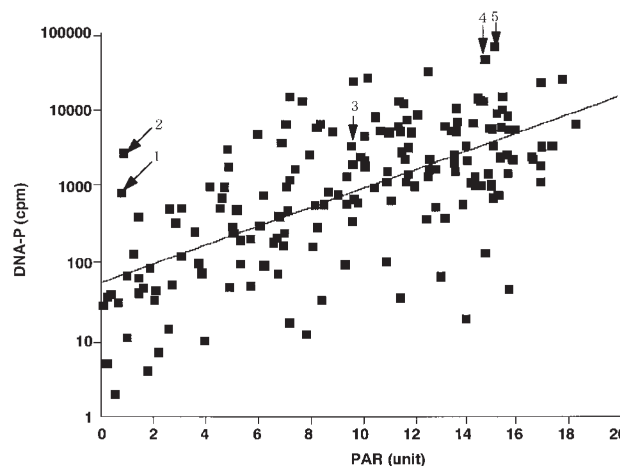

Fig. 1 Correlation between serum PAR and HBV DNA polymerase activities of HBe antigen-positive HBs antigen carriers. Numbers with arrows indicate patients selected for this study.

Footnote: This work was presented in part at the 49 th Annual Meeting of the American Association for the Study of Liver Diseases on November 6-10, 1998, at Chicago, USA.

patients 1 and 2 along with control patients and subtype adr (Genebank accession number M38454) are shown in Fig. 2. Compared with the adr wild-type sequence, several point mutations were detected: 5 in patient 1; 8 in patient 2; 5 in patient 3; 7 in patient 4; and 2 in patient 5. Also, patient 1 had 45 base deletions in the preS2 region, which spans 5' upstream to the middle of the putative polymerized human albumin binding site [10].

Amino acid sequences were deduced from nucleotide sequences (Fig. 3). The nucleotide point mutations seen in patients 3, 4, and 5 were silent, meaning that there was no amino acid replacement. In contrast, in patient 1 there were 2 missense mutations (^{90}Val to ^{90}Ala in preS1 region; ^{159}Ser to ^{159}Leu in preS2 region) and 15 amino acid-long deletions. In the case of patient 2, there were 3 missense mutations, one of which was located at the first codon of the preS2 region. The start codon (ATG, ^{120}Met) of the preS2 region was changed to ^{120}Val (GTG), and others were in the preS1 region: ^{77}Tryp to ^{77}Cys and ^{90}Val to ^{90}Ala.

To determine whether the mutations detected above were consistent in these patients, the same regions from sera collected at different times were sequenced: 2 and 4 years later in patient 1; 1, 2, 4, and 5 years later in patient 2. The nucleotide sequences from these samples were the same as the oldest ones shown in Fig. 2 (data not shown).

Discussion

Genomic mutations of several regions of the HBV genome have been reported thus far. In the envelope protein, the nucleotide sequence of the antigen-determining site of the S region is changed to a missense mutation, resulting in different antigenic properties. This mutation is referred to as an escape mutant, as this clone was discovered in successful HBs antigen vaccinees and escaped from neutralizing antibodies induced by the vaccine [11, 12]. Mutations in the preS region has also been reported [13-17].

The preS2 region consists of 55 amino acids, and its function and clinical significance has not yet been elucidated. It has, however, been hypothesized that this region might be essential for HBV to bind and enter into hepatocytes via polymerized human albumin in a specific manner [18-21]. The mutants found in our patients may relate to this function, although additional studies of preS proteins and *in vitro* expression will be necessary to confirm this hypothesis.

In patient 1 of our study, a part of the deletion of nucleotide sequences overlapped the putative PAR site, possibly destroying PAR function. In patient 2, we found a start codon mutant of preS2. The start codon mutant could result in an absence of preS2 protein. Although the preS2 sequence was contained in the large HBs antigen protein, which was translated from the preS1 start codon through preS1 and preS2, PAR function was lost in this mutant. As such PAR function might require the constitutional structure of this region.

In Santantonio's study, five out of 22 Italian HBV carriers had HBV without expression of the preS2 protein. Four showed a deletion of the start codon, and another had a point mutation of the start codon of the preS2 region [10]. Similar mutants have been reported by Fernholz [22] in chronic HBV carriers.

Interestingly, Pollicino [17] has reported a family cluster of fulminant hepatitis possibly caused by the preS2 start codon mutant. They have suggested that the absence of preS2 protein in early events in infection may lead to a failure to neutralize the virus and more severe liver damage. Our patients were chronic HBV carriers, and this sequence should be examined in acute hepatitis patients. No information regarding the relationship between mutant and PAR activity has been provided by the above studies, so the present results are the first to relate to both.

PAR activity has been used as a useful marker to determine the state of viral replication and disease activity of chronic hepatitis B and HBV carriers [5]. During the clinical course of hepatitis B, correlations were found between serum PAR activity and DNA-P in individual

Table 1 Clinical background of patients

	Age/sex	Range*			Subtype
		ALT	PAR (unit)	DNA-P (cpm)	
Patient 1	38/M	27-120	0.04- 1.48	4- 716	adr
Patient 2	41/M	41-525	0.24- 0.65	123- 2723	adwr
Patient 3	50/F	28- 48	10.20-17.29	151- 4522	adr
Patient 4	40/M	18-313	1.15-16.2	10-44346	adr
Patient 5	42/M	28-709	0.11-15.62	3-66553	adr

*, range of ALT, PAR, and DNA-P in studied patients during the observation period (1.4 years for patient 1, 1.9 years for patient 2). The sampling times for each patient were as follows: 23 in patient 1; 17 in patient 2; 19 in patient 3; 41 in patient 4; 82 in patient 5.

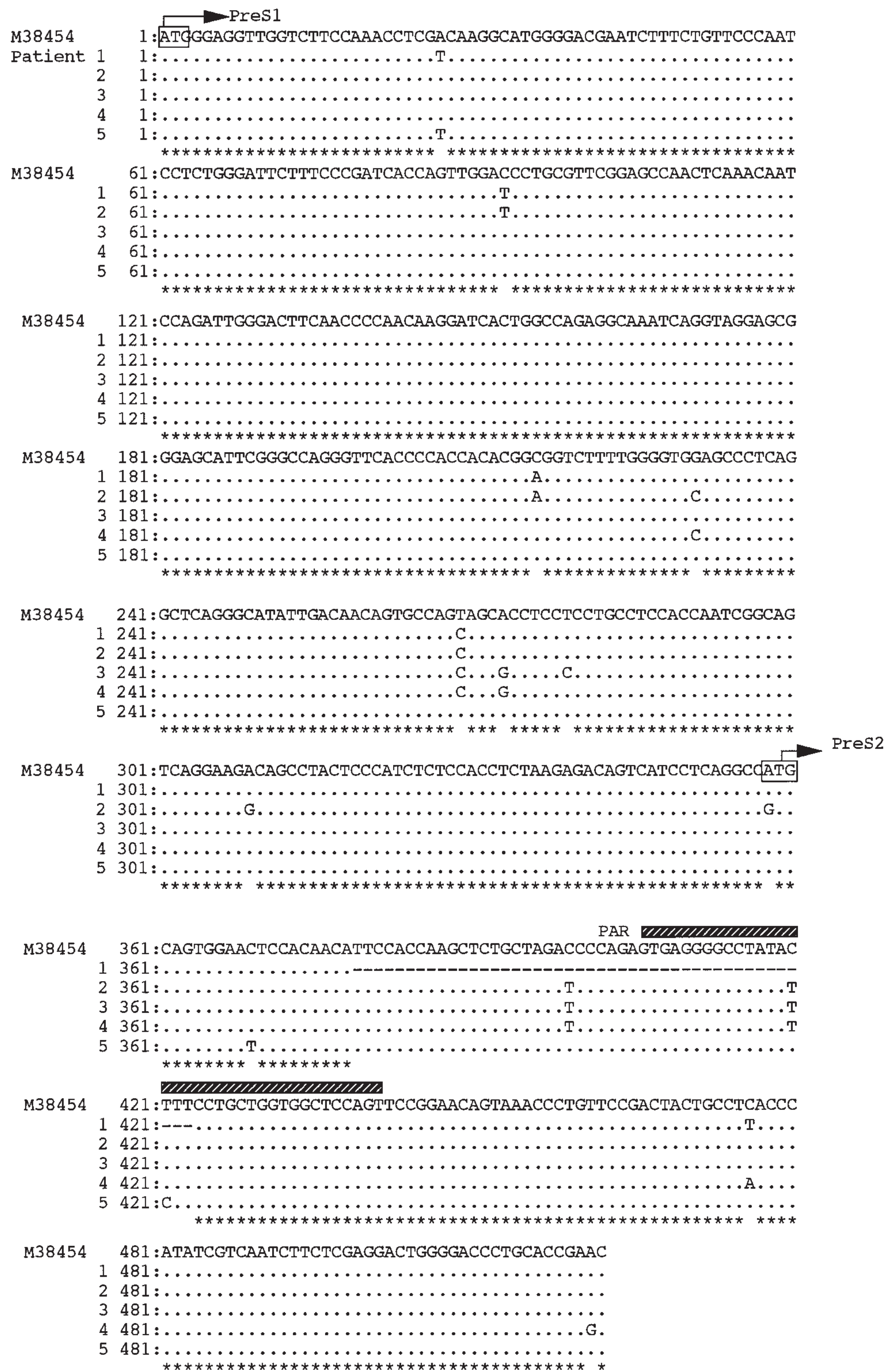

Fig. 2 Nucleotide sequence of the HBV preS region. Sequence of wild-type adr (Genebank M38454) and those of 5 patients are aligned. Putative sites for binding with polymerized albumin [10] are indicated by a shaded box.

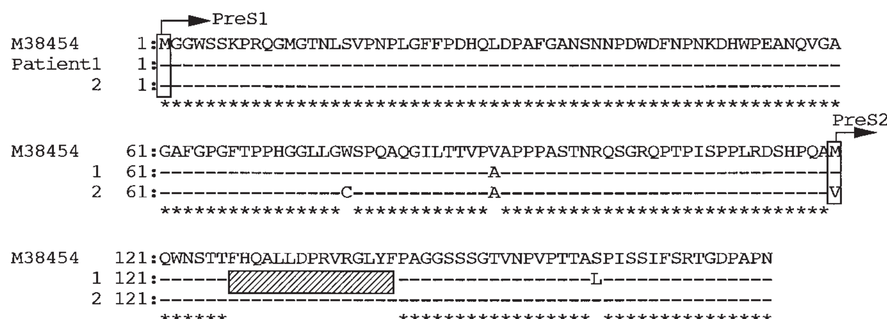

Fig. 3 Amino acid sequence of the PreS region deduced from the nucleotide sequences shown in Fig. 2. Deletion of amino acid sequences is indicated as a shaded box.

patients, except patients 1 and 2, in the present study (data not shown).

We found deviations from this significant correlation between PAR activity and HBV replication to be relatively rare (1.1% of 178 HBe antigen-positive HBV carriers) in this study, and the clinical significance of PAR activity did not change.

The subtype of HBV was adr in patient 1 and adwr in patient 2. A previous study of point mutations of compound subtypes [23] indicated no mutations at the start codon of preS2 in adwr, so this mutation does not appear to be related to subtype.

In conclusion, a mutation at the start codon or deletion in the preS2 region is suggested to cause a loss or lowering of PAR activity. The clinical significance of these mutants should be examined further.

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