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Comparison of Core Promoter Sequence and Activity Between Naturally Occurring Hepatitis B Virus Mutants from Patients with Acute Hepatitis B

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This study sought to identify the contribution of mutations in the core promoter region of the hepatitis B virus (HBV) to the pathogenesis of severe acute onset hepatitis B. Eighteen patients were enrolled in the study: 7 with fulminant hepatitis (FH), 5 with severe acute hepatitis (SAH), and 6 with acute self-limited hepatitis (AH). Sequences and transcriptional activity of core promoter region of HBV from the patients were studied. The mean number of base changes increased with the severity of the hepatitis: 4.6 ± 3.1 in FH, 3.4 ± 1.7 in SAH, and 0.5 ± 0.5 in AH. A double mutation, A (1762) → T and G (1764) → A, was found in 2 of FH, 3 of SAH, and none of AH. A C (1773) → T mutation was found in 5 of FH, 2 of SAH, and none of AH. There was no significant difference in chloramphenicol acetyl transferase activity between different mutated bases care promotor sequences. This finding suggests that the number of mutations in core promoter region was correlated with severity of liver disease, but development of fulminant hepatitis was not a consequence of enhanced core promoter activity.

Introduction

Four different promoter regions regulate the expression of hepatitis B virus (HBV) genes. The core promoter region is the one that controls the transcription of both core and precore RNA. Precore RNA and pregenome/core RNA are differentially regulated. Overlapping, but genetically distinct, regions of the core promoter regulate expression of precore RNA and pregenome/core RNA[1]. Therefore, mutations in the core promoter region may differentially influence the transcription of precore and pregenome/core RNA. A double mutation [A (1762) → T and G (1764) → A] has been found in patients with HBeAg-negative chronic hepatitis[2)3)] and fulminant hepatitis[4)5)]. It has been reported that this double mutation reduces precore transcripts, resulting in a decrease in precore-core protein[6)7)]. Furthermore, this double mutation increases pregenome/core RNA[8)9)] through expression of HNF-1 binding sites[6)]. These findings, together with the fact that precore-core protein suppresses the replication of HBV[10)～12)], suggest that this double mutation increases HBV replication and contributes to the pathogenesis of severe liver injury. However, recent reports have shown that this double mutation is not associated with fulminant hepatitis B[13)14)]. This study examined the frequency of mu-

Table 1 Demographics an clinical features of patients with acute hepatitis

Patients		Sex/Age	Outcome	Peak ALT (IU/ml)	HBeAg	Anti-HBe	G to A at nt 1896
FH							
	1	F / 41	Died	14826	+	+	A
	2	M / 70	Died	3480	−	+	A
	3	M / 35	Died	2025	+	−	A
	4	F / 34	Died	5980	+	+	G
	5	M / 31	Died	4980	−	+	G
	6	M / 50	Died	5040	−	+	A
	7	M / 23	Died	807	+	−	G
SAH							
	1	F / 48	Survived	1662	+	−	G
	2	M / 51	Survived	2277	−	+	A
	3	F / 48	Survived	8580	+	+	A
	4	M / 34	Survived	1327	−	+	A
	5	M / 27	Survived	4370	−	+	G
AH							
	1	M / 42	Survived	1656	−	+	G
	2	M / 29	Survived	545	−	+	A
	3	F / 32	Survived	3667	+	+	G
	4	M / 26	Survived	1095	+	−	G
	5	M / 22	Survived	2080	+	−	G
	6	M / 24	Survived	3309	−	+	A

ALT: alanine aminotransferase, FH: fulminant hepatitis, SAH: severe acute hepatitis, AH: acute self-limited hepatitis.

tations in the core promoter region in patiens with acute hepatitis B and correlated the frequency of mutations with disease severity. The transcriptional activity of different patterns of core promoter sequences also was investigated.

Patients and Methods

Patients

Eighteen patients were enrolled in the study: 7 with fulminant hepatitis (FH), 5 with severe acute hepatitis (SAH), and 6 with acute self-limited hepatitis (AH). The criteria for FH was development of hepatic encephalopathy and prolongation of the prothrombin time during the course of hepatitis[15], and SAH was defined as a prolonged prothrombin time without hepatic encephalopathy. HBV infection was established as the cause of acute of fulminant hepatitis by detection of immunoglobulin M antibodies (Ausria II, Abbott Laboratories, North Chicago, IL) to the hepatitis B core antigen. Assays for IgM-anti-HAV antibody (Havab-M, Abbott Laboratories) and anti-HCV antibody (Matrix HCV, Abbott Laboratories) were negative in all patients. Despite intensive care, all FH patients died; all other patients survived (Table 1). Serum samples were taken immediately after admission to our hospital and before administration of fresh frozen plasma and were stored at −80 ℃ until use.

Amplification and sequencing of the core promoter and precore regions of HBV DNA

A 50 μL sample of serum was diluted with 50 μL of sterile distilled water and mixed with 100 μL of a digestion mix containing 25 mM sodium acetate, 2.5 mM EDTA (pH 8.0), 1% SDS, 2 μg/mL of proteinase K, and 10 μg/mL of yeast t-RNA as carrier. Following digestion at 65 ℃ for 2 hours, the samples were extracted twice with phenol-chloroform, once with chloroform, and then

ethanol-precipitated before suspension of the viral nucleic acid in water. Extracted HBV DNA was used to amplify the core promoter and precore regions, an 808-bp sequence extending from nt 1653 to nt 2460, by the polymerase chain reaction (PCR). The primers used were 5'-CATAAGAGGACTCTTGGACT-3' (sense; nucleotides 1653 to 1672) and 5'-GGGATACTAACATTGAGATTCCCG-3' (antisense; nucleotides 2460 to 2437). Five microliters of the extracted DNA was mixed with 45 μL of a PCR reaction mixture containing 400 nM of the primer sets, and subjected to 35 cycles at 94 ℃ for 1 min, 55 ℃ for 1 min, and 72 ℃ for 3 min in an automatic DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR products were ligated into the vector pCRII using the TA cloning kit (Invitrogen, San Diego, CA), and clones carrying HBV DNA sequences were propagated. At least 5 clones of each sample of HBV DNA were then sequenced by the dideoxy-chain termination method with an AutoRead DNA sequencing kit (Pharmacia LKB Biotechnology, Uppsala, Sweden).

Quantification of promoter activity

The functional portions of the HBV core promoter were amplified by PCR using stored sera from FH patients 1, 4, 5, and 6; SAH patients 1 and 3; and AH patients 4 and 5, with representative mutations in the core promoter region. The primers used were 5'-AAGCTTCCAAGCTGTGCCTTG (sense; nt 1685–nt 1705 with the HindIII site underlined) and 5'-AGATCTCACAGCTTGGAGGC (antisense; nt 1887–nt 1868 with the XbaI site underlined). PCR products were digested with HindIII and XbaI (Takara Biochemicals, Kyoto, Japan). The resulting 203-bp fragment was ligated into a pCAT Enhancer Vector (Promega, Madison, WI) that had been cleaved with HindIII and XbaI, and clones carrying the HBV DNA sequence were propagated. For monitoring transfection efficiency, we used pCAT basic as negative control and pCAT control (Promega), which harbored both SV40 enhancer and promoter sequences as positive control. To examine promoter activity, we performed CAT (chloramphenicol acetyl transferase) assays by transfection using the hepatoma cell line Huh 7. The constructs were transfected into Huh 7 cells, and CAT activities in cell lysates were assayed using a QUAN-T Kit (Amersham, Buckinghamshire, UK).

Statistical analysis

Unless otherwise indicated, all data are expressed as mean ± SEM. Statistical comparison of two means was made using an unpaired Student's t test. Groups of data were considered significantly different if $p<0.05$.

Results

Precore mutation and HBeAg/HBeAb status

Of the patients with FH, two were positive for both HBeAg and HBeAb, two were HBeAg (+)/HBeAb (−), and the remaining three were HBeAg (−)/HBeAb (+). The patients with HBeAg (−)/HBeAb (+) had either the double mutation or a precore stop codon, or both. Among the patients with SAH, three were HBeAg (−)/HBeAb (+), one were HBeAg (+)/HBeAb (−), and one was HBeAg (+)/HBeAb (+). Of the patients with AH, two were HBeAg (+)/HBeAb (−), three were HBeAg (−)/HBeAb (+), and one was positive for both HBeAg and HBeAb. Except for the patient with AH, the HBeAg-negative patients had either the double mutation in the BCP (basic core promoter) or a precore stop codon, or both.

Sequence analysis of the core promoter region

The number of base changes in the BCP region correlated with the severity of acute hepatitis, with a mean of 4.6 ± 3.1 in FH patients, 3.4 ± 1.7 in SAH patients, and 0.5 ± 0.5 in AH patients (FH vs AH, SAH vs AH; $p<0.01$) (Figure). Five clones

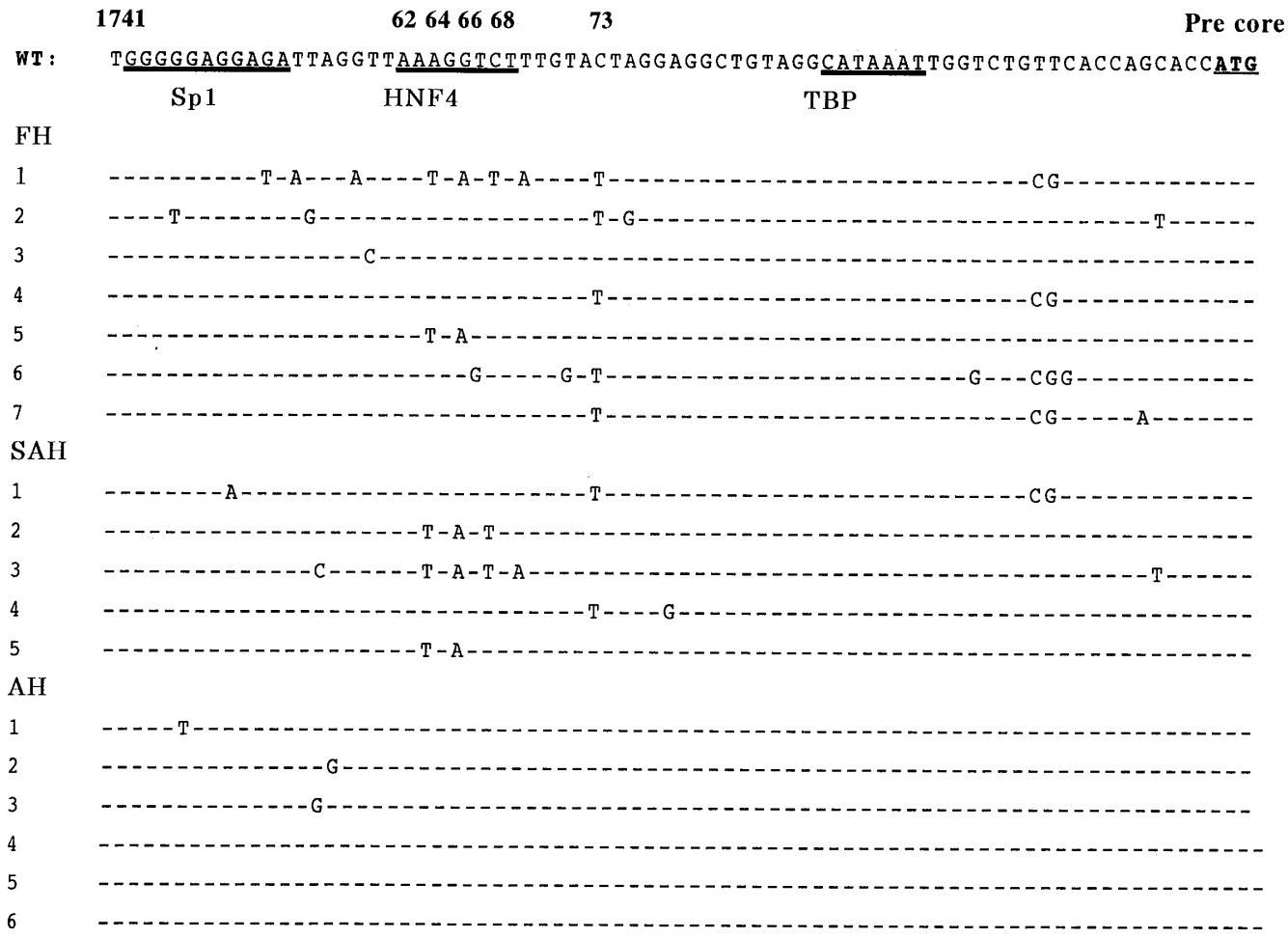

Figure Sequence analysis of the core promoter region of clones from 15 patients with acute hepatitis B

The consensus nucleotide sequence of the wild-type adr strain is shown in the first line. Dashes represent nucleotide sequences identical to reference sequences. Major nuclear protein binding sites are underlined. Bold letters indicate the precore start codon.

were analyzed in each patient in individual experiments, and the sequence in each patient was identical among all clones. Common mutations were not observed in any case of FH or SAH. A base change from C-to-T at nt 1773 was observed in 5 of 7 cases of FH and 2 of 5 cases of SAH. The double mutation at nt 1762 (T) and nt 1764 (A) was identified in 2 cases of FH and 3 cases of SAH. Mutated sequences in FH case 1 were almost identical with the mutated sequences reported in a case of nosocomial infection in Israel[16)17)].

CAT activity of mutated core promoter sequences

We analyzed the transcriptional activity of the naturally mutated core promoter regions from the HBV clones from the 15 patients (Table 2). The BCP regions of patients FH 1, FH 4, FH 6, and SAH 1 with the mutation C to T at nt 1773 (which was the most common mutation) were not associated with significantly higher CAT activity than the BCPs with a wild-type sequence at nt 1773. The BCP activity from patients FH 1, FH 5, and SAH 3, who had the double mutation at nt 1762 (T) and nt 1764 (A), was not different than

Table 2 Transcriptional activity of the BCP with naturally occurring mutation(s) from patients with differing severity of acute hepatitis B

	nt 1762*	nt 1764	nt 1766	nt 1768	nt 1773	CAT activity (cpm)
FH1	+	+	+	+	+	3625±746
FH4	−	−	−	−	+	3043±707
FH5	+	+	−	−	−	3699±122
FH6	−	−	−	−	+	4388±590
SAH1	−	−	−	−	+	3027±570
SAH3	+	+	+	+	−	2177±522
AH4	−	−	−	−	−	2439±373
AH5	−	−	−	−	−	3202±121
pCAT basic						220±32
pCAT control						3236±262

*: Positions where mutations in the BCP were detected. CAT: chloramphenicol acetyl transferase.

BCP activity in strains with wild-type sequences at those positions. Although several mutations other than those at nt 1762, nt 1764, and nt 1773 were observed in FH and SAH patients, BCP activity was unaffected.

Discussion

It recently has been reported that mutations in BCP are associated with an HBeAg-negative phenotype. Sato et al analyzed viruses from 25 FH patients and identified a double mutation at nt 1762 (T) and nt 1764 (A) in 20 patients[4]. In their report, the HBeAg(−)/HBeAb(+) phenotype was observed in patients without a G to A mutation at nt 1896, resulting in a stop codon in the precore region, with premature termination of HBeAg; those patients had the double mutation. Hence, the authors speculated that this double mutation might reduce the production of precore RNA. Subsequently, Laskus et al[18] and Kurosaki et al[2] reported that asymptomatic carriers and chronic persistent hepatitis patients with an HBeAg(−)/HBeAb(+) phenotype carry the double mutation at a higher frequency than other phenotypes. Sato et al's hypothesis was supported by an in vivo study in which the double mutation in the BCP reduced the level of precore transcripts[19]. In the present study, either the double mutation or a precore stop codon was observed in all HBeAg-negative patients except for one AH patient. In that AH patient, the HBeAg(−)/HBeAb(+) phenotype might have developed as part of the natural course of AH caused by wild-type HBV.

In the present study, common mutations were not found in the BCP, even though the number of base changes in the BCP increased with the severity of liver disease. This finding is not consistent with other data from Japan[4]. Common mutations in the BCP have not been detected in FH patients in Western countries[13][14]. Recent reports from Japan identified the double mutation in the BCP only in patients with chronic hepatitis B evolving into a fulminant form, not with acute FHB[5]. Furthermore, nearly wild-type BCP sequences were found in both source and infected patients in an outbreak of FH in a dialysis center in Tokyo[20]. These findings suggest that the double mutation may be associated with an HBeAg(−) phenotype, but does not correlate with severity of disese.

In the present study, C to T and T to A mutations at nt 1766 and nt 1768, respectively, were detected in FH case 1 and SAH case 3. These mutations previously have been identified in an FH

outbreak in Israel and were reported to enhance the efficiency of encapsidation of pregenomic RNA into core particles[21]. FH case 1 was a patient who contracted a nosocomial infection in a dialysis unit. Thus, although the mutations at nt 1766 and nt 1768 were not common in FH, HBV containing these two mutations may represent a virulent FH strain.

A C to T mutation at nt 1773 was detected in 5 of 7 FH patients (71%) and in 2 of 5 SAH patients (40%), and was the most common mutation among patients with severe hepatitis. This mutation was not described in FH patients in the reports cited above. However, a new TATA-like sequence, TATTA, resulted from the C to T mutation at nt 1773, creating a potential new nuclear protein binding site affecting BCP activity.

Consequently, we analyzed the transcriptional activity of the BCP using various naturally occurring mutations. CAT expression vectors containing the BCP region were constructed and assayed. However, promoter activity was not influenced by either the double mutation at nt 1762 and nt 1764 or by the C to T mutation at nt 1773. The double mutation partially overlaps with a TATA-like sequence from nt 1758 to nt 1762, which may regulate transcription of precore RNA[1]. Thus, the double mutation may reduce precore RNA[6][8][22]. Nishizono et al also failed to document an effect of this double mutation on the BCP activity using a CAT assay[23]. Thus, changes in BCP activity caused by mutations may be detectable only by using the whole HBV genome, not by a CAT expression vector, because the effect of mutation(s) on the BCP activity may be subtle.

Finally, the number of mutations in the BCP region increased with the severity of liver injury, while the BCP sequence in AH patients was very similar to wild-type sequences. It is still unclear whether these HBV variants with BCP sequence heterogeneity are virulent themselves or survive by selection under host immune pressure. This question must be addressed by transfecting HBV variant constructs to evaluate replication efficacy and, if possible, by establishing an animal model for the study of viral-host interactions.

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B型肝炎ウイルスのコアプロモーター活性と急性B型肝炎の重症度との関連

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［目的］劇症肝炎を含む，急性B型肝炎のcore promoterの塩基配列を解析し，さらに，肝炎の重症度と変異株のpromoter活性の相関につき検討を行った．

［対象と方法］劇症肝炎（FH）7例，重症急性肝炎（SAH）5例，急性肝炎（AH）6例を対象とし，core promoter, precore/coreを含む領域をPCR法で増幅したのち，増幅産物をpGEM-5Zへサブクローニングし，最低5クローンの塩基配列を調べた．また，すべての症例のcore promoterを含む領域のPCR産物をCAT enhancer vector（Promega）に挿入し，リポフェクチン法で培養肝細胞（Huh7）にトランスフェクションした後，QUAN-T CAT（Amarsham）を用いてpromoter活性を測定した．

［結果］core promoter領域の変異はFHで平均4.6個，SAHで3.4個，AHで0.5個と，重症化に伴い頻度が増加した．nt 1762とnt 1764のA→T, G→Aのいわゆるdouble mutation（MT62, MT64）は，FH 7例中2例，SAH 5例中3例に認めた．またFH 5例，SAH 2例にnt 1773のC→T（MT73）の変異を認めたが，FHあるいはSAHの全例に共通する変異はなかった．次にcore promoterの変異株のpromoter活性をCAT assayで調べたところ，野生株（WT）で3202±122cpm，MT62, 64, 73：3625±746cpm，MT62, 64：3699±122cpm，MT73：3027±570cpmであり，そのほかの変異のパターンを持つ症例でも，WTと比較しpromoter活性の低下，あるいは亢進のいずれも認められなかった．

［考察］core promoter領域の変異は，重症度と相関することから，肝炎の重症化との関連が示唆された．しかし，我々の用いたアッセイ系ではpromoter活性に差がみられず，重症化の要因は転写活性の変化とは別の機序によると考えられた．