Title Page

Nucleotide mutations associated with hepatitis B e antigen negativity

Short Title: Mutations in HB e antigen negativity

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Abstract

A total of 144 patients with chronic hepatitis B were analyzed to identify new mutations associated with hepatitis B e antigen (HBeAg) negativity, using a full genome sequence analysis. All the patients were Chinese and had hepatitis B virus infection of genotype C. Patients with none of the pre-core or core promoter mutations were significantly ($P< 0.001$) less common in the group with anti-HBe (13%) than in the group with HBeAg (56%). The complete nucleotide sequence was determined in four anti-HBe-positive patients who had neither pre-core nor core promoter mutations and in five HBeAg-positive patients who also had neither of these mutations (the groups were matched for age and sex). Six mutations were found to be significantly more common in the former group than in the latter: G529A (3/4 vs. 0/5), C934A (4/4 vs. 1/5), A1053G (4/4 vs. 1/5), G1915T/A (4/4 vs. 0/5), T2005C/A (4/4 vs. 0/5) and C3026T (3/4 vs. 0/5). Three of the six mutations were significantly more common in the four anti-HBe-positive patients who had neither pre-core nor core promoter mutations, compared to 11 HBeAg-positive patients who had pre-core and core promoter mutations, and also compared to 15 anti-HBe-positive patients who had pre-core and core promoter mutations, further suggesting the specificity of these mutations. Of the six mutations, two resulted in amino acid substitution in the polymerase protein, and one is located near the enhancer I region. The results suggest that the six newly-discovered mutations are associated with HBeAg negativity.

Key words:

hepatitis B e antigen (HBeAg); genotype; nucleotide mutation

Introduction

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide [Maynard, 1990; Maddrey, 2000]. Chronic HBV infection is the cause of up to 50% of cirrhosis and 70-90% of hepatocellular carcinoma (HCC) in China, South-East Asia and Africa [Lok, 1992; Fattovich, 1998], and in Asian countries, almost all patients with chronic HBV infection have been infected perinatally from hepatitis B e antigen (HBeAg)-positive mothers [Okada et al., 1976]. HBeAg is considered to be a marker for viral replication, but some HBeAg-negative patients remain viremic and continue to have active liver disease [Hadziyannis et al., 1983; Lok et al., 1984; Bonino et al., 1986]. Many of these patients are found to have a G to A change at nucleotide 1896, which creates a stop codon (TAG) in the precore (Pre-C) open reading frame, which in turn prevents translation of the Pre-C protein and aborts HBeAg production [Carman et al., 1989]. Other patients have mutations in the core promoter (CP) region, including an A to T mutation at nucleotide 1762 and a G to A mutation at nucleotide 1764 [Okamoto et al., 1994]. In vitro studies of this double mutation show decreased transcription of Pre-C messenger RNA and hence a resultant decrease in HBeAg production by 70% [Buckwold et al., 1996; Chan et al., 1999]. A recent follow-up study on Pre-C and CP mutations has also shown that the presence of these mutations is useful for predicting seroconversion [Yamaura et al., 2003].

Besides the G1896A mutation and the A1762T/G1764A mutation, a number of point mutations, as well as deletions and insertions of nucleotides, have been detected in the Pre-C region and CP region that could correlate with seroconversion [Okamoto et al., 1990; De Castro et al., 2001]. In the present study, we analyzed the complete HBV genome to search for other nucleotide mutations associated with

HBeAg negativity, in addition to mutations in the Pre-C and CP regions.

Materials and Methods

Patients

A cohort of 193 Chinese patients with chronic HBV infection who visited the Liver Disease Clinic of the Second Hospital of HeBei Medical University in Shijiazhuang city, North China, between June and August 2001 were enrolled in the study. These patients comprised 124 men and 69 women and had a median age of 29.1 years old (range: 5-73 years old). Patients who were co-infected with hepatitis C or D virus or human immunodeficiency virus and patients with other concomitant causes of chronic liver disease were excluded. According to the consensus diagnostic criteria for HBV infection, 182 patients were diagnosed with chronic hepatitis B. The remaining 11 patients had persistently normal alanine aminotransferase (ALT: normal range 10 - 21 IU/L) levels, suggesting an inactive HBV carrier stage. None of the 193 patients were treated with anti-viral agents such as interferon or lamivudine. Of the 193 patients, 169 (87.6%) were of genotype C, 21 (10.9%) of genotype B and 3 (1.5%) of genotype A. For the mutation analysis, 144 patients who were positive for either HBeAg or anti-HBe were selected from the 169 genotype C patients. Informed consent was obtained from each patient.

Conventional HBV markers and genotyping of HBV

Hepatitis B surface antigen (HBsAg), HBeAg and anti-HBe were measured using commercially available enzyme immunoassay kits (Abbott Japan, Tokyo, Japan). Serum concentration of HBV DNA was measured using the AMPLICOR HBV Monitor test (Roche Diagnostics K.K., Tokyo, Japan), which has a quantitative range

of 2.6 - 7.6 log copies/mL. When the concentration to be tested was beyond this range, the actual concentration was determined using a serum sample diluted 100-fold with normal human serum. The HBV genotype was determined using the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers [Mizokami et al., 1999].

Determination of Pre-C and CP mutations

The 1,896th nucleotide in the Pre-C region of G or A was detected with an enzyme-linked mini-sequence assay kit (Roche Diagnostics, Tokyo, Japan), and the results were expressed as the percentage mutation rate, as defined by Aritomi et al. [1998]. If the mutation rate was 0%, the genotype was considered to be Pre-C mutation-negative, while a Pre-C mutation-positive genotype was recorded when the mutation rate exceeded 0%. The double mutation in the CP region (A1762/T1764) was detected using an HBV CP mutation detection kit (Smitest: Genome Science Laboratories, Tokyo, Japan), and the results were classified into three categories: wild, mixed and mutant types. A wild type genotype was considered to be CP mutation-negative, while mixed and mutant types were recorded as CP mutation-positive genotypes. The detection limits of the pre-C and the CP mutation detection kits are both 1000 copies/ml.

Determination of nucleotide sequence

The complete genome sequence was determined according to the method reported by Rokuhara et al [2000]. Briefly, nucleic acids were extracted from a serum sample of 100µl with a DNA/RNA extraction kit (Smitest EX-R&D: Genome Science

Laboratories Co., Ltd., Tokyo, Japan). Two microliters of each DNA solution were used for amplification by PCR. The reaction was carried out in 25 µl of PCR-mixture containing 250 µmol/L of each dNTP, 1x PCR buffer [50mmol/L KCL, 10mmol/L Tris-HCL (PH 8.3), 1.5mmol/L MgCl2, 0.001% gelatin], 0.25U EX-Taq DNA polymerase (TaKaRa, Tokyo), and 0.25 µM of a primer pair. The PCR was initiated using the hot-start technique.

To determine the full-length nucleotide sequence of HBV, two fragments (fragments A and B) were amplified by PCR, using the primers shown in Table 1. Fragment A (1498 bases in length; nt 457 – nt 1954) was amplified with nested pairs of outer (SB1 and CB2) and inner primers (SB3 and CB4), while fragment B (2162 bases in length; nt 1611 – nt 557), was amplified with nested pairs of outer primers (es2 and PS4) and inner primers (is2 and PS3). The first round of PCR was performed with an outer primer set for 40 cycles (94˚C for 1.5 min, 55˚C for 1 min, and 72˚C for 2 min), and was followed by an extension reaction at 72˚C for 7 min. The second round was performed with an inner primer set for 30 cycles, and was also followed by an extension reaction. PCR products were subjected to electrophoresis on a 1.0% agarose gel with ethidium bromide staining and visualization with an UV transilluminator. The band containing the target sequence was removed and DNA was isolated using GFX™ PCR DNA and a Gel Band Purification kit (Amersham Pharmacia Biotech Inc., Piscataway, NJ). The nucleotide sequence was directly determined by the dideoxy method, using the sequencing primers shown in Table 1. The accuracy of the sequence was ensured by comparison of the sequence data for the complete genome obtained with sense-sequencing primers and that obtained with anti-sense-sequencing primers.

Statistical analysis

Mann-Whitney's U test was utilized for quantitative data, and Fisher's exact test and a Chi-square test were used for qualitative data. P values less than 0.05 were considered significant. Analyses were performed using SPSS version 10.0J (SPSS Inc., Chicago, IL).

Results

Of the 144 patients selected for the mutation analysis, 90 (62.5%) were HBeAg-positive and the remaining 54 (37.5%) were anti-HBe-positive. The clinical and virological backgrounds of the two groups of patients are compared in Table 2. The 90 HBeAg-positive patients tended to be younger and have a higher concentration of HBV DNA than the 54 anti-HBe patients. Patients with none of the Pre-C and CP mutations were significantly ($P< 0.001$) more common in the HBeAg-positive patients (56%) than in the anti-HBe-positive patients (13%).

A comparison of the clinical backgrounds of 7 anti-HBe-positive patients who had neither Pre-C nor CP mutations and 47 anti-HBe-positive patients who had at least one of the mutations is shown in Table 3. Distributions of age, gender, ALT level, and HBV DNA concentration did not differ between the two groups.

Nucleotide sequences of the complete genome were determined in 4 out of 7 anti-HBe-positive patients who had neither Pre-C nor CP mutations and in 5 out of 50 HBeAg-positive patients who also had neither mutation. All nine of the determined genome sequences had nucleotide lengths of 3215 bases, and thus there were no insertions or deletions. When the full genome sequences were compared, the six mutations shown in Table 4 were significantly more common in the 4 anti-HBe-positive patients than in the 5 HBeAg-positive patients. The positions of the

six mutations in the HBV genome are shown in Figure 1. Of the four mutations located in the polymerase gene, the G529A and C934A mutations cause amino acid substitutions in the polymerase protein. The C3026T mutation does not cause an amino acid substitution in the polymerase, but rather in the pre-S1 protein, while the A1053G mutation does not lead to an amino acid substitution, but the mutation is located near the enhancer I region. The G1915T/A and T2005C/A mutations are located in the core gene, but do not result in an amino acid substitution. Patients with at least one of the 3 mutations (G529A, C934A, and A1053G) which might affect HBV replication had a significantly (P=0.029) lower level of HBV DNA (n=22, median 5.3 copies/ml, range 3.8-8.9) than those patients who had no mutations (n=13, median 8.5 copies/ml, range 3.8-8.9).

To further analyze the specificity of the six mutations, these mutations were also determined in 11 HBeAg-positive patients who were positive for Pre-C and CP mutations and in 15 anti-HBe-positive patients who were also positive for Pre-C and CP mutations. The frequencies of the six mutations were compared between groups of patients classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status. Three (G1915T/A, T2005C/A and C3026T) of the six mutations were found to be significantly more common in anti-HBe-positive patients who had neither a Pre-C nor a CP mutation than in the two groups of patients with Pre-C and CP mutations, as shown in Table 5.

The nucleotide sequence data reported in this paper have been registered in the DDBJ/EMBL/GenBank nucleotide sequence databases, with the accession numbers AB198076-84.

Discussion

Studies to date have shown that the stop codon mutation in the Pre-C region (G1896A) and the double mutation in the CP region (A1762T/G1764A) are independently associated with the seroconversion of HBeAg, and that the Pre-C mutation is more directly associated with seroconversion than the core promoter mutation [Okamoto et al., 1994; Yamaura et al. 2003]. Only a small number of anti-HBe-positive patients (13%) were both negative for the Pre-C and CP mutations, and in the present study this rate was significantly lower than that (56%) in HBeAg-positive patients. These results are consistent with previous reports, suggesting that the two mutations are the main causes of seroconversion. However, there are also patients in whom HBeAg secretion discontinues without appearance of Pre-C and/or CP mutations. Thus, we speculated that some other mutations might be associated with HBeAg seroconversion. A variety of other mutations in the CP and Pre-C regions have been detected in previous studies [Carman et al., 1989; Tillmann et al., 1995; Baumert et al., 1996; Laras et al., 1998; Chan et al., 2000; De Castro et al., 2001; Yoo et al., 2003], but other regions of the HBV genome have not been analyzed sufficiently for mutations associated with HBeAg seroconversion.

When the full nucleotide sequences of HBV genomes of HBeAg-positive and anti-HBe-positive patients with neither Pre-C nor CP mutations were compared, six mutations (G529A, C934A, A1053G, G1915T/A, T2005C/A, C3026T) were found to be significantly more common in the anti-HBe-positive patients. The six mutations were also more common in anti-HBe-positive patients who had neither Pre-C nor CP mutations than in HBeAg-positive patients or in anti-HBe-positive patients who had Pre-C and CP mutations, with the results being statistically significant for three (G1915T/A, T2005C/A, C3026T) of the six mutations. These results suggest that the six mutations are associated with HBeAg negativity.

The mechanisms through which the six mutations facilitate HBeAg negativity were not investigated in the present study. However, some possible mechanisms can be speculated upon, based on the locations of these mutations in the HBV genome. The G529A and C934A mutations cause amino acid substitutions in the polymerase protein. Thus, these two mutations may attenuate HBV replication through changes in the enzymatic activity of the polymerase. The A1053G mutation is located near the enhancer I region, which may affect the replication of HBV [Bock et al., 2000]. Patients who had at least one of the 3 mutations associated with HBV replication tended to have a lower level of HBV DNA than those who had none of these mutations, providing further support for a replication-associated mechanism. It has been reported that amino acid substitutions in immunogenic epitopes in the core protein are found most frequently during or after seroconversion from HBeAg to anti-HBe [Carman et al., 1995; Akarca and Lok, 1995]. We found two mutations in the core gene, but these mutations did not cause amino acid substitutions. Thus, the mechanisms through which the G1915T/A and T2005C/A mutations exert their effects remains unclear.

In anti-HBe-positive patients, clinical backgrounds, including mean age, gender distribution, ALT level and HBV DNA level, were similar for patients with and without Pre-C and/or CP mutations. Although these comparisons were cross-sectional, the results suggest that mutations other than those in the Pre-C and CP regions have a similar impact in patients in whom seroconversion occurs, compared to Pre-C and CP mutations.

The six mutations identified in the present study have not been previously reported. These mutations are thought to be associated with HBeAg negativity because they were specifically found in anti-HBe-positive patients with neither a

Pre-C nor a CP mutation. However, several issues remain to be resolved to clarify the real significance of the six mutations, including the mechanisms through which they facilitate HBeAg negativity, their universality in genotypes other than genotype C, and their clinical relevance. Furthermore, it is possible that immune-based selection pressures that cause loss of HBeAg are responsible for the selection of the mutations identified in the present study [Locarnini, 2004]. Therefore, we are unable to conclude that the newly found mutations are definitely associated with seroconversion, but they do provide new clues regarding the nature of seroconversion.

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References

Akarca US, Lok AS. 1995. Naturally occurring core-gene-defective hepatitis B viruses. J Gen Virol 76(Pt 7):1821-1826.

Aritomi T, Yatsuhashi H, Fujino T, Yamasaki K, Inoue O, Koga M, Kato Y, Yano M, 1998. Association of mutations in the core promoter and precore region of hepatitis virus with fulminant and severe acute hepatitis in Japan. J Gastroenterol Hepatol 13:1125-1132.

Baumert TF, Rogers SA, Hasegawa K, Liang TJ. 1996. Two core promoter mutations in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. J Clin Invest 98:2268-2276.

Bock CT, Malek NP, Tillmann HL, Manns MP, Trautwein C. 2000. The enhancer I core region contributes to the replication level of hepatitis B virus in vivo and in vitro. J Virol 74:2193-2202.

Bonino F, Rosina F, Rizzetto M, Rizzi R, Chiaberge E, Tardanico R, Callea F, Verme G. 1986. Chronic hepatitis in HBsAg carriers with serum HBV-DNA and anti-HBe. Gastroenterology 90:1268-1273.

Buckwold VE, Xu Z, Chen M, Ou JH. 1996. Effects of a naturally occurring mutation in the hepatitis virus basal core promoter on precore gene expression and viral replication. J Viral 70:5845-5851.

Carman WF, Jacyna MR, Hadziyannis S, Karayiannis P, McGarvey MJ, Makris A, Thomas HC. 1989. Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. Lancet ii :588-590.

Carman WF, Thursz M, Hadziyannis S, McIntyre G, Colman K, Gioustoz A, Fattovich G, Alberti A. 1995. Hepatitis B e antigen negative Chronic active hepatitis: hepatitis B virus core mutations occur predominantly in known antigenic determinants. J

Viral Hepat 2:77-84.

Chan HLY, Hussain M, Lok ASF. 1999. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 29:976-984.

Chan HLY, Leung NW, Hussain M, Wong ML, Lok AS. 2000. Hepatitis B e antigen-negative chronic hepatitis B in Hong Kong. Hepatology 31:763-768.

De Castro L, Niel C, Gomes SA. 2001. Low frequency of mutations in the core promoter and precore regions of hepatitis B virus in anti-HBe positive Brazilian carriers. BMC Microbiology 1(1):10.

Fattovich G. 1998. Progression of hepatitis B and C to hepatocellular carcinoma in Western countries. Hepatogastroenterology 45:1206-1213.

Hadziyannis SJ, Lieberman HM, Karvountzis GG, Shafritz DA. 1983. Analysis of liver disease, nuclear HBcAg, DNA viral replication, and hepatitis B virus DNA in liver and serum of HBeAg vs. anti-HBe positive carriers of hepatitis B virus. Hepatology 3:656-662.

Laras A, Koskinas J, Avgidis K, Hadziyannis SJ. 1998. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. J Viral Hepatitis 5:241-248.

Locarnini S. 2004. Molecular Virology of Hepatitis B Virus. Semin Liver Dis 24 suppl 1:3-10.

Lok ASF, Hadziyannis S, Weller IVD, Karvountzis MV, Monjardino J, Karayiannis P, Montano L, Thomas HC. 1984. Contribution of low level HBV replication to continuing inflammatory activity in patients with anti-HBe positive chronic hepatitis B virus infection. Gut 25:1283-1287.

Lok ASF. 1992. Natural history and control of perinatally acquired hepatitis B virus

infection. Dif Sis 10:46-52.

Maddrey WC. 2000. Hepatitis B: an important public health issue. J Med Virol 61:362-366.

Maynard JE. 1990. Hepatitis B: global importance and need for control. Vaccine 8(Suppl):S18-20.

Mizokami M, Nakano T, Orito E, Tanaka Y, Sakugawa H, Mukaide M, Robertson BH. 1999. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS lett 450:66-71.

Okada K, Kamiyama I, Inomata M, Imai M, Miyakawa Y. 1976. E antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B virus to their infants. N Engl J Med 294:746-749.

Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, Tsuda F, Tanaka T, Miyakawa Y, Mayumi M. 1990. Hepatitis B viruses hosts along with seroconversion to the antibody against e antigen. J Virol 64:1298-1303.

Okamoto H, Tsuda F, Akahane Y**,** Sugai Y, Yoshiba M, Moriyama K, Tanaka T, Miyakawa Y, Mayumi M. 1994. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. J Virol 68:8102-8110.

Rokuhara A, Tanaka E, Yagi S, Mizokami M, Hashikura Y, Kawasaki S, Kiyosawa K. 2000. De novo infection of hepatitis B virus in patients with orthotopic liver transplantation: analysis by determining complete sequence of the genome. J Med Virol 62:471-478.

Tillmann H, Trautwein C, Walker D, Michitaka K, Kubicka S, Boker K, Manns M. 1995. Clinical relevance of mutations in the precore genome of the hepatitis B virus. Gut 37:568-573.

Yamaura T, Tanaka E, Matsumoto A, Rokuhara A, Orii K, Yoshizawa K, Miyakawa Y, Kiyosawa K. 2003. A case-control study for early prediction of hepatitis B e antigen seroconversion by hepatitis B virus DNA levels and mutations in the precore region and core promoter. J Med Virol 70:545-552.

Yoo BC, Park JW, Kim HJ, Lee DH, Cha YJ, Park SM. 2003. Precore and core promoter mutations of hepatitis B virus and hepatitis B e antigen-negative chronic hepatitis B in Korea. J Hepatol 38:98-103.

Table 1. Primers used for PCR and sequencing of HBV DNA

Primer		Sequence	nt position
Primers for PCR of fragment A			
SB1	sense	5-TGCTGCTATGCCTCATCTTC	(414-433)
CB2	anti-sense	5-GGAAAGAAGTCAGAAGGCAA	(1974-1955)
SB3	sense	5-AGGTATGTTGCCCGTTTCTC	(457-476)
CB4	anti-sense	5-AAAAGAGAGTAACTCCACAG	(1954-1935)
Primers for PCR of fragment B			
es2	sense	5-ACGTCGCATGGAGACCACCG	(1601-1620)
PS4	anti-sense	5-CAGTTTCCGTCCGAAGGTTTTG	(594-573)
is2	sense	5-GAGACCACCGTGAACGCCCA	(1611-1630)
PS3	anti-sense	5-GAAACATAGAGGTGCCTTGAGCAG	(557-534)
Primers for sequencing			
SB3	sense	5-AGGTATGTTGCCCGTTTCTC	(457-476)
as1	anti-sense	5-TGCGAAAGCCCAGGATGATG	(631-612)
s2	sense	5-TGCGAAAGCCCAGGATGATG	(760-783)
as2	anti-sense	5-AGTTGGCGAGAAAGTGAAAGCCTG	(1107-1084)
s3	sense	5-CTCTGCCGATCCATACTGCGGAA	(1256-1278)
as3	anti-sense	5-CGGGACGTAGACAAAGGACGT	(1434-1414)
is2	sense	5-GAGACCACCGTGAACGCCCA	(1611-1630)
ea1	anti-sense	5-TGAAAAAGTTGCATGGTGCTGGTG	(1827-1804)
s4	sense	5-TATCGGGAGGCCTTAGAGTCTCCG	(2012-2035)
as4	anti-sense	5-ATAGGGGCATTGGTCT	(2314-2298)
s5	sense	5-CGCAGAAGATCTCAATCTCGG	(2417-2437)
as5	anti-sense	5-GGATAGAACCTAGCAGGCAT	(2654-2635)
s6	sense	5-GGGTCACCATATTCTTGGGAA	(2814-2834)
as6	anti-sense	5-GGGTTGAAGTCCCAATCTGGATT	(2987-2965)
is1	sense	5-AAGCTCTGCTAGATCCCAGAGT	(18-39)
ea2	anti-sense	5-TAGAAAATTGAGAGAAGTCCACCA	(280-257)
s1	sense	5-CATCCTGCTGCTATGCCTCATC	(409-430)
as1	anti-sense	5-TGCGAAAGCCCAGGATGATG	(631-612)

Nucleotides are numbered from the unique *Eco*RI site of HBV.

Table 2. Comparison of clinical and virological backgrounds of patients with HBeAg and those with anti-HBe.

	HBeAg-positive n=90	Anti-HBe-positive n=54	P
Age [a]	25 (5-53)	36 (11-73)	< 0.001 [b]
Gender (M:F)	58:32	30:24	>0.2 [c]
ALT [a]	89 (11-2100)	62 (13-458)	>0.2 [b]
HBV DNA (log copies/mL) [a]	8.3 (4.4-7.9)	5.0 (3.2-8.8)	< 0.001 [b]
Pre-C/CP mutations			
Both negative	50 (56%)	7 (13%)	< 0.001[c]
Pre-C mutation only	13 (14%)	20 (37%)	
CP mutation only	12 (13%)	5 (9%)	
Both positive	15 (17%)	22 (41%)	

[a] Data are expressed as median values (range).

[b] Mann-Whitney test.

[c] Chi-square test.

Table 3. Comparison of clinical and virological backgrounds of anti-HBe-positive patients with neither Pre-C nor CP mutations and anti-HBe patients with at least one of these mutations.

	Pre-C and CP mutation-negative n=7	Pre-C and/or CP mutation-positive n=47	P
Age [a]	37 (18-60)	36 (11-73)	>0.2 [b]
Gender (M:F)	4:3	26:21	>0.2 [c]
ALT [a]	44 (18-86)	65 (13-458)	0.17 [b]
HBV DNA (log copies/mL)*	4.7 (3.3-5.5)	5.0 (3.2-8.8)	>0.2 [b]

[a] Data are expressed as median values (range).

[b] Mann-Whitney test.

[c] Chi-square test.

Table 4. Comparison of full nucleotide sequences of HBV with neither Pre-C nor CP mutations for HBeAg-positive and anti-HBe-positive patients.

Nucleotide mutation	Amino acid substitution (viral protein)	HBeAg Pre-C and CP mutation-negative n=5	Anti-HBe Pre-C and CP mutation-negative n=4	P
G529A	D480N (P) None (S)	0	3	0.048
C934A	L615I (P)	1	4	0.040
A1053G	None (P)	1	4	0.040
G1915T/A	None (C)	0	4	0.008
T2005C/A	None (C)	0	4	0.008
C3026T	A60V (Pre-S1) None (P)	0	3	0.048

Six mutation sites with significant differences are shown.

Data are expressed as the number of positives.

Statistical analysis was performed with a chi-square test.

P: polymerase protein, S: surface protein, C: core protein, Pre-S1: pre-surface 1 protein.

Table 5. Comparison of 6 mutations among three groups classified according to their HBeAg/anti-HBe and Pre-C/CP mutation status.

Mutation site	Anti-HBe Pre-C and CP mutation-negative n=4	HBeAg Pre-C and/or CP mutation-positive n=11	Anti-HBe Pre-C and/or CP mutation-positive n=15
G529A	3	3	3
C934A	4	6	10
A1053G	4	4	9
G1915T/A	4 [a]	3	1
T2005C/A	4 [b]	3	4
C3026T	3 [c]	0	1

Data are expressed as the number of positives.

Statistical analysis was performed with Fisher's exact test.

[a] P=0.026 vs. 11 patients with HBeAg, and P=0.001 vs. 15 patients with anti-HBe.

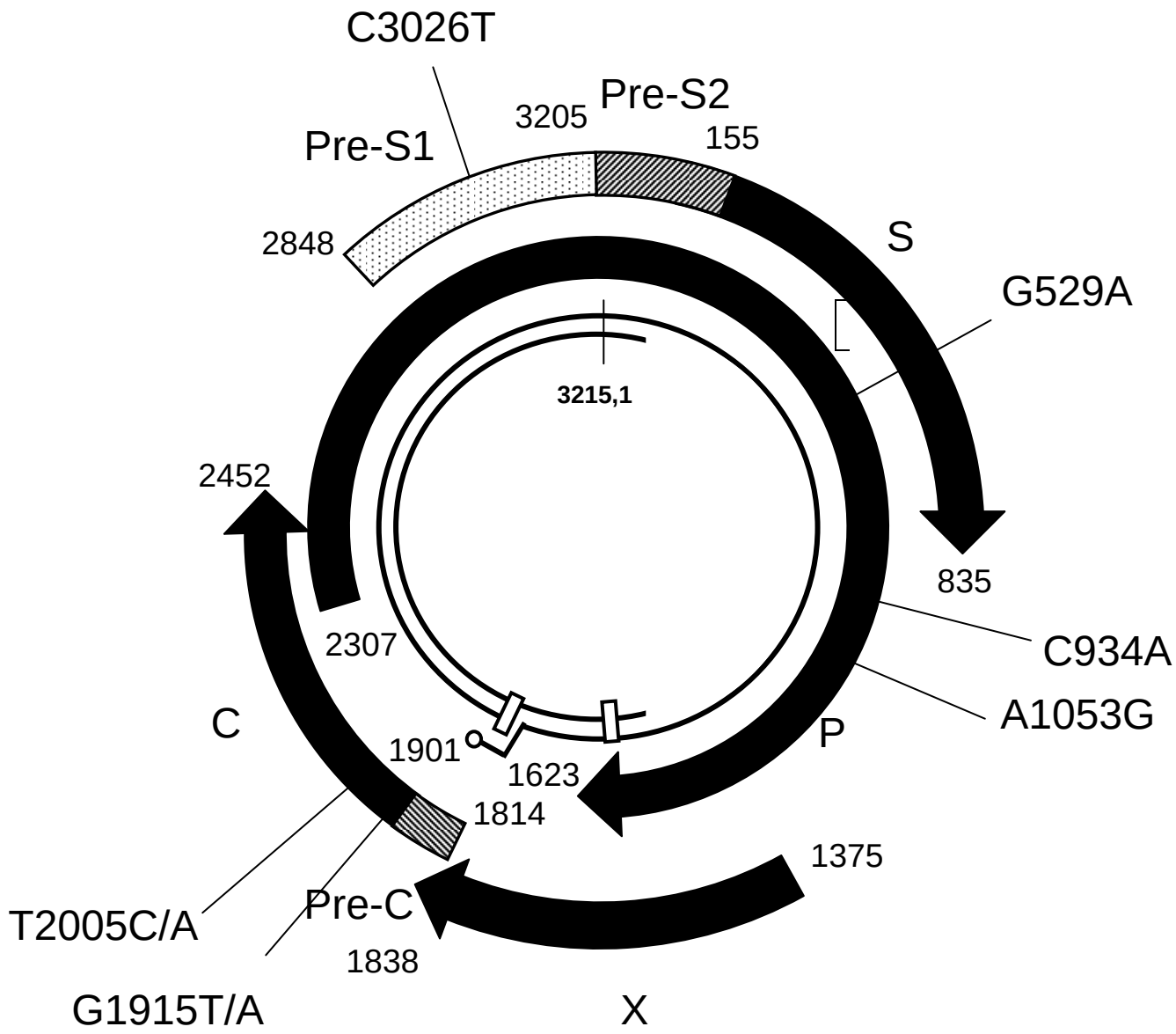
[b] P=0.026 vs. 11 patients with HBeAg, and P=0.018 vs. 15 patients with anti-HBe.

[c] P=0.009 vs. 11 patients with HBeAg, and P=0.016 vs. 15 patients with anti-HBe.

Other comparisons were not statistically significant.

Figure Legend

Fig. 1: Organization of the hepatitis B virus genome (genotype C) and the approximate positions of the 6 nucleotide mutations in the HBV genome. The inner circles represent the minus and plus DNA strands of the viral genome. The different open reading frames encoded by the genome, designated as S, C, P and X, are indicated by the arrows. Abbreviations: S, surface antigen; C, core; P, polymerase; Pre-C, precore.


C3026T
Pre-S2
3205
155
Pre-S1
2848
S
G529A
3215,1
2452
835
2307
C934A
A1053G
C
P
1901
1623
1814
1375
T2005C/A
Pre-C
1838
G1915T/A
X