Detection and significance of a G1862T variant of hepatitis B virus in Chinese patients with fulminant hepatitis

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The prevalence of a G1862T variant of hepatitis B virus (HBV) has been investigated in patients with fulminant hepatitis and chronic liver disease, using primer mismatch amplification, followed by restriction fragment length polymorphism analysis. This variant was five times more common in patients with fulminant hepatitis (13.7%, 7 of 52) than in chronic carriers (2.5%, 2 of 81). The G → T substitution at position 1862 leads to an amino acid change in codon 17 of the precore protein of the virus, which is part of a signal peptidase recognition motif. Variants with this mutation were only seen in patients infected with genotype B. In vitro translation experiments showed that this variant has greatly reduced capacity to produce hepatitis B e antigen (HBeAg) from its precore protein precursor. Furthermore, 88.5% of patients with fulminant hepatitis had mutations that are known to be associated with abrogated or reduced production of HBeAg. This suggests that, following HBV infection, the absence or reduced amounts of HBeAg may be a contributing factor in fulminant disease.

Introduction

Over the last 10 years, a number of mutations have been described which affect different regions of the hepatitis B virus (HBV) genome. These include nucleotide substitutions that may alter protein expression. An example of this is the translational stop codon in the precore region, which abrogates hepatitis B e antigen (HBeAg) production (Carman et al., 1989; Brunetto et al., 1989). Other nucleotide substitutions translate into amino acid changes that affect antigenicity, as in the case of the α antigenic determinant of the HBV surface antigen (HBsAg) (Carman et al., 1990; Yamamoto et al., 1994; Svedig-Tonekaboni et al., 2000), whilst others may affect regulatory elements of the virus, as does the double mutation A1762T and G1764A in the basic core promoter (BCP) (Okamoto et al., 1994; Sato et al., 1995; Takahashi et al., 1995).

Failure to produce HBeAg has also been attributed to mutations in the precore initiation codon, conversion of the second codon to a termination codon and to insertions or deletions in the precore region (Okamoto et al., 1990; Fiordalisi et al., 1990; Raimondo et al., 1990; Santantonio et al., 1991; Tran et al., 1991). The net result of these mutations is either failure to produce or premature termination of precore/core protein synthesis and, therefore, that of HBeAg. Another
mutation with possible repercussions on HBeAg production is a G1862T change, which has been described in one or two cases in studies investigating precore region variants (Sant-antonio et al., 1991; Tran et al., 1991; Laskus et al., 1993, 1994b; Li et al., 1993; Clementi et al., 1993; Horikita et al., 1994; Carman et al., 1995; Loriot et al., 1995; Valliammai et al., 1995; Zhang et al., 1996; Kramvis et al., 1997). This position affects one of the amino acids of the signal peptidase recognition motif in the precore peptide (Santantonio et al., 1991; Loriot et al., 1995; Valliammai et al., 1995; Kramvis et al., 1997). The prevalence of this mutation in a bigger cohort of chronic HBV carriers and patients with fulminant hepatitis has not been fully investigated. Moreover, the functional importance of this mutation remains unproved.

In this study, we have looked for this mutation in 133 patients with acute/subfulminant hepatitis or chronic HBV infection and correlated its presence with disease manifestation. We have also examined its association, or not, with other common mutations in this region of the genome. Moreover, the effect of this mutation on the processing of the precore protein to HBeAg was studied by in vitro translation and Western blot analysis following transfection of HepG2 cells.

**Methods**

| Patients. Serum samples were obtained from 52 patients with fulminant hepatitis B seen in four referral hospitals in China. Fulminant hepatic failure was defined as severe acute liver disease, with rapid onset of hepatic encephalopathy, in an individual without underlying liver disease (Hoofnagle et al., 1995). The demographic data for these patients are given in Table 1. All non-fulminant hepatitis patients were attendees at a regional hospital and these included 44 patients with chronic active liver disease and 37 patients with cirrhosis. All subjects studied were HBsAg-positive for more than 6 months and had been followed up at the hospital at intervals of 6–12 months. Diagnosis of chronic active hepatitis or cirrhosis was also documented by histological examination of liver biopsy material. All patients were negative for markers of hepatitis C virus (HCV), hepatitis delta virus (HDV) and human immunodeficiency virus (HIV). Patients were recruited following informed consent and with the approval of the protocol by the Institutional Review Board of the Nanfang Hospital, Guangzhou, China.

**Serological testing.** Serum stored at $\sim$ 70 °C was tested for HBV serological markers, including HBsAg, anti-HBs, HBeAg and anti-HBe, as well as anti-HCV, anti-HDV and anti-HIV, by commercially available enzyme immunoassays (Abbott Laboratories). HBV genotyping was performed by restriction fragment length polymorphism (RFLP) analysis, as described by Lindh et al. (1998, 1999).

**Amplification and RFLP analysis.** HBV DNA was extracted from serum as described previously (Hou et al., 1995). The G1862T mutation was detected by mismatch PCR amplification followed by RFLP analysis. First-round PCR was performed with primers P1 $5'\text{ATTAGGTTAAA-GGTCTTGTG, nt 1753–1772}$ and P2 $5'\text{CCAAACACAGAATAGCCTTTGCC, nt 2086–2067}$. Second-round PCR employed the nested primers P3 $5'\text{TGGGAGGCTGTAGGCATAAAC, nt 1774–1794}$ and P4 $5'\text{AAGGCCACACGTGGGAGCTTJAA, nt 1885–1863}$. The latter primer had a single nucleotide mismatch (underlined), which, in the presence of the G1862T change, creates a DraI (TITAAAA) restriction site. The digested PCR amplicons can thus be used to distinguish between wild-type and G1862T variants, following electrophoresis on a 3% agarose gel and visualization by ethidium bromide staining.

**Sequencing and sequence data analysis.** RFLP results were confirmed by direct sequencing using an ABI automatic sequencer. For this purpose, the precore/core and BCP regions were amplified using primers P7 $5'\text{TCCCTCGCCATCCATACTG, nt 1254–1273}$ and BC1 $5'\text{GGGAAAGTCAGAAGGCA, nt 1747–1755}$ or P8 $5'\text{TTCGGGATCCATACTGG, nt 1257–1276}$ and BC1, as described previously (Hou et al., 1999). P9 $5'\text{CAAGGGTCCTGTAAGGGA-CTCTT, nt 1643–1667}$ and BC1 were used as sequencing primers. Cloning, followed by sequencing of up to 10 clones, was undertaken in some cases to determine whether the virus was present as a mixed population of variants and wild-type. Nucleotide and amino acid sequence alignments were performed with the ﬁgure and protein software packages (Hitachi).

**In vitro translation.** The entire precore/core region was amplified from a wild-type isolate (pYN) and a G1862T variant (pLC202), using the primers and conditions described previously (Alba et al., 1997). These were cloned into the expression vector pTM3 (a gift of B. Moss, National Institutes of Health, Bethesda, Maryland, USA), which is under the control of the T7 promoter (Alba et al., 1997). A plasmid containing the core region from another wild-type isolate (pTH101C) was used as a control. In vitro translation was performed using a TNT Coupled Reticulocyte Lysate system (Promega) in the presence and absence of canine pancreatic microsomal membranes, according to the manufacturer’s instructions. The same amount of plasmid was used in all reactions, following spectrophotometric quantification of DNA.

**Cell transfection.** This was achieved using the EBO vector (a gift from F. V. Chisari, Scripps Research Institute, La Jolla, California, USA), which is an Epstein–Barr virus-based construct designed for stable expression in eukaryotic cells (Canfield et al., 1990; Guilhot et al., 1992). Initially, a construct containing wild-type genotype B precore/core sequences (accession no. AF282918) was used as template for PCR amplification of this region with primers Peel $5'\text{CTGAAGGTCTAC-CAATTTTTCACCTCTGC, nt 1814–1834}$ and Peer $5'\text{GCGGCTACAATATTGAGATTCGCC, nt 2452–2435}$, containing restriction sites for HindIII and KpnI, respectively (underlined). Amplification

<p>| Table 1. Demographic, biochemical, serological and genomic mutation data of the 52 patients with fulminant HBV infection |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group 1 (n = 18)</th>
<th>Group 2 (n = 34)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Outcome</td>
<td>Survived</td>
<td>Died</td>
</tr>
<tr>
<td>M/F</td>
<td>18/0</td>
<td>25/9</td>
</tr>
<tr>
<td>Median age (y)</td>
<td>35 ± 13</td>
<td>44 ± 13</td>
</tr>
<tr>
<td>HBeAg-positive</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Anti-HBe-positive</td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>HBeAg/anti-HBe-negative</td>
<td>7</td>
<td>10</td>
</tr>
<tr>
<td>Genotype: B/C/other</td>
<td>14/4/0</td>
<td>13/21/0</td>
</tr>
<tr>
<td>Median ALT (IU/l)</td>
<td>1129 ± 684</td>
<td>1001 ± 514</td>
</tr>
<tr>
<td>Median bilirubin</td>
<td>390 ± 143</td>
<td>340 ± 221</td>
</tr>
<tr>
<td>A1762T/G1764A</td>
<td>7</td>
<td>17</td>
</tr>
<tr>
<td>G1896A</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>G1862T</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>
conditions were 93 °C for 2 min, followed by 30 cycles of denaturation for 40 s at 93 °C, annealing for 40 s at 55 °C and extension for 70 s at 72 °C. The resulting amplicon was ligated into vector pGEM-T Easy and was then used as template to generate amplicons with various mutations by splice overlap extension PCR (Ho et al., 1989; Aiba et al., 1997). The mutated amplicons were next digested and then ligated into linearized EBO vector using the HindIII/KpnI sites. Plasmids constructed in this fashion included, apart from wild-type, the variants G1862T, G1896A, G1899A and G1896A/G1899A. The primers used to generate amplicons with these mutations were, respectively: M1 (5’ CATGTCCT- AICTTTCAAGCCTC) and M2 (5’ GAGGCTTGAAAAGTAGGACA- TG); M3 (5’ GGATGGCATTTAGGCACTGCAG) and M4 (5’ GTGCA- TGCCCTAAAGCCACCC); M5 (5’ GGGTTTGGGACATGGACAC- TTG) and M6 (5’ CAATGTCCATGCTCCAAAGCC); and M7 (5’ CCTTGGGTGGCTTTAGGACATGGACACCTG) and M8 (5’ GG- GTCATGTCCATGCTCCAAAGCCACCC). Mutated nucleotides are underlined. The presence of these mutations in the relevant constructs was confirmed by sequencing.

Lysates of HepG2 cells transfected with these constructs were collected 3 days after transfection and, after SDS–PAGE, analysed by Western blot. Proteins were transferred to nitrocellulose membranes, blocked in blocking solution for 1 h at room temperature and detected using a mouse anti-HBe monoclonal antibody (Ferns & Tedder, 1984; Gao & Yao, 1994) followed by horseradish peroxidase-conjugated goat anti-mouse IgG.

Results

Detection of the G1862T variant by mismatch PCR

The G1862T variant was detected by primer mismatch PCR amplification, followed by RFLP analysis of amplicons predigested with DraI. This restriction endonuclease had no effect on wild-type amplicons (111 bp), whilst the G1862T variant amplicons yielded two bands, 90 and 21 bp in length. The G1862T change was detected in 7 of 52 patients with fulminant hepatitis (13–5%), in 1 of 37 patients with liver cirrhosis (2–7%) and 1 of 44 patients with active liver disease (2–3%). No wild-type amplicons were obtained from any of the patients with the G1862T variant.

Verification of results

The reliability of the PCR/RFLP detection method was verified by sequencing all 52 fulminant hepatitis samples and 34 samples from the control groups, including the two that were positive for the G1862T change. The fragment that was sequenced extended from position 1742 to 1903 and included the BCP region and the entire precore region. These sequences have been deposited in GenBank (accession nos AF495662–AF495713). There was complete concordance between the two methods as far as the detection of the G1862T change was concerned.

The G1862T change and its relation to other genomic mutations

Table 1 summarizes the serological, biochemical, virological and genomic mutation data obtained from 52 patients with fulminant hepatitis. The A1762T/G1764A mutation was seen in 24 patients and two further patients had deletions in this region (Fig. 1). In addition, 17 of these 26 patients with core promoter mutations also had the G1896A stop codon change in the precore region. There were 30 patients in all with the
Table 2. Correlation of various genomic mutations with HBe status in patients with fulminant hepatitis

<table>
<thead>
<tr>
<th>Genomic variant</th>
<th>HBeAg-positive</th>
<th>Anti-HBe-positive</th>
<th>No e markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>G1862T</td>
<td>1</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>G1896A</td>
<td>2</td>
<td>4</td>
<td>7</td>
</tr>
<tr>
<td>A1762T/G1764A</td>
<td>2</td>
<td>2*</td>
<td>5</td>
</tr>
<tr>
<td>G1896A + A1762T/G1764A</td>
<td>9</td>
<td>7</td>
<td>1*</td>
</tr>
<tr>
<td>Died</td>
<td>12</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Survived</td>
<td>5</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* The figures include one patient in each group with a deletion in the region containing the double core promoter mutation.

latter mutation, nine of whom had the G1899A change also. All G1862T variants were free from either the BCP or precore stop codon mutations. Interestingly, all of them had the G1899A change and were of genotype B. None of the genotype C patients had this change. This was also true of the two patients in the control groups.

Genotype B versus C patients were more likely to survive (14 to 4) and, therefore, the reverse was true for those that died (13 to 21) (Table 1). This difference is statistically significant ($P = 0.007$). The G1862T variant, which, as already mentioned, was seen only in genotype B-infected patients was present in 1 of 14 survivors and in 6 of 13 of those that died ($P = 0.032$, Fisher’s two-tailed test).

Of the 52 patients with fulminant hepatitis, only six were found to have wild-type BCP and precore sequences. Some 35 patients had either the stop codon or the G1862T change and were therefore unable to produce HBeAg, as shown by their negative HBeAg status. Two of the HBeAg-positive patients were shown to be carrying a mixture of both the wild-type virus and the G1896A variant. These results are in agreement with previous findings (Sato et al., 1995; Hou et al., 1999).

The HBeAg status of the patients with fulminant hepatitis in relation to the BCP and precore mutations is summarized in Table 2. Of the 52 patients with fulminant hepatitis, 17 were HBeAg-positive. Nine of these patients carried variants with both the precore stop codon and BCP mutations in the absence of mixtures with the wild-type virus.

In vitro translation

Fig. 2 shows the results obtained from the in vitro translation experiments. The expression products obtained in the absence of microsomal membranes can be seen in Fig. 2(A). Both pLC202 and pYN expressed the precore/core protein as expected (p25), whilst a smaller protein representing core was expressed by the pTH101C construct (p21:5). In the presence of microsomal membranes (Fig. 2B), wild-type pYN is processed following removal of the signal peptide to yield HBeAg precursor (p22.5). In contrast, this process in the G1862T variant is greatly impaired but not entirely abolished. The constructs expressing the precore/core protein also express core but the intensity of this band is very weak.

Cell transfection

Fig. 3 shows the results obtained by Western blot analysis of HepG2 cell extracts, following transfection with wild-type and variant plasmid constructs. This experiment was performed on three separate occasions, with similar results. Both the wild-
type and the G1899A variant produced bands of the expected size, these being the precore protein (p25), the HBeAg precursor (p22.5) and HBeAg itself (p17). In contrast, none of these bands were detectable in cells transfected with constructs containing the precore stop codon (G1896A and G1896A/G1899A). The G1862T construct produced comparable amounts of p25 and p22.5 to the wild-type but no HBeAg was detectable.

**Discussion**

Fulminant hepatitis due to HBV infection has been linked in the past with the presence of genomic mutations resulting in abrogation of HBeAg synthesis. These involve the G1896A mutation creating a termination codon in the precore region (Carman et al., 1991; Liang et al., 1991; Omata et al., 1991; Kosaka et al., 1991) and the double mutation in the BCP region (Sato et al., 1995; Laskus et al., 1995). In the latter case, the double mutation leads to the downregulation of the mRNA encoding for the precore protein, which is the precursor of HBeAg production (Moriyama et al., 1996; Scaglioni et al., 1997). In recent years, a G1862T missense mutation in the precore region has also been described, primarily in patients with chronic hepatitis (active liver disease and asymptomatic) (Santantonio et al., 1991; Tran et al., 1991; Li et al., 1993; Laskus et al., 1994b; Horikita et al., 1994; Carman et al., 1995; Loriot et al., 1995; Valliammai et al., 1995; Zhang et al., 1996; Kramvis et al., 1997) but also in patients with hepatocellular carcinoma (Kramvis et al., 1998). Although there have been at least two reports of its presence in patients with fulminant hepatitis (Laskus et al., 1993; Clementi et al., 1993), the prevalence of this mutation in a larger cohort has not been investigated and its functional significance has remained a matter of speculation. Moreover, although this mutation has been detected mostly in anti-HBe-positive patients, it has been found in some HBeAg-positive ones also, leading to suggestions that the variant is rescued by wild-type virus in mixtures of the two.

In the present study, the G1862T nucleotide change was detected in 13.5% of patients with fulminant hepatitis and in only 2.5% of patients with active liver disease or cirrhosis, using a mismatch PCR/RFLP approach. Previous reports dealt with only single cases of this mutation in patients with fulminant hepatitis (Laskus et al., 1993; Clementi et al., 1993). One of these cases was the husband of a lady with reactivated disease due to the G1862T variant, indicating that this variant could be transmitted independently (Horikita et al., 1994). It was also apparent that in our patients this mutation was found in association with the G1899A mutation and in the absence of any association with the twin mutation in the BCP. This latter finding has not been reported previously. The G1862T change and that at position 1899 are found in the bulge and stem of the ε encapsidation signal, respectively, which overlaps with the precore region (Junker-Niepmann et al., 1990). The secondary structure of ε is of paramount importance for the replication of the virus (reviewed by Nassal & Schaller, 1996). Neither of these changes appear to destabilize the stem-loop structure of ε. In fact, the 1899 change allows for a stronger bond with the uridine at position 1855, with which it base-pairs.

The single nucleotide change at position 1862 affects codon 17 and leads to the substitution of valine for phenylalanine (V → F). This change is close to the signal peptide-cleavage site, which lies between residues 19 and 20. The presence of an aromatic residue like F at the −3 position of the signal peptidase recognition motif is ‘forbidden’ (von Heijne, 1983, 1984). This change may therefore prevent the removal of the first 19 amino acids of the precore/core protein during its processing into HBeAg in the endoplasmic reticulum (Ou et al., 1986). The in vitro translation experiments, which were performed in order to prove this, showed that the processing of the precore/core protein to HBeAg was indeed impaired, but not perhaps entirely abolished, in view of the presence of a very faint band at the level of the p22.5. HepG2 cell transfection studies confirmed these results. In these experiments, however, a p17 band representing authentic HBeAg was detectable in lanes 1 and 2 of Fig. 3, since the p22.5 precursor protein had undergone further processing at its carboxyl end, within the lumen of the endoplasmic reticulum. The lower band in lane 5 may represent precore protein processed at its carboxyl end alone.

Of interest is the finding that of the 52 patients with fulminant hepatitis studied, only six had wild-type BCP and precore sequences. The remaining 46 patients had variants that were either unable to produce (n = 30) or had impaired/downregulated production of HBeAg (n = 16). This is in accordance with the fact that only 17 patients were positive for HBeAg, the remainder being either anti-HBe-positive or -negative for both markers. However, nine of the HBeAg-positive patients were from the group with both the precore and BCP mutations. The sera tested were those collected on hospitalization or soon after. It was not therefore possible to ascertain whether the variants arose following infection with the wild-type or whether they were transmitted directly. The coexistence therefore of HBeAg- with non-HBeAg-producing variants may be due to the preexistence of the wild-type virus or due to degraded core, released as a result of the extensive liver tissue damage. Anti-HBe has been detected following transmission of the G1896A variant, even though in this case report, HBeAg was not detectable during the acute phase (Mphahlele et al., 1997). HBeAg epitopes can therefore be presented to the immune system by degraded core protein, and lead to the production of anti-HBe, in the absence of the authentic protein.

HBeAg has been suggested to act as a tolerogen in perinatal infection (Milich et al., 1990), thus favouring the establishment of a chronic phase. The absence of the tolerizing effect of HBeAg may therefore be a contributing factor in the development of fulminant disease. In the cohort of patients studied here, 46 patients in all were either unable to produce
HBeAg or had reduced expression as a result of the presence of the BCP mutations.

It has been suggested that the G1862T variant may be dependent on the presence of wild-type virus, which would rescue the variant genomes (Kramvis et al., 1997). And this is because the G1862T change in the bulge of the e encapsidation signal follows immediately after the fourth or third position of the proposed primer, depending on whether UUC or UUCA is used as template (Wang & Seeger, 1993; Nassal & Rieger, 1996). This primer is used during the reverse transcription step in the replication cycle of the virus, it base-pairs with the DR1 repeat at the 3’ end of the pregenomic RNA and then extends to form the complete negative-strand of viral DNA (Nassal & Schaller, 1996). Variation in nucleotide sequence at position 1862 would not therefore affect primer synthesis or reverse transcription. This is further supported by our data showing that this variant can exist alone and in the absence of mixtures with wild-type virus.

In summary, we have shown that almost 14% of patients with fulminant hepatitis carry a signal peptide variant of HBV. This variant appears to be replication competent. However, as a result of the amino acid substitution in the signal peptidase cleavage recognition motif, HBeAg production is impaired.

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References


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