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Significance of mutations in hepatitis B virus X gene for the pathogenesis of HB-associated glomerulonephritis

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Summary. – In this study, the significance of hepatitis B virus (HBV) X gene mutations for the pathogenesis of HBV-associated glomerulonephritis (HBV-GN) was investigated. DNA was extracted from 50 HBV-GN patients and 60 asymptomatic HBV carriers and subjected to PCR amplification and sequencing of HBV X gene. In HBV-GN patients, missense nucleotide mutations of C1653T, A1726C, A1727T, C1730G, T1753C, A1762T, and G1764A were detected in 84% of subjects, all located in the trans-acting regulatory region of the X gene. In control patients, missense nucleotide mutations of A1632C and A1635C were detected in 8% of subjects, both located in the non-functional region of the X gene. We conclude that, in most HBV-GN patients, X gene missense mutations occurred at some key sites playing an important role in the pathogenesis of HBV-GN.

Keywords: hepatitis B virus; glomerulonephritis; X gene; mutation

Introduction

Hepatitis B virus (HBV) is considered to be the main cause of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, which often combines clinical manifestations of extrahepatic diseases (Pyrsopoulos and Reddy, 2001). Due to the high incidence and prevalence of HBV in China (Linag *et al*., 2009), HBV-associated glomerulonephritis (HBV-GN) has become one of major secondary glomerular diseases in this country. HBV X gene has extensive trans-activating effects, and its mutations commonly lead to increased viral replication and enhanced infectivity. Thus, HBV X gene is closely related to the progress of liver lesions, especially the occurrence and development of hepatocellular carcinoma (Zhu *et al*., 2008). In this study, we explored the potential association of X gene mutation with HBV-GN.

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Abbreviations: BCP = basal core promoter; HBeAg = hepatitis B e antigen; HBV = hepatitis B virus; HBV-GN = HBV-associated glomerulonephritis

Materials and Methods

Patients. A total of 110 patients were selected from Shandong Peninsula, China, including 50 cases of HBV-GN without antiviral therapy and 60 asymptomatic cases carrying HBV (control group). The HBV-GN patients were confirmed pathologically by renal biopsy in the Department of Nephrology at The Affiliated Hospital of Qingdao University, and all of patients had ruled out chronic liver disease, cirrhosis, or liver cancer. Venous blood was collected from all patients; serum was separated and stored at -70°C until use.

DNA extraction. Serum DNA was extracted using a commercial kit (Sangon, Shanghai, China) as follows: 100 µl of serum was added with 400 µl of proteinase K buffer, and DNA was extracted with chloroform and phenol.

PCR. HBV DNA gene sequence was downloaded from the Genbank database. Primers were designed in the conserved regions of X gene by referring to the X gene primers of Zhu *et al*. (2008). The target gene fragment was between nt 1583 and nt 1793, 211 bp in total length. The upstream primer was located at the nt 1583–1600 (5'-GCACTTCGCTTCACCTCT-3'), and downstream primer was located at the nt 1793–1776 (5'-TATGCCTACAGCCTCCTA-3'). The PCR reaction (50 µl) was prepared with a commercial kit (San-

gon, Shanghai, China) following the manufacturer's instructions. The PCR products were checked by 2% agarose gel electrophoresis and stained with ethidium bromide. The DNA concentration was determined by spectrophotometry (>50 μg/ml) and PCR amplicons were sent to Sangon (Shanghai, China) for bidirectional sequencing.

Sequencing and sequence alignment. To determine the mutation sites, the obtained sequences were aligned and analyzed with the standard sequence retrieved from GenBank database (Acc. No. AB048705) in BioEdit7.0 and Clustal W.

Table 1. X gene missense mutations in HBV-GN and control patients

Patients	Nucleotide mutation	Codon change	Amino acid mutation
HBV-GN	C1653T	GCA→GTA	His94Tyr
	A1726C A1727T	AAA→ACT	Lys118Thr
	C1730G	GAC→GAG	Asp119Glu
	T1753C	ATT→ACT	Ile127Thr
	A1762T	AAG→ATG	Lys130Met
	G1764A	GTC→ATC	Val131Ile
Control	A1632C	CAT →CCT	His87Pro
	A1635C	GAT →GCT	Asp88Ala

Results

After 2% agarose gel electrophoresis, the PCR products were compared with DNA marker. All PCR products contained the 211-bp target gene band as shown in Fig. 1. The obtained HBV X gene sequences were aligned with C-genotype adr-serotype HBV genome sequence. Results showed that X gene point mutations occurred in 42 out of 50 patients in the HBV-GN group (84%), resulting in amino acid substitutions. X gene point mutations were not detected in the remaining 8 patients. The nucleotide sequences were translated into amino acid sequences, and the fragment detected comprised the aa 70–139 of X protein. Missense nucleotide mutations C1653T, A1726C, A1727T, C1730G, T1753C, A1762T, and G1764A resulted in amino acid mutations of His94Tyr, Lys118Thr, Asp119Glu, Ile127Thr, Lys130Met, and Val131Ile, respectively. In the control group, missense nucleotide mutations were detected in 5 out of 60 patients (~8%); these were the mutations A1632C and A1635C, resulting in amino acid mutations His87Pro and Asp88Ala, respectively (Table 1).

Discussion

X gene is the smallest open reading frame of HBV genes, which is located at nt 1374–1838 and is 452–465 bp in total length (the size of X gene often varies with the genotype of HBV DNA). HBX protein encoded by X gene comprises 145–154 aa, with a relative molecular mass of 16–17 kDa (Hsia *et al*., 1997); the antigenicity of HBX protein is relatively weak, and the function of the protein is closely related to virus replication. The target fragment of our study, the HBV gene sequence at nt 1583–1793 comprises or partially comprises multiple key regulatory elements of HBV DNA replication, including the basal core promoter (BCP nt 1742–1849), the core upstream regulatory sequence (CURS nt 1643–1742), a negative regulatory element (NRE nt 1457–1628), and an enhancer II (EnhII nt 1685–1773). The amino acid sequence showed that the aa 70–139 of the X protein comprise a number of T- or B-cell immunodominant epitopes involved in immune response, including 3 TH-cell epitopes: aa 61–75, aa 91–105, and aa 111–135 (most important); 5 TC-cell epitopes: aa 91–100, aa 99–108, aa 115–123 (most important), aa 126–134 and aa 133–141; and one B-cell epitope: aa 89–102 (Chun *et al*., 2003; Hwang *et al*., 2003). HBV X gene is a transcription factor required for HBV replication, which has anti-activating function. HBV X gene can induce mitochondrial permeability transition and cause calcium effusion into the plasma, promotes viral replication (Tan C *et al*., 2009), induces apoptosis (Clippinger *et al*., 2009), and causes inflammatory reactions (Xia *et al*., 2009), thus playing an important role in cell transformation and proliferation. HBV X gene can activate relevant transcription factors (e.g. ERKs and NF-κB) and upregulate the expression of cell proliferation-associated cytokine TNF-α, further promoting mesangial cell proliferation (Lu *et al*., 2013). HBX protein can block cyclin B1 degradation and simultaneously downregulate the expression of cyclin A, but upregulate the expression of negative regulatory protein of

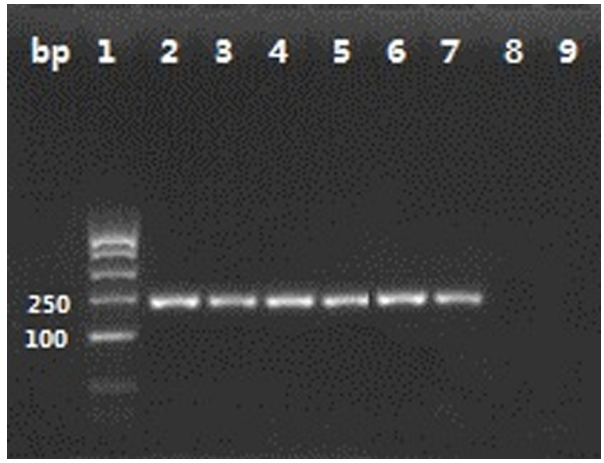

Fig.1
PCR amplification of HBV X gene from HBV-GN patients sera
Agarose gel electrophoresis. DNA size markers (lane 1), patient sera (lanes 2–7), and negative controls (lanes 8–9).

cell cycle p21; these processes would retard cell cycle at the G2/M phase, thereby inhibiting podocyte proliferation. HBV X gene upregulates CD59 and Crry expression in mouse podocytes through P38 pathway, decreasing formation of membrane attack complex in renal tissues and thus inhibiting complement-mediated podocyte lysis. This mechanism may be beneficial to latent infection of HBV in podocytes, which is difficult to be cleared and leads to continued renal damage (Yin and Zhou, 2010). After transfection into HK-2 cells, HBX plasmid can cause the changes in cell number and morphology, as well as promote epithelial-mesenchymal transdifferentiation in renal tubular epithelial cells (Zhou *et al*., 2013). Additionally, HBV X gene upregulates Bax/Bcl-2 ratio by activating JAK2/STAT3 pathway, thereby inducing apoptosis in renal tubular epithelial cells (He *et al*., 2013). In recent years, the effects of X mutations on the transcription, replication, and pathogenesis of HBV have become a research hotspot. Among the most frequently and most in-depth studied is the A1762T/G1764A double mutation in BCP. Many *in vitro* experiments have confirmed that A1762T/G1764A double mutation can increase HBV replication and reduce hepatitis B e antigen (HBeAg) expression by inhibiting the transcription of pre-core mRNA (Moriyama K *et al*., 1996; Gunther S *et al*., 2000; Milich D and Liang TJ, 2003). *In vivo* studies found that A1762T/G1764A mutation is often associated with a high level of serum HBV DNA and is related to seroconversion of HBeAg (Lindh M *et al*., 1999). Previous work has compared the HBV genome sequences between patients with chronic hepatitis and HBV-associated hepatocellular carcinoma; results showed that the incidence of A1762T/G1764A double mutation was significantly higher in hepatocellular carcinoma than in chronic hepatitis, suggesting its relation with cancer (Zhu *et al*., 2008). Because X gene mutations are closely related to viral replication, disease progression, or even cancer development, whether X gene mutations are associated with the occurrence and development of HBV-GN is the focus of our research. Our study demonstrated that point mutations occurred at multiple sites in patients with HBV-GN, which resulted in the substitution of amino acids; amino acid mutations mainly involved aa 87 (nt 1632), aa 88 (nt 1635), aa 94 (nt 1653), aa 118 (nt 1726, 1727), aa 119 (nt 1730), aa 127 (nt 1752), aa 130 (nt 1762), and aa 131 (nt 1764). In the 50 HBV-GN patients, A1762T/G1764A double mutation was detected in 19 cases, with A1762T single mutation in 7 cases. Although A1762T/G1764A double mutation is closely related to the progression of liver diseases, its relationship with HBV-GN has not been reported to date. Many *in vitro* experiments have confirmed that A1762T/G1764A double mutation in BCP can lead to high-level viral replication; such variation resulted from specific choice of chronic HBV infection, which increases the survival and integration probabilities of the virus and further increases the incidence of HBV-GN. Additionally, studies suggested that HBX protein expression in podocytes has a significant anti-proliferative effect, whereas failure of proliferation, apoptosis, and shedding are the main causes for the quantitative decrease in podocytes. Podocyte lesions are the predominant manifestation of HBV-GN; amino acid mutation Lys130Met/Val131Ile, resulting from the double mutation A1762T/G1764A, is located in the important TH-cell epitope aa 111–135 and TC-cell epitope aa 126–134. Mutation-induced immune escape may be the main cause for the pathogenesis of HBV-GN. On the one hand, mutation-induced immune escape increases latent HBV in glomerular podocytes and tubular epithelial cells, which are difficult to be cleared; on the other hand, X gene mutations can stimulate mesangial cell proliferation and inhibit podocyte proliferation, further aggravating renal tubulointerstitial injury and promoting the apoptosis of renal tubular epithelial cells, ultimately promoting the development of relevant lesions. Missense nucleotide mutation A1762T, which may exist as an intermediate process of the double mutation, is also closely related to the incidence of HBV-GN. Additionally, missense nucleotide mutation T1753C was detected in 8 cases, which was located in the trans-acting regulatory region of HBX protein; thus, associated mutations may play a role in the occurrence and development of HBV-GN by influencing the trans-activating function of HBX. Missense nucleotide mutation C1653T was detected in 8 cases as well; its relationship with the development of HBV-GN needs to be further studied. Finally, we observed that missense nucleotide mutations of A1762T/G1764A and C1730G were associated other mutations but did not occur alone. Previous research on liver disease has reported that A1762T/G1764A and C1730G may be associated with low replication of virus (Zhu *et al*., 2008), thus may be unrelated to the pathogenesis of HBV-GN but related to the degree of the lesion. In the control group, missense nucleotide mutations of A1632C and A1635C were detected in 5 patients. Being located at non-functional sites, these mutations were unrelated to occurrence of HBV-GN.

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