

# Relationship between genotypes and clinical manifestation, pathology, and cccDNA in Chinese children with hepatitis B virus-associated glomerulonephritis

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**Background:** Hepatitis B virus-associated glomerulonephritis (HBV-GN) is one of the extrahepatic manifestations after HBV infection, which would cause great clinical harm to people. The present study was undertaken to investigate the HBV-GN genotypes and its clinical relevance in Chinese children.

**Methods:** A total of 41 HBV-infected children diagnosed with HBV-GN were enrolled in the study. All patients underwent liver and kidney biopsy. The genotypes and cccDNA were detected in their serum samples to analyze the relationship between HBV genotypes and clinical characteristics, cccDNA, and pathology.

**Results:** Among the 41 children with HBV-GN, 29 (70.7%) had genotype C, 10 (24.4%) had genotype B, 2 (4.9%) had genotype B/C, and none of them had genotype non-B/C. Most children had genotypes B or C; moreover, the genotype C was the most frequent one. The incidence of hematuria and albuminuria, reduction in complement C3, increase in serum alanine aminotransferase levels and renal insufficiency in the children with genotype C were significantly higher than those in the children with genotype B ( $P < 0.05$ ); however, there was no statistically significant difference in hypertension and hepatomegaly ( $P > 0.05$ ). The frequency of HBV cccDNA positive in the genotype C group was significantly higher than that in the genotype B group (72.4% vs. 30.0%,  $P < 0.05$ ). No difference was observed in hepatic inflammation grades and stages of fibrosis between the two groups ( $P > 0.05$ ).

**Conclusion:** Genotype C was the most frequent genotype in the described group of patients with HBV-GN, and the liver and kidney damage indicators were more likely to occur in patients with genotype C.

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**Key words:** cccDNA; genotypes; glomerulonephritis; hepatitis B virus; pathology

## Introduction

Hepatitis B virus (HBV) is one of the world's most widespread infectious agents, and China has a high incidence of HBV infection. As a clinically common disease, HBV-associated glomerulonephritis (HBV-GN) is one of the extrahepatic manifestations after HBV infection, which would do great clinical harm to the people.<sup>[1]</sup> At present, the possible pathogenesis of HBV-GN are as follows<sup>[2-5]</sup>: (a) immune complex deposit is the generally acknowledged nosogenesis; (b) HBV directly infects the kidney; as the immune function of the host is deficient after HBV infection, the virus could not be eliminated; and (c) the genetic mutation of HBV could change the pathogenicity of the virus. However, the specific pathogenesis of HBV-GN is still unclear. The widely accepted view is that persistent viral infections could lead to immune complex-mediated nephritis.<sup>[6]</sup>

Studying the genotypes of HBV-GN in children is very useful for research into the pathogenesis of HBV-GN, evaluation of disease condition, and assessment of prognosis as well as devising treatment strategy. The present study at first analyzed the relationship between HBV-GN genotypes and cccDNA, its clinical manifestations, and its pathology by genotyping the serum samples of children with HBV-GN, discussed its clinical significance, and provided some clinical guidance.

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## Methods

### Patients and samples

Peripheral blood serum was collected from 41 patients with chronic HBV-GN (24 boys and 17 girls). The diagnostic criteria of hepatitis B were in conformity with *The Guidebook for Prevention and Cure of Chronic Hepatitis B* issued in 2010.<sup>[7]</sup> None of the patients had other hepatitis virus infection, autoimmune liver disease, drug-induced liver damage, and alcoholic liver damage. The diagnosis of HBV-GN was confirmed pathologically, and the reference diagnostic criteria used in China are as follows:<sup>[8]</sup> (a) presence of hepatitis B surface antigen (HBsAg)-positive serum; (b) presence of glomerular nephritis, excluding lupus nephritis and other secondary glomerular diseases; and (c) presence of HBV antigens including HBsAg or HBcAg, or HBV DNA measured using polymerase chain reaction (PCR) in the nephridial tissue. Among these criteria, the third criterion is required for the final diagnosis. In this study, this diagnostic criterion was also used. HBsAg >0.5 µg/L, HBsAb >10 IU/L, HBeAg >0.03 NCU/mL, HBeAb >1.5 NCU/mL, and HBcAb >0.1 NCU/mL were defined as positive (+), otherwise were defined as negative (-). The patients were newly diagnosed and treatment-naïve (these patients have never received any antiviral treatment). Among these 41 patients, 38 were in immune tolerance phase, 2 in immune clearance phase, one in inactive or low (no) copy phase, and no one in the phase of reactivation.

At the time of diagnosis, the patients were enrolled at the People's Hospital of Gansu Province and Lanzhou University First Hospital from April 2008 to April 2012 after obtaining informed consent. The age of the patients ranged from 4 years and 6 months to 17 years and 2 months (13.4±3.5 years). Under the guidance

of ultrasound, all the patients underwent liver and kidney biopsies to obtain hepatic and nephridial tissue respectively for the diagnosis and subsequent research, and the samples were stored at -70°C for further use. The cofactors such as liver function test, serum C3 level, proteinuria, hematuria, and renal insufficiency are listed in Table 1. When the level of serum creatinine was >1.5 mg/dL and glomerular filtration was <60 mL/min/1.73 m<sup>2</sup>, the patients were diagnosed with renal insufficiency.

### Pathological classification and diagnostic criteria

The lesions of glomerular nephritis were classified in accordance with the 1990 World Health Organization criteria.<sup>[9]</sup> Sections taken from all biopsy specimens were stained routinely with hematoxylin and eosin, periodic acid-silver methenamine, Masson's trichrome, and antibodies against immunoglobulin A (IgA), IgG, IgM, and the C3 complement component. Fluorescently labelled goat antihuman IgA (alpha) was purchased from Kirkegaard & Perry Laboratories, Inc (Gaithersburg, Maryland, USA). Fluorescein isothiocyanate (FITC)-labelled mouse antihuman IgG1 (Fc) and FITC-labelled goat anti-human IgM were purchased from Abcam Limited (Cambridgeshire, UK). FITC-labelled rabbit anti-human C3c antibody was purchased from Dako A/S (Glostrup, Denmark).

### Liver puncture biopsy

Liver biopsy is the standard procedure for obtaining hepatic tissue for histopathological examination.<sup>[10]</sup> Patients infected with hepatitis B were indicated for this biopsy to understand the evolution of liver disease including acute and chronic hepatitis, the activity of chronic hepatitis, whether it is evolved into liver cirrhosis or liver cancer, etc. The liver biopsy was performed under B-ultrasound guidance to collect and preserve the hepatic tissues, and liver biopsy specimens were obtained with two-point puncturing. The length of each hepatic tissue was required to be >1.0 cm. After the liver tissue was fixed, dehydrated, paraffin-embedded, and sectioned, it was used for HE staining and Masson's trichrome, and finally its histological structure was observed under a light microscope. The histological structure was graded based on the classification of Desmet<sup>[11]</sup> and Batts<sup>[12]</sup> for chronic hepatitis inflammation grading and fibrosis staging.

### Extraction of HBV DNA and HBV-GN genotyping

HBV DNA was extracted from 400 mL of serum by QIAamp UltraSens Virus Kit (Qiagen GmbH, Hilden, North Rhine-Westphalia, Germany), then resuspended in 50 mL of water and stored at -20°C

**Table 1.** The clinical characteristics of HBV-GN genotypes

Variables	Genotypes		Total (n=39)	P value
	C (n=29)	B (n=10)		
Sex (male/female)	18/11	4/6	24/15	0.282
Age (≤7 y/>7 y)	15/4	14/6	29/10	0.716
HBeAg (+/-)	24/5	7/3	31/8	0.399
Hematuria	25	3	28	0.002
Albuminuria	20	3	23	0.031
Renal insufficiency	21	2	23	0.007
Hypertension	7	5	12	0.232
C3 decreased	23	4	27	0.043
Hepatomegaly	5	3	8	0.653
ALT increased	24	4	28	0.017
cccDNA (+/-)	21	3	24	0.027

HBV-GN: hepatitis B virus (HBV)-associated glomerulonephritis; ALT: alanine aminotransferase; HBeAg: hepatitis B e antigen, HBeAg >0.03 NCU/mL was defined as positive (+), otherwise was negative (-); cccDNA: Ax value >2.21 was defined as positive (+), otherwise negative (-).

until analysis. Fasting blood sample (5 mL) was collected, centrifuged for 10 minutes at 3000 rpm, 1-2 mL of serum was separated and stored at  $-70^{\circ}\text{C}$ . HBV genotyping kit (PCR assay) was provided by Shanghai Clone Biological High Technology Co., Ltd, and the genotyping was based on the manufacturer's instructions. HBV genotyping was performed for all PCR-positive samples using a reverse hybridization line probe assay (INNO-LiPA HBV Genotyping assay; Innogenetics NV, Ghent, Belgium).

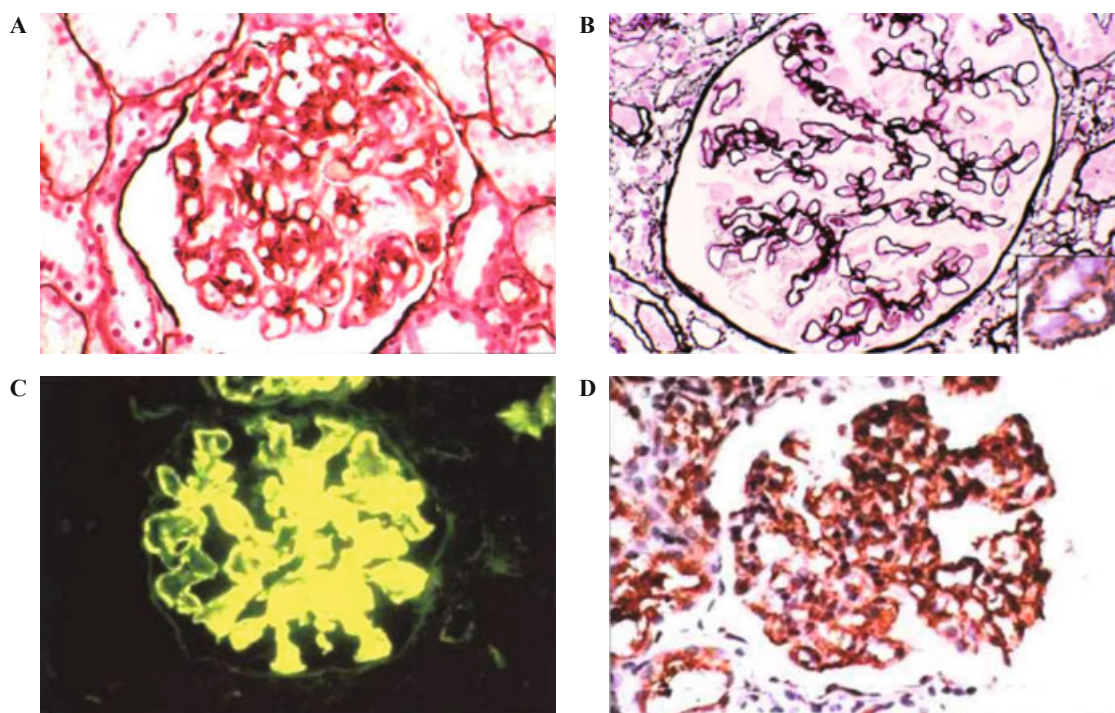
#### Detection of HBV cccDNA

PCR fluorescence molecular beacon technology was used for detecting cccDNA. Blood was collected in the morning using a disposable syringe, from which 200  $\mu\text{L}$  of serum was separated and stored at  $-18^{\circ}\text{C}$  till using for detection. PCR amplification: 1.2  $\mu\text{L}$  of DNA polymerase was obtained and added to the reaction tube (both DNA polymerase and the reaction tube needed to be centrifuged for a few seconds). Supernatant of the treated samples and negative controls as well as standard preparations I, II, and III (5  $\mu\text{L}$  each) were obtained and added to the reaction tube, blended together, centrifuged for a few seconds, and followed by amplification. Meanwhile, a blank control tube was prepared (samples were replaced with 5  $\mu\text{L}$  of

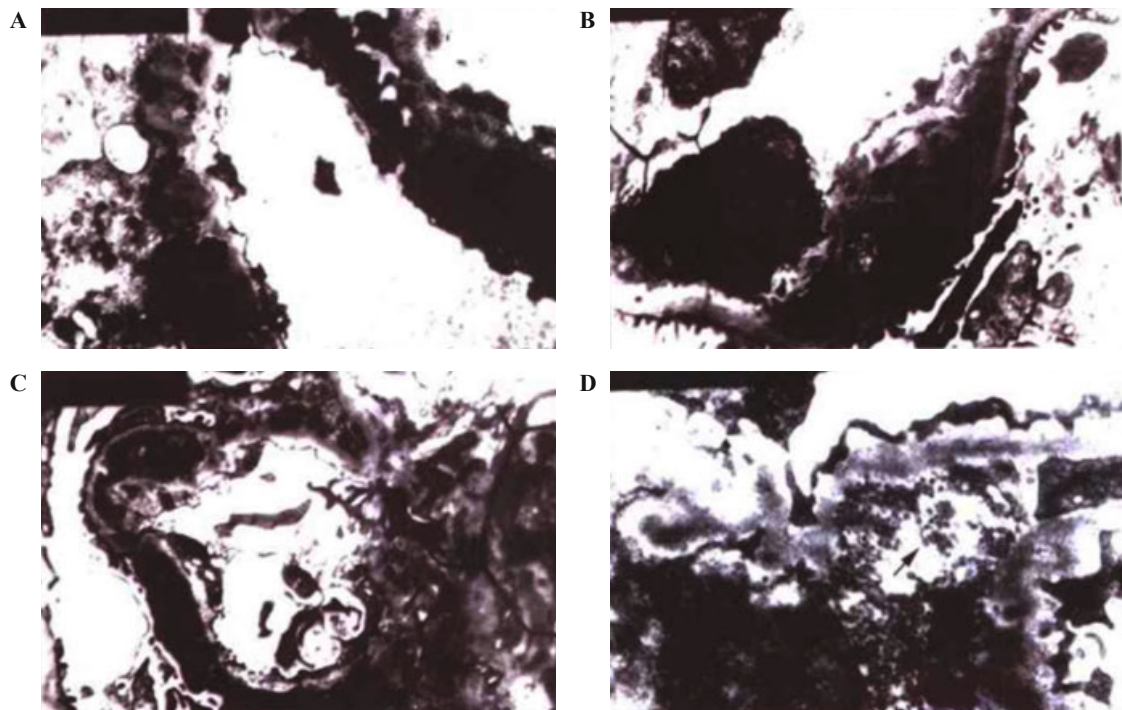
deionized water in the blank control tube), blended, and centrifuged for 10 seconds at 10 000 rpm. The conditions of the program for PCR were  $94^{\circ}\text{C}$  for 3 minutes, followed by 38 revolutions at  $94^{\circ}\text{C}$  for 35 seconds. Then the sample was added to the reaction tube at  $55^{\circ}\text{C}$  for 35 seconds,  $72^{\circ}\text{C}$  for 40 seconds, and  $72^{\circ}\text{C}$  extension for 3 minutes. All the PCR reaction tubes were centrifuged briefly, before these tubes were cooled down to  $28^{\circ}\text{C}$ . The reaction tubes were placed into a fluorescence detector, and the fluorescent value was read and recorded. Fluorescence excitation wavelength was 487 nm and emission wavelength was 518 nm. Ax value was determined by the fluorescence intensity of the sample or blank control. If the Ax value was above 2.21, the cccDNA was positive, otherwise it was negative.

#### Statistical analysis

Data analyses were performed using the SPSS 17.0 software (SPSS, Chicago, IL, USA). Pearson's product-moment correlation coefficient, the Chi-square analysis and Fisher's exact test were used to compare the difference in categorical variables between the two groups. For all analyses, two-tailed *P* values of 0.05 or less were considered statistically significant.



**Fig. 1.** Pathological findings of membranous nephropathy renal biopsy. **A:** hematoxylin and eosin staining (original magnification  $\times 400$ ), GBM thickening and false dual-track formation, mesangial proliferation; **B:** PASM staining (original magnification  $\times 400$  and  $\times 800$ ), GBM thickening, spikes formed (right corner); **C:** Immunofluorescence (original magnification  $\times 400$ ), Ig G fluorescence, IgG mass in particle deposition in mesangial area and the capillary wall; **D:** The HBsAg levels in glomeruli in frozen biopsy slices (original magnification  $\times 400$ ), and HBsAg in glomeruli. GBM: glomerular basement membrane; PASM: periodic acid-silver methenamine.



**Fig. 2.** Electron microscopy findings of membranous nephropathy. **A:** giant subepithelial deposits (original magnification  $\times 8000$ ); **B:** subendothelial deposits (original magnification  $\times 8000$ ); **C:** Giant block electron dense deposit on subendothelial, GBM and glomerular mesangium (original magnification  $\times 8000$ ); **D:** Virus-like particles in the electron dense of subendothelial (original magnification  $\times 10\ 000$ ). GBM: glomerular basement membrane.

## Results

### Results of renal biopsy

Among the 41 patients with HBV-GN, 32 had membranous nephropathy (MN), 5 had mesangial proliferative glomerulonephritis (MPGN), and 4 had minimal change disease (MCD). The number of renal glomerulus biopsies was 4-19 ( $10\pm 6$ ). More than six renal glomeruli were observed in 87.8% (36/41) of the patients. In the remaining five patients, two were found to have four renal glomeruli and another two children were found to have renal glomerulus, and only 1 child was observed to have two renal glomeruli. In these patients, the typical changes in the renal glomerulus of patients with MN were observed (Figs. 1 and 2): thickening of the glomerular basement membrane, formatting of the false dual-track and a proliferating mesangium (Fig. 1A), and the appearance of spikes (Fig. 1B). The immunofluorescence showed the particle deposition of IgG mass in the mesangial area and the capillary wall (Fig. 1C). A few findings of the electron microscopy are shown in Fig. 2.

### Distribution of genotypes

In the 41 patients with HBV-GN, 29 (70.7%) had genotype C, 10 (24.4%) had genotype B, and 2 (4.9%) had mixed genotype B/C. In these patients, no genotype none-B/C was found.

### Differences in clinical characteristics between genotypes B and C

No significant difference was observed in sex, age ( $\leq 7$  years/ $>7$  years), HBeAg-positive rate, and HBV-GN genotypes B and C ( $P>0.05$ ; Table 1). The incidence of hematuria and albuminuria, reduction in complement C3, and increase in levels of serum alanine aminotransferase (ALT) and renal insufficiency in the children with genotype C were significantly higher than those in children with genotype B ( $P<0.05$ ; Table 1). However, there were no differences in the frequency of hypertension and hepatomegaly between genotypes B and C.

### Difference in cccDNA between genotypes B and C

The frequency of HBV cccDNA positive was identified in 3 patients of the genotype B group (3/10, 30%), and in 21 patients of the genotype C group (21/29, 72.4%); the frequency of HBV cccDNA positive in genotype C was higher than that in genotype B ( $P<0.05$ ; Table 1).

### Association of HBV-GN genotype B and C in hepatic pathological grading and staging

No significant difference was observed in hepatic inflammation grades and stages of fibrosis between genotype B group and C group ( $P=0.891$  and  $0.870$ , respectively; Table 2).

**Table 2.** The relationship of inflammation and fibrillation degree with genotype

Genotypes	Genotype C (n=29)	Genotype B (n=10)	Total (n=39)	P value
Inflammation grades				0.891
G <sub>0</sub>	7	3	10	
G <sub>1</sub>	11	3	14	
G <sub>2</sub>	4	2	6	
G <sub>3</sub>	4	1	5	
G <sub>4</sub>	3	1	4	
Fibrillation stages				0.870
S <sub>0</sub>	7	4	11	
S <sub>1</sub>	13	3	16	
S <sub>2</sub>	3	1	4	
S <sub>3</sub>	4	1	5	
S <sub>4</sub>	2	1	3	

## Discussion

HBV infection is an important public health problem in the world, especially in the developing countries, and HBV-GN remains one of the most common secondary glomerular diseases.<sup>[13]</sup> This study showed that genotype C, B, B/C, and none-B/C accounted for 70.7%, 24.4%, 4.9%, and 0, respectively, and that genotype C was predominant. The lower proportion of the mixed genotype B/C was probably due to superinfection or partly transformed from other genotypes.<sup>[14]</sup> There was no genotype none-B/C found in this study, which can be explained by the limited number of specimens used.

Currently, HBV is considered as a noncytopathic virus, and HBV-associated renal damage is thought to be the consequence of a long lasting cytolytic immune response against infected renal tissue. The pathological pattern of HBV-GN is varied. Zhou et al<sup>[15]</sup> reported that toll-like receptor 4 may be involved in an immune inflammatory reaction by inhibiting the replication of HBV in human kidney 2 cells, which could have an antiviral effect during HBV infection in the kidney. MPGN, mesangioproliferative glomerulonephritis, and MN are the different pathological types of glomerular lesions, which have been described in association with HBV infection. The present study showed that MN was the most common pathological subtype, and that mesangial proliferative nephritis and MCD accounted for a small part. These results were in accordance with the distribution in Chinese adults.<sup>[16]</sup> However, they were inconsistent with the results from the study of Zhang et al.<sup>[17]</sup> MN as a chronic progressive glomerulonephritis is common in adults, characterized by subepithelial immune deposits inducing nonselective proteinuria. The result was probably due to the fact that the kids' immune system is not fully matured and the immune complex is more likely to deposit in their bodies.<sup>[18]</sup>

In our study, the incidence rate of hematuria and albuminuria, reduction of complement C3, increase of ALT, and renal inadequacy of the children with genotype C were higher than those of children with genotype B. This result indicated that the patients with genotype C were more likely to have serious renal injury. Therefore, early detection of hepatitis B genotypes would be helpful in detecting HBV-GN, thus providing opportunities for early treatment.

A previous study<sup>[19]</sup> showed that patients with genotype C were more likely to have liver damages and high level of liver enzymes at early time, but the relationship between genotype distribution and the degree of liver damage had not yet been clarified. This study has the following limitations: only the genotypes and clinical characteristics were analyzed in the 41 patients with HBV-GN. As a result, the degree of liver damage and the incidence of ALT in children with genotype C were much higher than those in children with genotype B. Obviously, HBV genotype had some relationship with the activity, severity, and occurrence of HBV-GN. The natural pathogenesis of HBV infection is associated with HBV genotypes.

The natural history of chronic HBV infection including immune tolerance, immune clearance, inactive or low (no) copy, and reactivation are the four phases of hepatitis natural pathogenesis.<sup>[20]</sup> Usually, the immune tolerance phase lasts up to 10 to 20 years. HBV-infected children are often found in the immune tolerance phase especially in genotype C. HBV infection in genotype C is associated with a higher risk of reactivation of hepatitis B and progression to cirrhosis than in genotype B.<sup>[21]</sup> The results of the present study showed that liver damage, increased levels of liver enzymes, and rapid replication of DNA occur more commonly in patients with genotype C than in those with genotype B, which indicates that the immune tolerance phase of genotype C is shorter than that of genotype B, and that it reaches the active period earlier than genotype B. However, further study is needed to confirm the results.

HBV cccDNA exists in the nuclei of hepatic cells. It is an indicator that virus has successfully infected the liver cells and replicated persistently in the patients. We found that the positive rate in genotype C was higher than that in genotype B. Moreover, the replication of HBV and its genotype were reported to be correlated with liver damage.<sup>[22]</sup> In children with genotype C with HBV DNA, rapid replication was more likely to have HBV-GN.

In conclusion, genotype C was predominant in the patients described, and HBV-GN was more severe in genotype C than in genotype B.

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**Ethical approval:** This research was approved by People's Hospital of Gansu Province ethics committee.

**Competing interest:** The authors reported no potential conflicts of interest.

**Contributors:** SYH and LXY designed the study, analyzed the data and wrote the manuscript; SYP, CJH, SYC contributed to the collection of samples and clinical data; GX performed the study and wrote the manuscript. All authors read and approved the final version. SYH and LXY contributed equally to this study.

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