Expression of Foxp3 in renal tissue of patients with HBV-associated glomerulonephritis and their clinical and pathological characteristics

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Received March 9, 2017; Accepted June 15, 2017

DOI: 10.3892/etm.2017.5111

Abstract. Our study retrospectively investigated the expression of forkhead/winged helix transcription factor (Foxp3) in renal tissue and clinical features of patients with hepatitis B virus (HBV)-associated glomerulonephritis (HBV-GN). A total of 58 patients with HBV-GN were assigned to group A; 45 serum and renal tissue HBsAg-negative patients with nephritis were group B; 24 serum HBsAg-positive and renal tissue HBsAg-negative patients with slightly increased serum creatinine without nephritis were group C. Clinical manifestations, laboratory indices and renal biopsies were recorded. Expression of Foxp3, CD4 and CD25 in renal tissue was detected by immunohistochemistry. In group A, 74.1% were serum HBeAg-negative, with serum complement C3 level of 0.99±0.27 g/l, and deposition rates of renal complement C3 and C1q in renal tissue of 34.9 and 16.3% respectively; 25.9% were serum HBeAg-positive, with serum complement C3 level of 0.19±0.17 g/l, and deposition rates of renal complement C3 and C1q in renal tissue of 80 and 46.7%, respectively. A significant difference was found in C3 and C1q between HBeAg-negative and HBeAg-positive group (P<0.05). Increased urinary protein and decreased serum albumin were found in patients in group A with moderate levels of HBV DNA compared with patients with low levels of HBV DNA in the same group over 24 h (P<0.05). The numbers of $Foxp3^+$ lymphocytes, $CD4^+$ T cells and $CD25^+$ T cells in the tubulointerstitium of patients in groups A and B were 3.41±1.16 vs. 3.52±1.27, 2.78±0.15 vs. 3.12±0.17 and 2.90±0.20 vs. 3.09±0.18, respectively. The clinical manifestation of HBV-GN is nephrotic syndrome, and HBV DNA is correlated with urinary protein and serum albumin levels. Activation of C3 and C1q may be related to the pathogenesis of HBV-GN in serum HBeAg-positive patients. Downregulation of Foxp3 expression in regulatory T cells is related to the development and progression of HBV-GN.

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Key words: hepatitis B, glomerulonephritis, clinical manifestations, pathology, forkhead/winged helix transcription factor

Introduction

Hepatitis B virus (HBV)-associated glomerulonephritis (HBV-GN) is also known as hepatitis B immune complex nephritis, or HBV antigen-related glomerulonephritis. HBV-GN has not been clearly defined, it usually refers to the disease of direct or indirect infection caused by HBV glomerulonephritis confirmed by renal biopsy immunofluorescence and serum immunology, on this basis, other secondary glomerulonephritis must be excluded (1). In China, 10-15% of the population are HBV carriers, and incidence of glomerulonephritis in HBV carriers is around 6.8-20% (2,3). HBV-GN can occur in both children and adults, and the incidence is higher in males than in females. The most common clinical manifestation of HBV-GN is nephrotic syndrome, the most common histological types being membranous nephropathy (MN), membranoproliferative glomerulonephritis (MPGN), mesangial proliferative glomerulonephritis (MsPGN) and IgA nephropathy (4). However, the mechanism of pathogenesis is not yet clear. It is proposed that HBV-GN is mainly due to immune injury caused by interactions between the host, virus and environmental factors. A large number of clinical studies and evidence-based medicine have shown that antiviral therapy can effectively relieve proteinuria and promote negative conversion rate of serological HBeAg (5).

Regulatory T cells (Tregs) are an important subset of $CD4^+$ T cells. They can inhibit the activation and proliferation of other immune cells. They also play pivotal roles in maintaining the stability of the internal environment and preventing the occurrence of autoimmune diseases, transplantation rejection and other diseases (6); a reduction in Treg number and function is associated with a variety of autoimmune diseases (6). Tregs can be divided into several subtypes, among which $CD4^+CD25^+$ Tregs that express Foxp3 are the most studied. Foxp3, also known as forkhead/winged helix transcription

factor, is currently identified as a $CD4^+CD25^+$ Treg-specific molecular marker, and plays pivotal roles in the development and function of $CD4^+CD25^+$ Tregs (7,8). Studies have shown that $Foxp3^+CD25^+CD4^+$ Tregs, which are natural immune cells that regulate self-tolerance, are associated with a variety of immune injury diseases. However, tissue detection of Foxp3 is mainly applied in the field of tumor and organ transplantation research. Studies on the infiltration of $Foxp3^+$ Tregs in autoimmune disease target organs are still lacking (9-11).

To further explore the clinical pathological features of HBV-GN, data from 58 patients with HBV-GN diagnosed by renal biopsy in Qingdao No. 6 People's Hospital were collected and retrospectively analyzed. The expression of Foxp3 protein in renal tissue was detected by immunohistochemistry to explore the pathogenesis of HBV-GN.

Materials and methods

Subjects

Inclusion criteria. Data from 58 patients diagnosed as HBV-GN by pathologic examination of renal biopsy in Qingdao No. 6 People's Hospital between February 2013 and August 2016 were collected, and designated as group A (40 males and 18 females; ratio, 2.2:1; mean age, 26.7±10.3). Diagnostic criteria were in accordance with the advice on diagnosis established by the Beijing symposium on HBV-GN in 1989: i) serum HBV markers should be positive, ii) patients should have glomerulonephritis but not secondary glomerular disease and iii) renal biopsy is positive for HBV antigen or HBV DNA can be detected in renal tissue sections. At the same time, 45 serum and renal tissue HBsAg-negative patients with complete case data and nephritis diagnosed by renal biopsy were selected and designated as the non-HBV-GN group (group B). In addition, 24 serum HBsAg-positive and renal tissue HBsAg-negative patients with slightly increased serum creatinine but no nephritis (per biopsy) were designated as the HBV-infection control group (group C). This study was approved by the Ethics Committee of Qingdao No. 6 People's Hospital. Written and signed informed consent was obtained from all the patients.

Exclusion criteria. The patients with secondary renal injury, including lupus nephritis, hypertensive nephropathy, purpuric nephritis, diabetic nephropathy, and tumor-related nephropathy. Patients with incomplete case data or obvious errors in medical records were also excluded.

Clinical data

Observation indices. The following indices were assessed and recorded for all patients: sex, ethnicity, age, routine urine test, the five index of HBV, HBV DNA, liver and kidney function, 24-h urine protein quantification, coagulation, blood lipids, immunological item test (C3, C4, IgA, IgG, IgM, C1q), abdominal type-B ultrasound, renal biopsy report and renal tubular interstitial injury degree.

Renal tissue pathological data. All the fresh renal biopsy specimens were fixed with PB-FA fixation solution, and treated with ethanol and xylene to make the tissue transparent. After paraffin embedding, continuous sections were prepared with a thickness of 2 μm. The sections were dewaxed with xylene, hydrated with ethanol and then stained with H&E, PAS and PASM-Masson. Morphological changes of the glomeruli and tubulointerstitium were observed under a light microscope (BX-42; Olympus, Tokyo, Japan). IgM, IgM, IgG, C3, C4 and C1q were stained by two-step immunohistochemical staining (kit purchased from Beijing Beyotime Biological Products Co., Ltd., Beijing, China), and the levels and distributions of IgA, IgM, IgG, C3, C4 and C1q were detected under a light microscope. Fresh renal tissue specimens were collected, and treated with renal infiltration electron microscopy fixative in Qingdao No. 6 People's Hospital. The tissue was cut into 1 mm^3 pieces, and then immersed in glutaraldehyde solution to remove the fixative from the surface. After rinsing with PBS buffer, the tissue was fixed with osmium acid, and then PBS buffer was used to rinse the samples again, followed by dehydration with ethanol and acetone. After infiltration with acetone and the 618 embedding agent, the samples were then embedded in resin. Ultrathin sections were used for double staining with lead and uranium. The ultrastructure of the kidney and the deposition sites of the electron dense material were observed under an electron microscope.

The classification of renal pathology was in accordance with the glomerular pathological classification criteria established by the World Health Organization in 1995, including MN, MPGN, MsPGN and IgA nephropathy, minimal change disease, focal segmental glomerulosclerosis, segmental MsPGN, sclerosing glomerulonephritis (SGN). The degree of renal tubular injury was determined using the semi quantitative Katafuchi integral method, including interstitial inflammatory cell infiltration (0-3 points), interstitial fibrosis (0-3 points), tubular atrophy (0-3 points), mild injury (1-3 points), moderate injury (4-6 points) and severe injury (7-9 points).

Research methods

Detection of serum HBV markers. HBV antigen-antibody was detected by enzyme-linked immunosorbent assay (Beijing Beyotime Biological Products Co., Ltd.). Serum HBV DNA content in the serum HBsAg-positive patients was detected by quantitative real-time PCR using ABI PRISM® 7000 fluorescence quantitative PCR instrument (Applied Biosystems, Waltham, MA, USA), HBV DNA PCR detection kit was purchased from DaAn Gene Co., Ltd. of Sun Yat-Sen University (Guangdong, China). Positive and negative controls were set up for each PCR.

Detection of HBV markers and Foxp3, CD4, CD25 protein in renal tissue. HBV antigens and Foxp3, CD4, CD25 protein in renal tissue paraffin sections were detected by a two-step method (EnVision system; Agilent Technologies, Santa Clara, CA, USA) immunohistochemical staining. Mouse anti-human HBsAg monoclonal antibody and rabbit anti-human HBcAg polyclonal antibody were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. (Beijing, China). Concentrated mouse anti-human Foxp3 monoclonal antibody, mouse anti-human CD4 monoclonal antibody, and mouse anti-human CD25 monoclonal antibody were purchased from eBioscience Inc. (San Diego, CA, USA) (working titer 1:100 for all). The secondary antibody was purchased from Shanghai LongIsland Biotec. Co., Ltd. (Shanghai, China). The positive control was immunohistochemical staining of liver tissue sections, and negative control was renal sections from the patients who showed negative signal for all HBV markers in peripheral blood serum (group B). Yellow or brown yellow particles in nuclei staining

Table I. Comparison of general data and laboratory test indices of patients in three groups.

	Group A	Group B	Group C	F-value or χ^2 value	P-value
N	58	45	24	-	-
Age (years)	26.7±10.3	24.5±5.5	27.8±17.1	1.02	0.36
Sex (male, %)	40 (68.9%)	32 (61.6%)	14 (58.3%)	1.09	0.57
Nationality (Han, %)	50 (86.2%)	45 (86.5%)	21 (87.5%)	0.04	0.98
Alanine aminotransferase (U/l)	31.23±12.12	34.37±10.23	28.17±9.14	0.06	0.76
Urinary protein >0.3 g (g/24 h)	53 (91.4%)	40 (78.1%)[a]	-	6.108	0.017
Serum creatinine (μmol/l)	4.4	41.3±14.4	37.7±19.8	0.5	0.61

[a]Represents the comparison with group A, P<0.05.

were treated as positive signals of Foxp3, CD4, and CD25. Cell count of $Foxp3^+$, $CD4^+$, $CD25^+$ lymphocytes was assessed as the number of positive cells counted in five randomly selected high-power (x400) visual fields for each sample. Quantitative determination was based on the quantitative ratio of the positive cells and the intensity of staining.

Specific steps of two-step immunohistochemical staining. Samples were soaked in 80% ethanol for 10 min, then 10 min in 95% ethanol three times, 15 min in anhydrous ethanol three times, 10 min in xylene twice, followed by the first wax immersion (56-58˚C paraffin for 20 min), and then the second wax immersion (56-58˚C paraffin for 60 min). Continuous sections with a thickness of 4 μm were prepared and immersed in xylene three times for dewaxing (5 min for each immersion), and then treated with 100, 95 and 75% ethanol for 5 min, respectively. Samples were then immersed in 0.01 M citrate buffer (pH 6.0) and treated with ready-to-use trypsin at 37˚C for 10-15 min as a digestion step, followed by three 2-min 6.5% PBS washes. Samples were then treated with the first antibody at 37˚C for 1 h followed by three 2-min PBS washes, and then treated with polymer enhancer at 37˚C for 20 min, followed by three 2-min PBS washes. Samples were then treated with the second antibody (HRP-poly-chelate-mouse anti-human IgG) at 37˚C for 30 min, followed by three 2-min PBS washes, then stained with ready-to-use AEC color reagent, washed for 2 min in water, then treated with ethanol-free hematoxylin for 1-3 min, washed for 3 min with water and then sealed with AEC seal reagent.

Quality control. Inclusion criteria of patients with HBV-GN was in accordance with the diagnostic criteria and exclusion criteria established by the Beijing symposium on HBV-GN in 1989. The inclusion criteria of the control group was also in accordance with the pathological diagnosis and exclusion criteria and checked by senior physicians in our department. Laboratory indices such as routine urinalysis, hepatitis B five items tests, HBV DNA, liver and kidney function, 24-h urinary protein quantification, blood coagulation, blood lipid, immunological eight items tests (C3, C4, IgA and IgG), coagulation and abdominal type-B ultrasound were all double checked by physicians in the Department of Infectious Disease of Qingdao No. 6 People's Hospital to ensure the accuracy of the results.

Statistical analysis. Data were statistically processed using SPSS 17.0 (IBM SPSS, Armonk, NY, USA) software. The t-test was used to compare the mean values between two samples. One-way ANOVA analysis was used to compare the mean values among multiple samples. The countable data were analyzed by chi-square test and Fisher's exact test. Statistical significance was set at P<0.05.

Results

General information. There were 40 males (69%) and 18 females (31%) in the HBV-GN group (group A), with an average age of 26.7±10.3. Overall, 50 patients were of Han nationality and 8 cases were of minority ethnicity. All 58 patients were diagnosed as HBV-GN for the first time, and all patients were positive for serum HBV markers; among them, 42 patients (72.4%) were HBV carriers, 15 patients (25.9%) had chronic hepatitis B, and 1 patient (1.7%) had occult HBV infection. Of the 58 patients, 33 (56.9%) had edema as the main manifestation. Hematuria and proteinuria were found in 10 patients (17.3%) by physical examination, and 9 patients (15.5%) had hematuria as the main manifestation. Six patients (10.3%) with headache and dizziness were found to have high blood pressure. The clinical type of HBV-GN was nephrotic syndrome in 27 patients (46.6%), nephritic syndrome in 24 patients (41.4%), chronic renal insufficiency in 4 patients (6.9%), and simple hematuria or proteinuria in 3 patients (5.1%). The non-HBV-GN group (group B) included 32 males and 20 females, with an average age of 24.5±5.5. Overall, 45 patients were of Han nationality and 7 patients were of minority ethnicity. Nephrotic syndrome was the main manifestation in 26 patients (50%), and nephritis syndrome was the main manifestation in 21 patients (40.4%); both syndromes were present in 5 patients (9.6%). The HBV infection control group (group C) contained 10 females and 14 males, with an average age of 27.8±17.1. In total, 21 patients were of Han nationality and 3 patients were of minority ethnicity. There were no significant differences in age, sex or ethnic origin between the three groups (Table I). There was no significant difference in serum creatinine between the three groups (F=0.05; P=0.61), but there was a significant difference in urinary protein level (>0.3 g/24 h) between groups A and B (P<0.05).

Laboratory tests for patients in group A. There were four types of serum hepatitis B markers in patients, the highest proportion in group A being HBsAg, anti-HBe and anti-HBc (Table II). Patients were divided into HBeAg-positive group

Table II. Four types of hepatitis B markers in patients.

Serum hepatitis B markers	Cases	Percentage
HBsAg, HBeAb, HBcAb(+)	33	56.90
HBsAg, HBeAg,HBcAb(+)	15	25.90
HBsAg, HBcAb(+)	9	15.50
HBsAb, HBcAb(+)	1	1.70

(n=15) and HBeAg negative group (n=3), and clinical indices were compared between the two groups (Table III). Significant differences in serum urea nitrogen, aspartate aminotransferase, glutamyl transpeptidase and complement C3 levels were found between the two groups. Patients were divided into three groups according to serum HBV DNA levels: 32 cases were allocated to the low replication group (HBV DNA <107 copies/ml), 11 cases to the moderate replication group (≥105 HBV DNA <107 copies/ml) and 15 cases to the high replication group (HBV DNA ≥107 copies/ml); there were no significant differences in serum creatinine, urea nitrogen and uric acid between the three groups (P>0.05), but compared with the low replication group, the moderate replication group showed significantly higher levels of 24-h urine protein and lower levels of serum albumin (P<0.01) (Tables II and IV).

Features of renal pathology. Of the 58 patients with HBV-GN, MN was found in 25 cases (43.1%), MsPGN was found in 16 cases (27.6%) MPGN was found in 9 cases (15.5%), IgA nephropathy was found in 6 cases (10.3%), and SGN was found in 2 cases (3.4%). Two-step immunohistochemical staining for HBeAg-positive and HBeAg-negative groups (Table V) showed that the HBeAg-positive group had the highest deposition rates of renal complement C3 and C1q, significantly higher than those in the HBeAg-negative group (P<0.05). HBsAg was the most common deposition type in 21 (36.2%) of the 58 patients with HBV-GN, and was mainly found in the mesangial area and rarely in the capillary loops, tubule and interstitium. HBcAg deposition accounted for 25 cases (43.1%) and was mainly found in the tubule and interstitium, and rarely in the mesangial area and capillary loops; both of the deposition types were found in 12 cases (20.7%).

There was no significant difference in pathological types between the groups A and B (χ^2=1.086; P=0.99), but there was a statistically significant difference in the degree of renal tubulointerstitial injury between the groups (χ^2=9.15; P=0.027) (Table VI).

Semiquantitative analysis of Foxp3$^+$ lymphocytes, CD4$^+$ T cells and CD25$^+$ T cells. Significant differences were found in the expression level of Foxp3$^+$, CD4$^+$ and CD25$^+$ between the three groups (F=23.00; P=0.00) and the expression levels in groups A and B were significantly lower than those in group C (P<0.01).

Table III. Difference in laboratory indices between HBeAg-positive and HBeAg-negative groups.

Clinical indices	HBeAg(+) group (n=15)	HBeAg(-) group (n=43)	P-value
Urea nitrogen (μmol/l)	4.94±1.50	6.79±5.80	0.04
Serum uric acid (μmol/l)	366.05±83.98	349.38±103.22	0.615
Urine protein (g/24 h)	4.96±4.30	3.64±4.15	0.391
Alanine aminotransferase (U/l)	53.53±47.99	45.10±65.99	0.936
Aspartate aminotransferase (U/l)	42.54±31.27	31.71±17.82	0.035
Glutamyltranspeptidase (U/l)	42.00±59.44	35.70±22.42	0.039
Triglyceride (mmol/l)	2.34±1.17	2.38±2.02	0.208
Total cholesterol (mmol/l)	6.58±1.58	5.91±2.29	0.111
Complement C3 (g/l)	0.19±0.17	0.99±0.27	0.041
Complement C4 (g/l)	0.24±0.09	0.29±0.32	0.508
IgG (g/l)	10.26±8.62	7.09±3.44	0.508

Table IV. Comparison of clinical indices between different HBV DNA replication groups.

Clinical indices	Low replication group (n=32)	Moderate replication group (n=11)	High replication group (n=15)
Serum creatinine (μmol/l)	78.75±50.83	2.81±3.50	77.78±21.00
Urea nitrogen (μmol/l)	7.15±6.55	5.33±2.43	5.27±1.79
Serum albumin (g/l)	29.33±8.39	19.35±4.82[a]	24.60±11.28
Serum uric acid (μmol/l)	363.32±96.83	309.39±96.12	365.63±99.85
Urine protein (g/24 h)	2.81±3.50	6.62±5.45[a]	4.53±3.77

[a]Represents the comparison with the low replication group P<0.01. HBV, hepatitis B virus.

Table V. Comparison of immunological test results between HBeAg-positive and HBeAg-negative groups.

Group	N	C3	C4	IgG	IgM	IgA	C1q
HBeAg(+)	15	12 (80%)	6 (40.0%)	20 (66.7%)	11 (73.3%)	13 (86.7%)	7 (46.7%)
HBeAg(-)	43	15 (34.9%)	11 (25.6%)	10 (67.4%)	27 (62.8%)	36 (83.7%)	7 (16.3%)
χ^2	-	6.98	4.4	4.91	5.17	2.33	3.62
P-value	-	0.0003	0.231	0.597	0.341	0.575	0.025

Table VI. Comparison of pathological type composition and degree of renal tubulointerstitial injury between groups A and B.

Pathological types	Group A, n (%)	Group B, n (%)	χ^2	P-value
MN	25 (43.1)	18 (34.62)	5.21	0.23
MPGN	9 (15.52)	8 (15.38)	4.12	0.13
IgAN	6 (10.34)	7 (13.46)	4.31	0.11
MsPGN	16 (27.62)	10 (19.23)	5.13	0.101
SGN	2 (3.45)	2 (3.85)	2.35	0.56
Degree of renal tubulointerstitial injury				
Mild injury	22 (37.93)	20 (38.46)	9.15	0.027
Moderate injury	23 (39.66)	11 (21.15)	3.57	0.032
Severe injury	4 (6.9)	1 (1.92)	4.13	0.025
No renal tubular injury	9 (15.52)	20 (38.46)	3.17	0.034

MN, membranous nephropathy; MPGN, membranoproliferative glomerulonephritis; MsPGN, mesangial proliferative glomerulonephritis; SGN, sclerosing glomerulonephritis.

Table VII. Comparison of the expression of Foxp3, CD24 and CD25 among the three HBV-GN groups.

Groups	N	$Foxp3^+$ T cells	$CD4^+$ T cells	$CD25^+$ T cells
Group A	58	3.41±1.16	2.78±0.15	2.90±0.20
Group B	45	3.52±1.27	3.12±0.17	3.09±0.18
Group C	24	5.73±2.12[a]	5.25±0.25[b]	5.9±0.35[c]

[a]$Foxp3^+$ lymphocyte level in group C was significantly different from that in groups A and B (P=0.011); [b]$CD4^+$ T cell level in group C was significantly different from that in groups A and B (P=0.021); [c]$CD25^+$ T cell level in group C was significantly different from that in groups A and B (P=0.004). Foxp3, forkhead/winged helix transcription factor; HBV-GN, hepatitis B virus-associated glomerulonephritis.

No significant difference was found in expression levels of $Foxp3^+$, $CD4^+$ and $CD25^+$ between groups A and B (Table VII).

Discussion

HBV-GN is one of the main types of secondary renal damage in China. It is also one of the causes of chronic renal failure, which is often neglected by clinicians and patients due to occult disease development (12). Large sample epidemiological research and standardized diagnosis and treatment are still lacking. It remains a difficult problem in the treatment of chronic hepatitis B. Strengthening HBV-GN research and improving the level of diagnosis and treatment will have significant value for the improvement of the prognosis of these patients.

Treg cells are a subset of T cells with immunomodulatory properties that were recently identified (13,14). Abnormal development and dysfunction of Treg cells may lead to the development of a variety of diseases including autoimmune diseases, inflammatory responses, acute and chronic infections, immune tolerance of tumors, transplant rejection, and allergies (13). There are many subgroups of Treg cells, among which $CD4^+$ $CD25^+$ Treg cells play an important role in inhibiting the proliferation of autoreactive T cells and maintaining immune stability (13,14). Foxp3, which is a $CD4^+$ $CD25^+$ Treg cell-specific molecular marker (10), can reflect the level and functional activity of Treg cells (15). Foxp3 is also the core intracellular marker in maintaining the inhibitory function of human natural Treg and can downregulate the expression of pro-inflammatory factors and upregulate the expression of immunosuppressive factors to

inhibit the immune response (16). Decreased Foxp3 expression suggests a decreased number or decreased functioning of Treg cells, which in turn can lead to autoimmune diseases. The number of Treg cells in the peripheral blood of patients with autoimmune hepatitis is significantly lower than that of healthy people (16-18). From BXSB Lupus experiments in an animal model, Lan *et al* (18) found that the reduction of Treg cells was associated with tubulointerstitial damage in lupus nephritis. The number of $CD4^+$ $CD25^+$ Treg cells recovered to normal levels after induction of mucosal immune tolerance in SNF1 mice, and the symptoms and pathologic changes associated with glomerulonephritis were significantly improved (19). Therefore, it can be extrapolated that the reduction in Treg cells may be one of the causes of renal tubulointerstitial damage in patients with HBV-GN (18,19). The surface of Treg cells can express three kinds of IL-2 receptors: CD25, CD122 and CD132, with CD122 and CD132 being important components of the IL-2 high affinity receptor complex. Studies have shown that Treg cells can inhibit the proliferation of Foxp3-responsive T cells by competing with effector T cells to recruit IL-2, and induce apoptosis of T effector cells through the apoptotic factor Bim-mediated pathway to achieve immunosuppression (20).

In this study, a total of 134 patients were included. There were 58 patients with HBV-GN, with a male to female ratio of 2.2:1, suggesting that HBV-GN is more common in males. Patients in our study mostly visited the doctor due to facial or limb edema (accounting for 56.9% of the included patients), but there were a considerable number of patients who visited the doctor due to hematuria and urinary protein was then found on examination, suggesting that occult development of HBV-GN can be missed easily, and that routine urinalysis is necessary for patients with HBV infection. The main clinical manifestation was nephrotic syndrome (46.6%), and MN was the main pathological type (43.1%), consistent with previous reports (21-23). Among the 58 patients, 72.4% of the patients were HBV carriers and only 25.9% were patients with chronic hepatitis B. This suggests that the development of HBV-GN does not appear to be correlated with liver disease progression. HBV-infected patients with normal liver function also need to look out for the development of HBV-GN. One patient with HBV-GN was HBsAg-negative but positive for HBV markers by renal immunohistochemistry, suggesting that renal biopsy is of great value in the diagnosis of HBV-GN. Routine urinalysis also cannot be ignored for patients with occult HBV infection. We found that serum C3 levels were lower in the HBeAg positive group, while the C3 and C1q deposition levels were significantly higher in the renal tissue compared with the HBeAg-negative group, suggesting that the pathogenesis of HBV-GN in HBeAg-positive patients may be achieved through the complement activation pathway. Antigen-antibody complex deposition in the glomerular capillary basement membrane can lead to kidney damage. The findings in our study are largely consistent with previous reports (24).

Most of the HBV-GN patients in the present study are HBeAg-negative. We therefore believe that different immune states of HBV-infected individuals cause different mechanisms of HBV-GN. The occurrence of HBV-GN in HBeAg-negative patients may be related to HBV pre-C mutations, which confer to the virus the ability to evade host immune surveillance; it may also be related to sustained direct damage caused by the virus as well as genetic susceptibility. HBeAg-positive patients had higher levels of aspartate aminotransferase and glutamyltranspeptidase than HBeAg-negative patients, which may be due to the fact that all 15 HBeAg-positive patients are in the immune clearance phase. To answer the question of whether HBeAg positivity is specific to HBV-GN requires data from a larger sample. High serum HBV DNA levels indicate high HBV replication rate *in vivo*. Increased HBV in the circulatory system can cause an increase in HBV-related antigens, followed by a large amount of circulating immune complex deposition in renal tissue, which may cause renal injury. In this study we found that serum HBV DNA levels in patients are correlated to the levels of urinary protein and serum albumin. The 24-h urinary protein levels in the HBV DNA moderate replication group were significantly higher than those in the low replication group, and serum albumin levels in the moderate replication group were significantly lower than those in the low replication group ($P<0.05$), consistent with previous observations (25). Increased HBV DNA replication levels were followed by increased HBV antigen deposition and more serious renal damage. As an independent factor for HBV-GN (26,27), HBV replication is critical. Cabrera *et al* (28) reported that HBV DNA can effectively be removed by 1-year lamivudine treatment. Lamivudine treatment can also lead to the reduction or even complete remission of proteinuria. In this study, 24-h urinary protein quantitation and serum albumin levels were not significantly altered in the high replication group, which may be explained by the small sample size and the different pathological types.

In addition, we also found that the degree of tubulointerstitium injury of patients in the HBV-GN groups was higher than in the non-HBV-GN group; consistent results were also found by comparing 24-h urinary protein between these two groups. A possible explanation is that the renal damage of HBV-GN was caused by a variety of pathogenic mechanisms: in addition to the classic renal damage caused by antigen-antibody complex deposition in the glomerular capillary basement membrane, renal damage could also be caused by autoimmune lesions and direct damage caused by HBV, so early diagnosis of HBV-GN is very important, and anti-HBV therapy is likely to be beneficial. The expression of Foxp3 in the HBV-GN group and the non-HBV-GN group was significantly lower than that in the non-nephritis control group, which indicated that the decrease of Treg cells might be one of the pathogenic mechanisms of nephritis, and support the involvement of immune dysfunction in the development of HBV-GN. In this study, no significant differences were found in Foxp3 expression levels between the HBV-GN group and the non-HBV-GN group, indirectly suggesting that renal injury in these two groups were caused by the same mechanism, which induced the reduced expression of Foxp3, leading to autoimmune injury. In the HBV infection control group, the expression of Foxp3 did not decrease, and no HBV-GN occurred, which may be related to genetic susceptibility. In the pathogenesis of HBV-GN, Tregs can inhibit the activity and proliferation capacity of $CD4^+$ and $CD8^+$ T cells (27-29). Our data showed that Foxp3 expression decreased in the tubulointerstitium of the patients with HBV-GN, which may be related to the weakened inhibition ability on $CD8^+$ cytotoxic T lymphocytes and further lead to renal tubulointerstitium

injury through the $CD8^+$ cytotoxic T lymphocyte-mediated perforin pathway. In conclusion, we believe that $Foxp3^+$ Tregs play a pivotal role in maintaining the balance between the host and the pathogen.

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