Letter to the Editor

The Role of HBx Gene Mutations in PLA$_2$R Positive Hepatitis-B-associated Membranous Nephropathy[*]

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China is a higher-intermediate hepatitis B virus (HBV) endemicity country, and HBV is among the most important infectious diseases[1,2]. Hepatitis-B-associated glomerulonephritis (HBV-GN) is also one of the main forms of secondary kidney diseases seen in China[3]. The main pathological type is hepatitis-B-associated membranous nephropathy (HBV-MN). In the recent years, it has been found that human renal podocyte membrane M-type phospholipase 2 receptor (PLA$_2$R) is the main autoantigen in idiopathic membranous nephropathy (IMN)[4]. At present, it is found that PLA$_2$R and its antibodies are closely related to the pathogenesis of IMN, but the specific mechanism is still unclear. PLA$_2$R expression also exists in secondary MN such as HBV-MN. A previous study confirmed that hepatitis B virus X gene (HBx) mutation plays an important role in the occurrence and development of HBV-GN[5]. Therefore, this paper will further study the expression of PLA$_2$R in HBV-MN and the effect of HBx gene mutation on PLA$_2$R expression and HBV-MN and explore the possible mechanism of HBx gene mutation in PLA$_2$R expression and pathogenesis in HBV-MN.

A total of 103 patients with biopsy-proven HBV-MN were included in the study. All patients were divided into two groups according to the results of PLA$_2$R immunofluorescence staining in renal tissue: 66 cases were PLA$_2$R-positive and 37 cases were negative. The present study was approved by the Ethics Committee of The Affiliated Hospital of Qingdao University and an informed written consent was obtained.

Laboratory tests were as follows: 24 h urine protein quantity, serum creatinine, albumin, HBV DNA titer, C3, cholesterol, serum HBV biomarkers including HBsAg, HBsAb, HBcAg, HBeAg, and HBeAb,

and immune index. Total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and platelet counts were normal. A standard kidney biopsy procedure, including light, immunofluorescence (IF), and electron microscopy, was used for renal pathological diagnosis. MN was divided into 3 phases: MN stage I, MN stage II, and MN stage III. Positive PLA$_2$R was characterized as granular staining along the capillary loops and was scaled from 0 to 3+. HBsAg, HBcAg, and HBeAg were regularly detected in kidney tissue by IF microscopy.

To determine the mutation sites, the obtained sequences were aligned and analyzed with the standard sequence retrieved from GenBank database. SPSS 19.0 software was used for the statistical analysis. Quantitative data were expressed as mean ± SD. One-way ANOVA was used to compare the data among three groups or more, while t-test was used to compare the data between two groups. Chi-square test was used for the comparison of qualitative data. Spearman's rank correlation coefficient was used for correlation analysis.

There were 103 cases of HBV-MN. All patients with HBV genotype were C-genotype. 66 cases (64%) had positive PLA$_2$R in the renal tissue, and 37 cases (36%) were PLA$_2$R-negative. There were 45 males and 21 females who had positive PLA$_2$R in the renal tissue. The male-to-female ratio was 2.14:1, with an average age of 42.2 ± 13.8 years. There were 25 males and 12 females with negative PLA$_2$R in the renal tissue. The male-to-female ratio was 2.08:1, with an average age of 43.8 ± 14.4 years. There was no significant difference in age and male-to-female ratio between the two groups ($P > 0.05$). All patients had proteinuria while some patients experienced hematuria (Table 1; Figure 1).

The main laboratory indexes of the two groups were compared, including 24 h urinary protein quantity, serum albumin, serum creatinine, cholesterol, and C3. It was found that the 24 h urinary protein quantity in the PLA_2R-positive group was significantly higher than that in the PLA_2R-negative group. The difference was statistically significant ($P < 0.01$). There was no significant difference in other clinical indexes ($P > 0.05$) (Table 2).

Renal pathological injury was compared between the two groups. Each group was divided into two groups according to the pathological stage of HBV-

Table 1. Baseline demographic and clinical characteristics in subjects with patients

Characteristics	n	PLA_2R+	PLA_2R-
Age		42.2 ± 13.8	43.8 ± 14.4
Gender (n, %)			
Male	70	45 (64.3)	25 (35.7)
Female	33	21 (63.6)	12 (36.4)
Pathology (n, %)			
MN stage I	43	21 (48.8)	22 (51.2)
MN stage II	44	33 (75.0)	11 (25.0)
MN stage III	16	12 (75.0)	4 (25.0)
HBsAg+	28	20 (71.4)	8 (28.6)
HBcAg+	31	20 (64.5)	11 (35.5)
HBeAg+	103	66 (64.1)	37 (35.9)
C-genotype	103	66 (64.1)	37 (35.9)

MN: MN stage I (mild pathological injury) and MN stages II–III (severe pathological injury). The changes of the pathological injury were compared between the two groups. MN stage I patients accounted for 31.8% and MN stages II–III patients accounted for 68.2% in the PLA_2R-positive group. In the PLA_2R-negative group, MN stage I patients accounted for 59.5%, and patients of MN stages II–III accounted for 40.5%. There was a significant difference between MN stage I and stages II–III ($X^2 = 7.449$, $P < 0.01$), and there was also a significant difference between the two groups in MN stages II–III ($X^2 = 10.150$, $P < 0.05$). (Supplementary Figure S1 available in www.besjournal.com). It is suggested that the kidney injury in the PLA_2R-positive group is more severe than that in negative group. Secondly,

Table 2. Clinical index in different groups (mean ± SD)

Index	PLA_2R+ ($n = 66$)	PLA_2R- ($n = 37$)
Urinary protein (g/d)	4.7 ± 2.2[#]	3.1 ± 2.3
Serum albumin (g/L)	29.6 ± 8.7	32.2 ± 8.8
Serum creatinine (μmol/L)	94.9 ± 23.1	96.8 ± 23.7
Serum cholesterol (mmol/L)	5.5 ± 0.8	5.4 ± 0.8
C3 (mg/dL)	96.8 ± 16.9	94.5 ± 16.2
BIL-T (μmol/L)	10.8 ± 3.4	9.6 ± 3.7
ALT (U/L)	18.0 ± 4.5	17.0 ± 4.7
AST (U/L)	23.0 ± 6.2	24.0 ± 6.1

Note. PLA_2R: M-type phospholipase 2 receptor. [#]$P < 0.01$ as compared with PLA_2R-negative group.
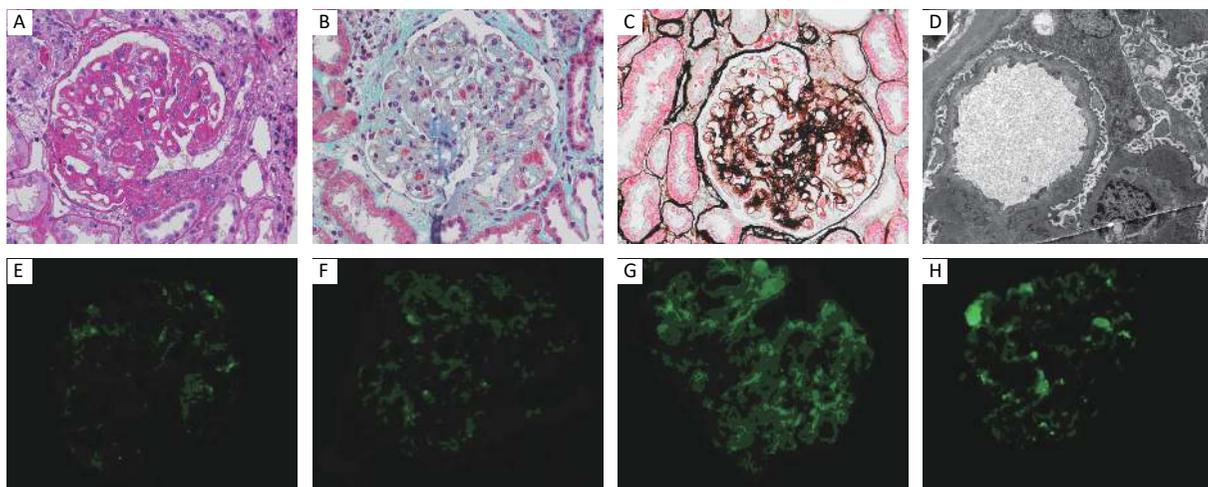
Figure 1. Pathology of HBV-MN. (A) PAS stain (×400), (B) Masson stain (×400), (C) PASM stain (×400), (D) Electron microscopy (×10,000), (E) IF staining for HBsAg (×400), (F) IF staining for HBcAg (×400), (G) IF staining for HBeAg (×400), (H) IF staining for PLA_2R (×400).

Spearman's rank correlation analysis was performed according to different PLA$_2$R IF staining intensity and different MN pathological stages in the PLA$_2$R-positive group. It was found that with the increase of PLA$_2$R expression intensity, MN stage was gradually increasing and the renal pathological injury was aggravated. The difference was statistically significant (r = 0.325, P < 0.01). (Supplementary Table S1 available in www.besjournal.com).

The mutation of HBx gene impacts the corresponding amino acid changes in two groups. The X gene sequencing was compared with C-genotype adr-serotype HBV genome sequence. It was found that only seven patients had no mutation in the two groups. Nucleotides mutation occurred in the other 96 patients, which led to the replacement of amino acids. Nucleotides missense mutations include the following sites: nt1653, nt1726, nt1727, nt1730, nt1753, nt1762, and nt1764, which resulted in amino acid mutations of aa94, aa118, aa119, aa127, aa130, and aa131, respectively. nt1726/1727 double mutation and nt1762/1764 double mutation often existed at the same time. (Supplementary Table S2 available in www.besjournal.com) .

Hepatitis B is among the most important infectious diseases in China. An estimated seroprevalence of HBsAg was 5.49% in 2013[6], compared with 7.18% in 2006 by the national serosurvey. According to a population-based prospective cohort study of 0.5 million Chinese adults[7], HBsAg positivity was significantly associated with a higher risk of incident chronic kidney disease (CKD), and the association was stronger in men than in women. HBV-GN is one of the main secondary kidney diseases in China. In our previous studies, it was found that the proportion of HBV-MN in HBV-GN was as high as 75%[3]. PLA$_2$R is a glycoprotein, and its expression has been reported in the podocytes of the kidney. PLA$_2$R was characterized as a major target antigen of IMN in 2009[8]. A recent study showed that PLA$_2$R was detected in the kidney in the majority of patients with IMN, but not in non-MN patients or normal control patients[9]. It was hypothesized that the antigenicity of PLA$_2$R on podocytes may be altered in IMN, which leads to podocyte injury and disease progression. The cause of the change of PLA$_2$R in IMN remains unclear. Whether the expression of PLA$_2$R is increased in secondary MN and is involved in the pathogenesis of the disease is not clear. Renal PLA$_2$R was detected in 66 patients from the 103 HBV-MN patients we had studied. A previous study had reported the presence of renal PLA$_2$R in HBV-MN[10]. Xie et al.[11] reported

that renal PLA$_2$R was positive in 25 of 39 patients with HBV-MN. Our study analyzed the clinical indexes and renal pathological changes of PLA$_2$R-positive and PLA$_2$R-negative groups and discussed the significance of PLA$_2$R in HBV-MN.

Comparing the clinical indexes in two groups, it was found that the 24 h urinary protein quantity in the renal PLA$_2$R-positive group was significantly higher than that in the PLA$_2$R-negative group, and the difference was statistically significant. However, there was no significant difference in serum creatinine, serum albumin, cholesterol, and C3 levels. According to the pathological stage of MN, we found that the proportion of patients with MN stages II–III in the renal PLA$_2$R-positive group was significantly higher than that in MN stage I group, while the proportion of MN stage I in the renal PLA$_2$R-negative group was higher than that in MN stages II–III. Combined with the urinary protein quantity and renal pathological stage, it was shown that the renal injury in PLA$_2$R-positive group was more severe than that in the PLA$_2$R-negative group, and the difference was statistically significant. Secondly, according to the IF intensity in renal tissue, PLA$_2$R was divided into 1+ to 3+. The relationship between different intensities and renal pathological stage was compared. It was found that the higher the PLA$_2$R intensity, the more serious the pathological stage. Some studies have found that PLA$_2$R IF exhibited a granular staining along the capillary loops and was colocalized with HBsAg, which suggests that PLA$_2$R and HBV antigen may have crosstalk in the glomerular capillary wall leading to podocyte injury. However, the specific mechanism by which this occurs is unknown. In our study, the positive HBsAg in renal tissue was 30.3% in the PLA$_2$R-positive group and 26.6% in the PLA$_2$R-negative group, with no significant difference between the two groups. However, whether there is a correlation between both of them needs to be studied in a larger sample. In conclusion, the increase of PLA$_2$R expression was correlated with the progression of HBV-MN.

Our previous study found that in most HBV-GN patients, X gene missense mutations occurred at some key sites, which plays an important role in the pathogenesis of HBV-GN[5]. We explored the question of whether the mutation of HBx gene related to the expression of PLA$_2$R in renal tissue, which leads to the progress of disease. We analyzed the mutation sites of X gene in HBV-MN patients and further clarified the relationship between X gene mutation and PLA$_2$R. We found that most of HBV-

MN had mutations in X gene, including 64 cases (97.0%) in renal PLA$_2$R-positive group and 32 cases (86.5%) in renal PLA$_2$R-negative group. Further analysis of the mutation sites between the two groups showed that almost all patients had X gene mutations, which led to the substitution of amino acids. The main missense mutations included aa94 (nt1653), aa118 (nt1726, nt1727), aa119 (nt1730), aa127 (nt1753), aa130 (nt1762), and aa131 (nt1764). Seven patients had no X gene mutations, two cases in the PLA$_2$R-positive group and five cases in the PLA$_2$R-negative group. Most patients had mutations in two sites or more. In the two groups, most of the patients had T1762/A1764 double mutation or nt1762 mutation. T1762/A1764 double mutation caused the change of aa130/131. Both TC cell epitopes (aa126–aa134) and TH cell epitope (aa111–aa135) are involved in the immune response[12,13], which contain aa130/131. Immune escape caused by mutation of aa130/131 may be an important aspect in the pathogenesis of HBV-MN. Most of the PLA$_2$R-positive groups had site mutations in nt1753. It was found that nt1753 was located in the transregulatory region of HBx protein. The mutation of this site could affect the transactivation of X protein, and the enhancement of transactivation might have an effect on renal podocytes, which leads to the exposure of PLA$_2$R specific target antigen on the surface of podocytes and the increase of PLA$_2$R expression, thus leading to renal injury. However, most of the patients in the PLA$_2$R-negative group had mutations in nt1726/1727 and nt1730. In previous studies, it was considered that nt1726/1727 and nt1730 mutations might be accompanied by low replication of virus[14], which was related to the degree of lesion. This may explain why the degree of proteinuria and kidney injury was mild in PLA$_2$R-negative patients.

In conclusion, we found that renal PLA$_2$R was positive in most of patients with HBV-MN and PLA$_2$R in renal tissue is associated with proteinuria and affects the degree of renal injury. The presence of PLA$_2$R in renal tissue was associated with HBx gene mutation in nt1753. These results provide valuable information for understanding the mechanism of PLA$_2$R in HBV-MN.

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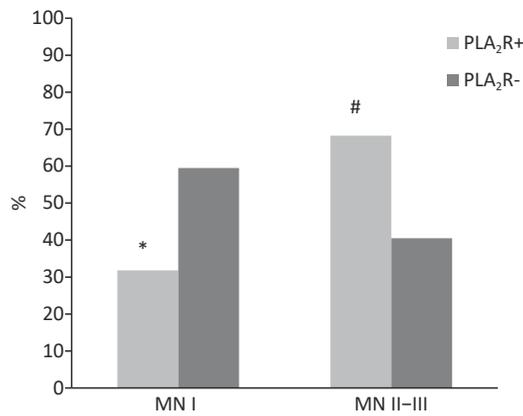
Supplementary Figure S1. Percentage of MN stage in different groups, [#]$P < 0.05$ and [*]$P < 0.01$ as compared with PLA$_2$R-negative group.

Supplementary Table S1. Relationship between HBV-MN and PLA$_2$R

	MN stage I	MN stage II	MN stage III
PLA$_2$R1+	10	6	3
PLA$_2$R2+	8	15	2
PLA$_2$R3+	3	12	7

Note. Spearman correlation coefficient between PLA$_2$R and HBV-MN ($r = 0.325$, $P < 0.01$).

Supplementary Table S2. HBx gene mutations in two groups

PLA$_2$R+	PLA$_2$R-	Nucleotides mutation					
		nt1653	nt1726/1727	nt1730	nt1753	nt1762	nt1762/1764
2	5	−	−	−	−	−	−
1	7	+	+	+			
5	4		+		+		
1	8			+			+
1	6		+	+			+
1	4		+	+		+	
2	1	+					
10	0				+		
17	1				+	+	
26	1				+		+

Note. -, Wild +, Mutant.