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Over IFI16 expression increased inflammation in Hepatitis B Virus-Associated Glomerulonephritis by Regulating Caspase-1 and IL-1 ß

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Research

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Abstract

Aims & background: IFI16 plays an important role in innate immunity against invasive microbial infection by sensing double-stranded DNA viruses due to caspase-1-dependent inflammasome activation and subsequent maturation and secretion of IL-1β. However, the role of IFI16 in regulating the immune response to viruses in vivo and in vitro, especially in sensing hepatitis B virus (HBV), has not been examined. We hypothesized that the expression of IFI16 increases corresponding to HBV-mediated inflammation in patients with hepatitis B virus associated glomerulonephritis (HBV-GN), a condition which activates inflammatory mechanisms and causes renal damage. To test this hypothesis, we therefore analyzed the expression of IFI16 and inflammatory factors in HBV-GN tissues and cell lines relative to the inflammatory response to HBV infection.

Methods: A total 75 patients with chronic nephritis(CN) including 50 with HBV-GN and 25 with chronic glomerulonephritis (CCN) involved in this study. Each CN patient received renal biopsy, and immunohistochemistry(IHC) was used to detect the expression of IFI16 and inflammatory factors Caspase-1 and IL-1β in the biopsy specimens. Following IFI16 was transfected in HBV-infected and HBV-uninfected human glomerular mesangial (HGM) cell line and HEK-293T cell line, expression of Caspase-1 and IL-1β were detected by Western blot and qRT- PCR. Results: IFI16 expression in HBV-GN biopsies (80.0%) was significantly higher than in CGN (24.0%) and positively correlated with caspase-1 and IL-1β expression in HBV-GN. In vitro, over expression IFI16 increased caspase-1 and IL-1β expression in HBV - infected HGM and HEK-293T.

Conclusions: The elevation of IFI16 during HBV infection or replication may contribute to renal damage due to inflammation, thus providing a putative therapeutic target and a new avenue for researching the pathogenesis of HBV-GN.

1. Introduction

Hepatitis B virus (HBV) infection is an important public health problem worldwide. HBV infection leads both hepatic and extrahepatic organ injures. Hepatitis B virus-associated glomerulonephritis (HBV-GN) is common extrahepatic diseases of HBV-infection and remains one of the most common secondary glomerular diseases [1]. Ever since HBV-associated-glomerular diseases was first reported by Combes et al. in 1971 [2], more HBV-GN cases have been described all over the world. The common sense is persistent HBV infection could induced to immune complex-mediated nephritis [3]. However, the specific pathogenesis of HBV-GN remains unclear.

IFI16 (Interferon-g-inducible protein 16) belongs to Pyrin-Hin200 family(PHI-200), playing important role in innate immune and oncogenesis[4, 5]. As innate immune sensors, IFI16 recognizes both cytosolic and nuclear double-stranded DNA (dsDNA) from invaded DNA viruses such as vaccinia virus (VACV), herpes simplex virus 1 (HSV-1), and Kaposi sarcoma-associated herpesvirus (KSHV)[6, 7, 8]. DNA recognition by IFI16 triggers downstream stimulator of interferon genes-TANK-binding kinase 1-interferon regulatory

factor 3 (STING-TBK1-IRF3) signaling to induce type I interferon (IFN-I) or apoptosis-associated speck-like protein containing a CARD (ASC)-caspase-1-dependent inflammasome to produce interleukin-1ß (IL-1ß)[8, 9]. Both IFN-I and IL-1ß are important inflammatory cytokines playing critical roles in the host immunity against viral infection. However, over activation of IFN-a/b receptor (IFNAR)or caspase-1 induced excessive production of IFN-I or IL-1ß and lead to autoimmune diseases such as systemic lupus erythematosus (SLE) and Sjo¨ gren syndrome[10, 11]. Although IFI16 induces the ASC-dependent inflammasome pathway and the IFN-b pathway through the STING-TBK1-IRF3 axis is well understood, the specific pathogenesis associated IFI16 against HBV invaded is unclear. HBV contains a circular and partially double-stranded DNA(dsDNA), and HBV particles have been shown to be detectable in the kidneys of HBV-GN patients [12]. Cytoplasmic HBV-DNA in the kidneys has a high possibilities to be recognized by IFI16. The potential binding of IFI16 to HBV-DNA may lead to the activation of caspase-1 and subsequent maturation and secretion of IL-1β. This cascade of events leads to the development of the inflammasome, which may then be responsible for the renal damage seen in HBV-GN patients.

To test this hypothesis, we compared the expression of IFI16 in HBV-GN and chronic glomerulonephritis (CGN) and further explored the relationship between the expression of IFI16, caspase-1 and IL-1β. Moreover, The effects of IFI16, caspase-1 and IL-1β expression status in primary human glomerular mesangial (HGM)cells and HEK-293T cells transfected with HBVDNA and IFI16 or vector control or empty were also investigated. Our results showed that IFI16 expression is high in HBV-GN compared with CGN, IFI16 expression was positive correlation with caspase-1 and IL-1β expression. Furthermore, over-expression of IFI16 increased expression of caspase-1 and IL-1β in vitro. Thus, IFI16 induces IL-1β releasing though activation of caspase-1 axis may playing an important role in the development and progression of inflammation in HBV-GN.

2. Materials And Methods

2.1 Patients

Our retrospective study was approved by the ethics committee of Jinan Infectious Disease Hospital(JCLL-2016-04). A total of 75 patients diagnosed with chronic nephritis, identified between 2008 and 2016 at Jinan Infectious Disease Hospital and QiLu Hospital of Shandong University Shandong, China, were included in the study. The experimental group consisted of 50 HBV-GN patients, the negative control group consisted of 25 CGN patients, Subjects received either kidney (CGN)puncture biopsy under ultrasound guidance to attain nephridial tissue, respectively, for diagnosis and subsequent research. Participation was dependent upon fulfillment of the following criteria:(1) patients must not have used an immune agent or antiviral agent in the past three months; (2) patients must not have HAV, HCV, HDV, HEV or HIV co-infection; (3) patients must not have a history or current evidence of secondary glomerulonephritis; and (4) consent for participation must have been obtained from those who participated.

2.2 Diagnosis of HBV-GN, CGN and pathological classification of HBV-GN

The diagnostic criteria used for CGN and HBV-GN were in accordance with the 2002 Kidney Disease Outcome Quality Initiative (K/DOQI), edited by the National Kidney Foundation (NKF) [13]. The diagnosis of HBV-GN was confirmed by pathology. The pathological classification of and diagnostic criteria used for HBV-GN were in accordance with 1990 WHO classification criteria [14]. Frozen slices from biopsies of the 50 HBV-GN patients were kept in a low-temperature freezer. Monoclonal goat-anti-human HBsAg and HBcAg antibodies were purchased from Dako (Denmark), and immunohistochemical staining for HBsAg and HBcAg in renal biopsies was used or electron microscope detection for HBV to confirm the diagnosis (Figs. 1 and 2A). For HBV-GN patients with undetectable HBsAg or HBcAg in nephridial tissue, HBV was detected using the JCM-6000 scanning electron microscope from Jeol, Ltd. (Japan). Sections from all biopsy specimens were stained routinely with hematoxylin and eosin (H&E), periodic acid-sliver methenamine (PASM), Masson's trichrome and antibodies against IgA, IgG, IgM, C3 and C1q complement component. Fluorescently-labeled IgA, IgG, IgM, C3 and C1q rabbit-anti-human antibodies were purchased from Dako.

2.3 Immunohistochemistry and scoring

Nephridial and hepatic tissue specimens were first fixed in 10% formalin, then the tissue was cut, dehydrated, dipped in wax, embedded and sectioned. These sections were then placed on slides, baked, placed into xylene, cleared of the wax, rehydrated using graded ethanol and immersed in 0.3% hydrogen peroxide for five minutes to reduce non-specific background staining caused by endogenous peroxidase. The slides were then washed with PBS buffer three times for five minutes each, placed in citrate buffer solution at a pH of 6.0 and then into a high temperature pressure pot to recover the tissue antigen. After being heated, the slides were cooled and restored at room temperature, washed three more times in PBS buffer and incubated with AIM2 (ab93015, rabbit anti-human polyclonal antibody; Abcam, USA), caspase-1 (sc-56063,mouse anti-human polyclonal anti- body; Santa Cruz Biotechnology Inc., USA) and IL-1β antibodies (ab2105, rabbit anti-human polyclonal anti-body; Abcam), respectively. The slides were then placed in a 4 °C refrigerator overnight. The next day, the slides were washed with PBS buffer three times, each time lasting longer than five minutes, then incubated with the secondary antibody PV-9000 (universal antibody) at 37 °C for 10 minutes, washed with PBS buffer, and DAB staining was applied. The stain was terminated using running water, then the slides were washed with hydrochloric acid alcohol for differentiation. Lastly, the slides were washed with distilled water, cleared with xylene and mounted. Appearance of a tan stain in the cytoplasm signaled positive expression of the protein. After staining, scores were assigned based on stain intensity and percentage of positive cells as follows: For stain intensity, a score of 0 was given for no brown staining (i.e., no cells stained), 1 for light brown, 2 for brown and 3 for dark brown; for percentage of positive cells, a score of 0 was given for fewer than 5% positive cells, 1 for 5–30%, 2 for 30–60% and 3 for greater than 60%. Scores for stain intensity and percent positive were then added together, and a negative sign (−) was assigned for scores totaling 0, mildly

positive (+) for scores between 1 and 3, mode- rately positive (++) for scores between 4 and 6 and strongly positive (+++) for scores greater than 7.

2.4 Cell lines and reagents

The human glomerular mesangial (HGM) cell line and HEK-293T cell line used in this study were purchased from the cell bank of the Chinese academy of sciences (Shanghai, China) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Life Technologies, Carlsbad, CA, USA), ampicillin and

streptomycin at 37 °C, 5% CO_2 conditions. HBV expression plasmids were constructed with a pcDNA3.0 vector. The 1.1-fold over length HBV genome was cloned into the pcDNA3 vector to generate pcDNA3.0-1.1HBVDNA, 1.1HBV as the expression gene and ampicillin resistance for antibiotic selection(Amresco, Penn- sylvania, USA). Caspase-1, IL- 1β, antibodies were obtained from Cell Signaling Tech Abcam (Cambridge, MA,USA).

2.5 Cell transfection

HGM cell line and HEK-293T cell line were seeded into 12-well plates and transfected with pcDNA3.0-1.1HBVDNA- IFI16 or negative control for indicated time at a concentration of 1 ng/mL by Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.6 Western blots

According to the manufacturer's instructions, the whole cell protein extracts were prepared and were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis and 10% gel. Proteins were then transferred to a polyvinylidene difluoride membrane (Millipore, Bed- ford, MA, USA), according to the instruction manual. Filters were blocked overnight in 5% w/v low- fat dry milk in 10 mmol/L Tris- HCl, pH 7.5, 0.1 mol/L NaCl and 0.1% Tween- 20 and incubated with primary antibodies overnight at 4 °C. After washing with TBST buffer, the blots were then incubated with HRP- conjugated secondary antibody for 2 hours at room temperature. After washing with TBST buffer, the blots were visualized using the ECL- Plus reagent (Millipore, Billerica, MA, USA). GAPDH was used as the loading control in the Western blotting.

2.7 qRT-PCR

Quantitative real- time RT- PCR was performed, and the expression levels of IFI16, caspase-1 and Il-1ß were normalized to GAPDH for gene expression. The primer:
IFI16FP:AGACTGAAGACTGAACCTGAAGA;RP:GAACCCATTGCGGCAAACATA.caspase-1F:GCTTTCTGCTCTTCCACACC; R: CATCTGGCTGCTCAAATGAA. Il-1ßF:GCACAAGGCACAACAGGCTGC;R:CAGGTCCTGGAAGGAGCACTTCA

2.8 Statistical Analysis

The SPSS program (version 19.0) was used for analysis. Measurement data was described as mean ± standard deviation. Background factors were compared using Student's-test (numerical data) or the

Chisquare test (categorical data). Spearman's two-tailed test was used for correlation analysis, and differences were regarded as significant if the p value was less than 0.05 on either side.

3. Results

3.1 Expression of IFI16 was significantly high in HBV-GN compared with CGN

We investigated and compared the expression of IFI16 in biopsied kidney tissue from 50 HBV-GN and 25 CGN patients. The results showed that IFI16 expression was exclusive to the cellar cytoplasm of glomerular endothelial cells and mesangial cells in the tissue(Fig. 2B). Statistical analysis revealed that the positive expression rate of IFI16 in HBV-GN patients was significantly higher than in CGN patients (80% versus 24%, $p<0.01$); (Table1) To further clarify the potential correlated factor associated with expression of IFI16, we analyzed the correlation with status of HBV-associated antigen deposited in kidney and pathological type of HBV-GN. the results indicated that expression have no correlation with the status of HBV-associated antigen deposited in kidney and pathological type of HBV-GN, respectively(p = 0.510, p = 0.997) (Table2).

Table 1

Expression of IFI16 was significantly high in HBV-GN compared with CGN

group	Tissue	Cases(n)	Age	Gender(M %)	IFI16				Positive Rate(%)
					-	+	++	+++	
HBV-GN	K	50	36.1 ± 12.7	35(70%)	10	14	26	0	80
CGN	K	25	38.2 ± 15.5*	18(72%)**	19	6	0	0	24***

HBV-GN: Hepatitis B viral associated glomerulonephritis; CGN: Chronic glomerulonephritis; K: kidney; M: Male; *compared with HBV-GN,(t = -0.072,p = 0.943); **compared with HBV-GN, (x2 = 0.032, p = 0.85); ***compared with HBV-GN, N, (x2 = 30.091, p < 0.01)

Table 2

Expression of IFI16 was negatively correlated with HBV antigen

	Expression of IFI16					
	N(%)	-	+	++	χ^2	p
s-Ag+,c-Ag+	20(40%)	5	5	10		
s-Ag+,c-Ag-	24(48%)	4	6	14		
s-Ag-,c-Ag+	3(6%)	1	2	0		
s-Ag-,c-Ag-	3(6%)	0	1	2		
Total	50	10	14	26	5.125	0.510

3.2. Expression of IFI16 was positively correlated with Caspase-1 and IL-1ß in HBV-GN tissue

We investigated the expression of Caspase-1、IL-1ß in biopsied kidney tissue from 50 HBV-GN, The results showed that Caspase-1、IL-1ß expression were exclusive to glomeruli, renal tubules and interstitial foci(Fig. 2C, D). The correlation between expression of IFI16 and Caspase-1、IL-1ß was analyzed. Statistical analysis revealed that the expression of IFI16 was positively correlated with that of Caspase-1($r = 0.998$, $p＜0.01$) and IL-1ß ($r = 0.953$, $p＜0.05$). (Table3).These results suggested that the expression of IFI16 was correlated with inflammation in HBV-GN tissue and elevation of IFI16 may responsible for inflammatory damage of HBV-GN.

Table 3

Expression of IFI16 was no correlation between pathological types of HBV-GN.

	Expression of IFI16				χ^2	p
PT	**n(%)**	**-**	**+**	**++**		
MsPGN	15	3	4	8		
MPGN	8	1	2	5		
MN	23	6	6	11		
MCG	2	0	1	1		
FSS	2	0	1	1		
Total	50	10	14	26	2.927	0.997

PT,Pathological type;;MsPGN, Mesangioproliferative glomerulonephritis; MPGN, Membranoproliferative glomerulonephritis; MN, Membranous nephropathy; MCG, Minimal change glomerulopathy; FSS, Focal segmental sclerosis.

Table 4
Expression of IFI16 was positively correlated with Caspase-1 and IL-1ß in HBV-GN tissue

IFI16	**Caspase-1**				**Il-1ß**			
	-	**+**	**++**	**+++**	**-**	**+**	**++**	**+++**
+++	0	0	0	0	0	0	0	0
++	5	5	14	2	3	10	12	1
+	0	4	10	0	1	4	9	0
-	6	4	0	0	5	4	1	0
rs	0.998				0.953			
p	< 0.01				< 0.05			

A; Subepithelial granular electron compact deposited in the basement membrane of capillary wall in HBV-GN. Transmission electron microscopy, 7500×

B; Organelles were abundant and swollen in the cytoplasm, and more virus-like particles were deposited. Transmission electron microscopy, 7500×

C; Intracytoplasmic globular virus-like particles. Transmission electron microscopy, 30000×

A; The immunohistochemical staining of HBcAg was positive, and the brown-yellow granules were distributed along the capillary wall of the glomerulus. IHC ,400×

B; IFI16 was positively expressed in the cytoplasm and nucleus of glomerular cells in HBV-GN. IHC ,400×

C; The positive expression of caspase-1 in HBV-GN. IHC ,400×

D; The positive expression of IL-1β in HBV-GN. IHC ,400×

3.3. Over expression of IFI16 promoted inflammation in vitro

HGM cells and HEK-293T cells were divided into three groups: IFI16 and HBV DNA cotransfected group (over expression group, OE), HBVDNA-transfected group without IFI16 (negative control group, NC), and empty plasmids transfected group (blank group, Blank). Expression of IFI16, Caspase-1and IL-1ß significantly elevated in OE group compared those in NC and Blank group, respectively. These similar changes were noted by both Western blot measured and qRT-PCR detection(Fig. 3).

4. Discussion

Hepatitis B virus associated glomerulonephritis (HBV-GN) is one of most common HBV infection associated diseases. However, the specific pathogenesis and role of HBV in HBV-GN remains unclear. The widely accepted view is that immune complex including circulating and in situ immune complexes

depositing in basement membrane mediated immune damage[2, 15]. lin[16] proved that CD4 + T subgroup decreased in HBV-GN, suggesting cellular immunity deficiency existing in HBV-GN. Moreover, The autoimmune response may play a important role in the pathogenesis of HBV-GN[17, 18]. Increasing immunological evidence and electron microscope finding have suggested HBV-DNA replication was detected directly in HBV-GN kidney tissue[19, 20]. Therefore, besides HBV-mediated immune injure, HBV directly infecting renal tissues may also be involved in the pathogenesis.

IFI16 is a member of the HIN 200 family, playing crucial part in against invaded DNA virus in innate immunity[4]. It can sense dsDNA from some viruses to activate STING-TBK1 pathway for the production of IFN- β for anti-virus defence and on the other hand, it has also been uncovered to bind DNA and form an inflammasome—a cytoplasmic protein complex(ASC) which induces pyroptosis and proteolytic maturation of inflammatory cytokines IL-1β and IL-18[7, 8]. IL-1β is not only essential to innate immune defense, but is also an important mediator of adaptive immune response to viral infections[21]. Therefore, it is critical for the clearance of pathogens or damaged cells. However, its role related to HBV infection in HBV-GN pathogenesis is still unknown.

Our study found that expression of IFI16 significantly elevated in HBV-GN kidney tissues compared with that in CCN kidney tissues, and its exclusive expression in glomerular endothelial cell and mesangial cell cytoplasm in the kidney tissues. In addition, expression of IFI16 positively correlated with expression of Caspase-1and IL-1ß, these results suggested that IFI16 may sense HBV-DNA leading to the activation of caspase-1 and subsequent maturation and secretion of IL-1β. This cascade of events leads to the development of the inflammasome, which may then be responsible for the renal damage seen in HBV-GN patients. To further clarify the factors affecting the expression of IFI16 in HBV-GN patients, we considered the potential influence of HBV associated antigen deposited in kidney tissues and clinical pathological type. The results revealed that IFI16 expression negatively correlated to HBV associated antigen status and pathological type.

To further verified the IFI16 induces the ASC-dependent inflammasome pathway through the Caspase-1and IL-1ß axis leading kidney injure, we analyzed the expression of IFI16, Caspase-1and IL-1ß in over expression IFI16 cells transfected HBV, the results demonstrated that both protein and mRNA of IFI16, Caspase-1and IL-1ß significantly elevated in OE group compared those in NC and Blank group, respectively. The results suggested that this inflammation signal transfer pathway was related to IFI16 levels in HBV-GN and, subsequently, that IFI16 may play an important role in the pathogenesis of HBV-GN.

Conclusions

In summary, we found the expression of IFI16 to be significantly increased in HBV-GN patients. In vitro, over expression IFI16 leads to significantly elevated of Caspase-1and IL-1ß. Collectively, these results suggest that the elevation of IFI16 during HBV infection or replication may contribute to renal damage due to inflammation. Our findings may help provide a new therapeutic target and a new avenue for HBV-GN.

Abbreviations

HBV
Hepatitis B virus; HBV-GN:Hepatitis B virus associated glomerulonephritis; CN:chronic nephritis; CCN:chronic glomerulonephritis; PHI-200:Pyrin-Hin200 family; IFN-I:typeI interferon; IFI16:Interferon-g-inducible protein 16; ASC:Apoptosis-associated speck-like protein; IL-1β:Interleukin-1β; HGM:human glomerular mesangial; VACV:vaccinia virus; HSV-1:herpes simplex virus 1; KSHV:Kaposi sarcoma-associated herpesvirus; dsDNA:double-stranded DNA; HAV:Hepatitis A virus; HCV:Hepatitis C virus; HEV:Hepatitis E virus; HIV:Human immunodeficiency virus; K/DOQI:Kidney Disease outcome quality initiative; NKF:National Kidney Foundation; H&E:Hematoxylin and eosin; PASM:Periodic acid-sliver methenamine; MsPGN:Mesangioproliferative glomerulonephritis; MPGN:Membranoproliferative glomerulonephritis; MN:Membranous nephropathy; MCG:Minimal change glomerulopathy; FSS:Focal segmental sclerosis.

Declarations

Ethical Approval and Consent to participate

This study was approved by the ethics committee of Jinan Infectious Disease Hospital(JCLL-2016-04).

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

None of the authors has an affiliation or conflict of interests.

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Authors’ contributions

Study concept and design: DWJ, Acquisition of data: DWJ and ZZQ, Analysis and interpretation of data: ZZQ and LYY, Drafting of the manuscript: LYY, Critical revision of the manuscript for important intellectual

content: DWJ, Statistical analysis: YJH and LJ, Administrative, technical or material support: YJH, LJ and CSJ, Study supervision: LQ. All authors read and approved the final manuscript.

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Authors' information

See information below the heading.

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Figures

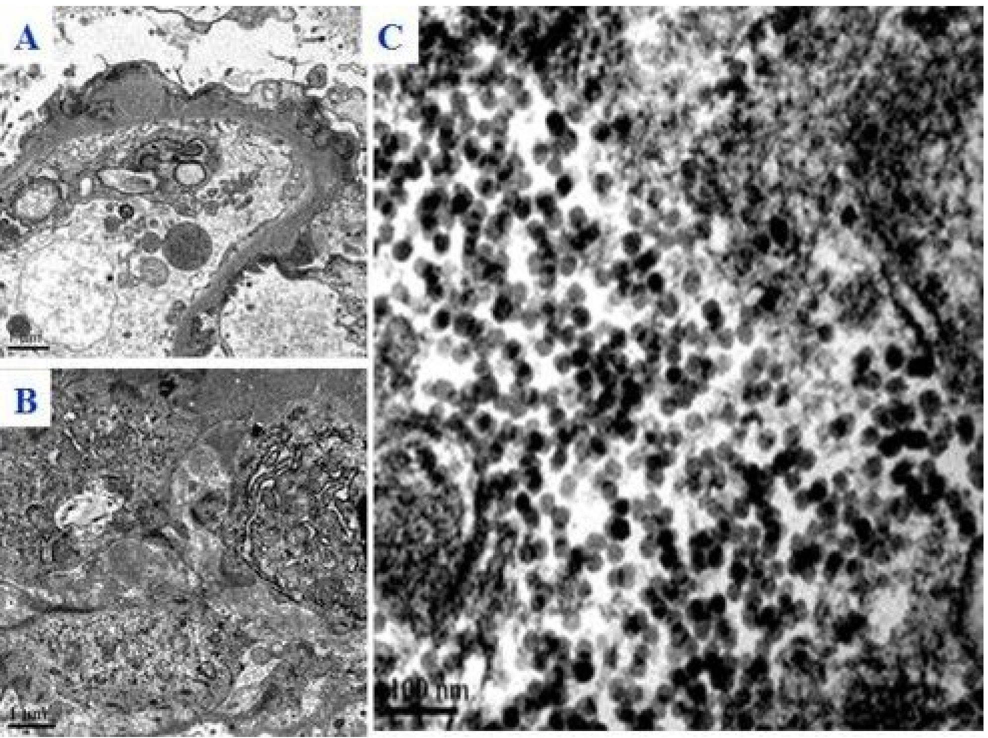

Figure 1

A; Subepithelial granular electron compact deposited in the basement membrane of capillary wall in HBV-GN. Transmission electron microscopy, 7500× B; Organelles were abundant and swollen in the cytoplasm, and more virus-like particles were deposited. Transmission electron microscopy, 7500× C; Intracytoplasmic globular virus-like particles. Transmission electron microscopy, 30000×

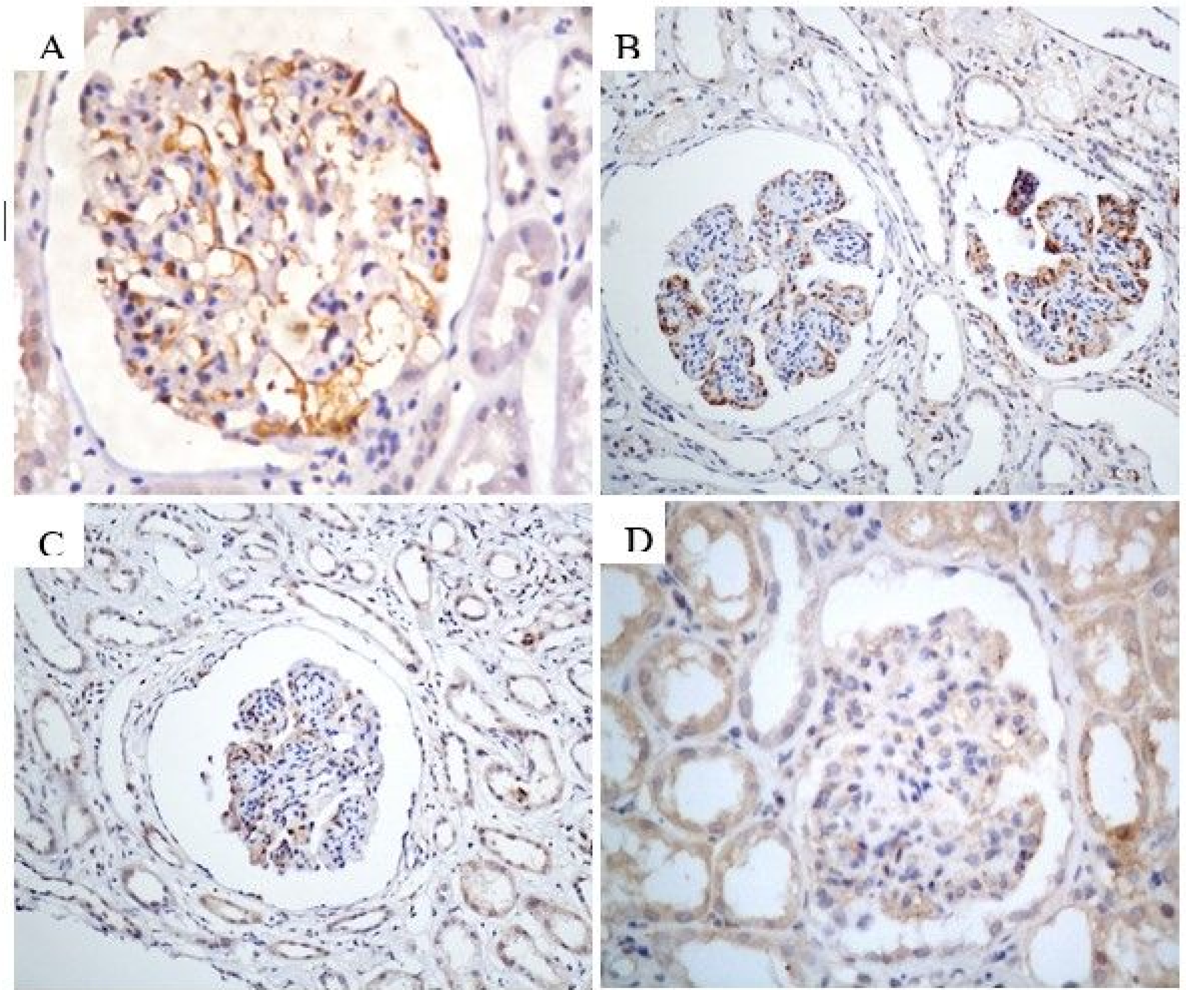

Figure 2

A; The immunohistochemical staining of HBcAg was positive, and the brown-yellow granules were distributed along the capillary wall of the glomerulus. IHC 400× B; IFI16 was positively expressed in the cytoplasm and nucleus of glomerular cells in HBV-GN. IHC 400× C; The positive expression of caspase-1 in HBV-GN. IHC 400× D; The positive expression of IL-1β in HBV-GN. IHC 400×

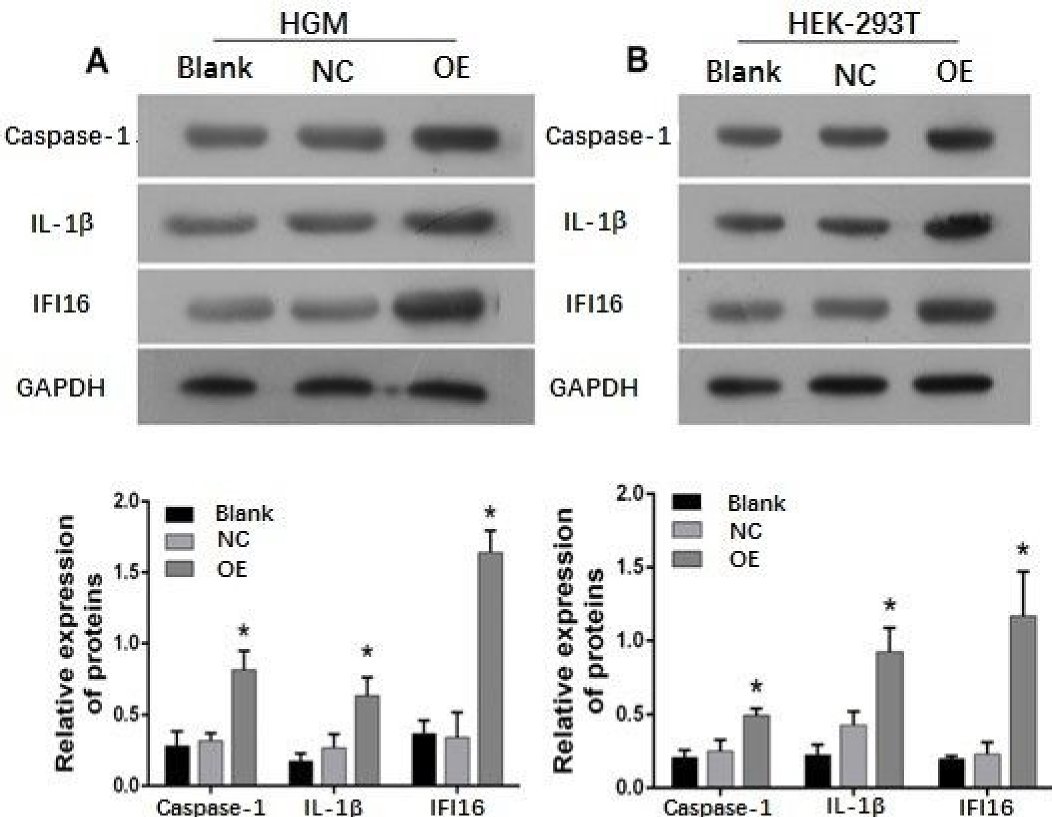

Figure 3

Western blot measured and qRT-PCR detection(