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Original Article

Profile and clinical relevance of autoantibodies in patients with chronic hepatitis B infection

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Abstract: Objectives: This study aims to assess the prevalence of non-organ specific autoantibodies (NOSA) in patients with chronic hepatitis B virus (HBV) infection and the potential association between NOSA with clinical parameters, specifically those with pathological stages. Materials and methods: Serum samples were obtained from 312 adult patients with treatment-naive, histologically-proven chronic hepatitis B. NOSA, including antinuclear antibody (ANA), anti-smooth muscle antibody (ASMA), anti-mitochondrial antibody (AMA), anti-liver/kidney microsomal antibody type 1 (LKM-1) and anti-liver cytosol antibody type 1 (LC-1) were detected by indirect immunofluorescence. The clinical data, including serum biochemistry, serum HBeAg status, HBV DNA loads, histological features and percentage of T cell subsets were observed. Results: Overall 45 patients (14.4%) had at least one autoantibody, of which 40 (12.8%) was positive for ANA, 4 (1.3%) for ASMA and 1 (0.3%) for AMA, respectively. The geometric mean titer of ANA was 1:174. A significantly higher frequency of NOSA positivity was found in female patients with chronic HBV infection compared with those in males. There were no differences in age, liver function tests, HBeAg status, HBV DNA levels, and T cell subsets. Seropositivity for NOSA was significantly and slightly negatively associated with fibrosis scores and inflammatory stages, respectively. Conclusion: Chronic HBV infection can result in occurrence of NOSA. Further investigations are need to evaluate the potential role of NOSA in the pathogenesis of HBV infection.

Keywords: Autoantibodies, chronic HBV infection, antibody profiling, pathological states, clinical features

Introduction

Infection with hepatitis B virus (HBV) is a major cause of chronic liver disease worldwide, with over one million annual deaths from complication of HBV-associated end-stage liver diseases [1]. Although the precise mechanism determining the disease outcomes is still not fully understood, it has been stated that HBV is not directly cytopathic, and infected hepatocytes injury is mainly caused by immune-mediated host-virus interactions [2]. Several immunological abnormalities have been described in patients who are chronically infected with HBV. Antigen-specific $CD8^+$ T cells, a crucial mediators of the antiviral responses to HBV, displayed exhausted phenotypes [3, 4], while the imbalance of Treg/Th17 cells and their relative cytokines was associated with the disease development of HBV-induced liver cirrhosis [5]. Recently, a few of studies have also shown that the prevalence of autoimmune diseases is increased in patients infected with HBV [6, 7], indicating that autoimmune reactions may participate in the pathogenesis of HBV infection.

It is well known that autoantibodies are a hallmark feature of autoimmune disorders. Autoantibodies are mainly classified into two entities: one is non-organ specific autoantibodies (NOSA), such as antinuclear antibodies (ANA), smooth muscle antibodies (SMA), antimitochondrial antibodies (AMA), and antibodies to liver kidney microsome type-1 (LKM-1). Another is organ-specific autoantibodies such as antibodies to soluble liver antigen and asialoglycoprotein receptor [8]. Circulating NOSA have been found in patients with hepatitis virus-related diseases. A number of studies have reported a higher prevalence of NOSA to nuclear, smooth muscle and anti-mitochondrial anti-

Table 1. Baseline characteristics of 312 chronic hepatitis B patients

Patient characteristic	No. of patients
Median age (years)	33.5 ± 10.8
Gender, n (%)	
Male	204 (65.4%)
Female	108 (34.6%)
ALT (n, %)	
≤ULN*	87 (27.9%)
>ULN, ≤2ULN	97 (31.1%)
>2ULN	128 (41.0%)
HBeAg-positive, n (%)	249 (79.8%)
HBV DNA (log_{10} copies/mL, mean ± SD)	6.6 ± 1.4
Inflammatory activity, n (%)	
<G2	161 (51.6%)
≥G2	151 (48.4%)
Fibrosis stage, n (%)	
<S2	180 (57.7%)
≥S2	132 (42.3%)

*ULN, upper limit of normal. Normal range ALT: <40 U/L.

gens [9-14], and the relationship between NOSA and clinical, biochemical and virological evidence of liver disease in patients with chronic hepatitis C virus (HCV) infection [15]. However, only a few studies investigate the prevalence of NOSA in patients with chronic HBV infection (CHB). Moreover, there have been no conclusive findings concerning the relevance of NOSA to the severity of liver necroinflammation and fibrosis, as well as immune status in CHB patients. In the current study, we examined the frequency of NOSA and evaluated the association of NOSA profiles with clinical features, specifically liver necroinflammatory and fibrosis stages in patients with CHB.

Methods

Study subjects

A total of 312 patients with treatment-naive, histologically-proven CHB from the Fifth Hospital of Shijiazhuang, Hebei, China between October 2012 and July 2015 were included in this study. Chronic HBV infection was defined as a positive hepatitis B surface antigen for at least 6 months prior, and the diagnosis of CHB was based on clinical and laboratory findings according to the Guidelines of Prevention and Treatment for Chronic Hepatitis B (2010 version) [16]. Acute hepatitis B as well as hepatitis A virus, hepatitis C virus, hepatitis D virus, hepatitis E virus infection, drug-induced hepatitis, and alcoholic hepatitis were all excluded.

Autoantibodies detection

Indirect immunofluorescence assay (IIFs) was employed to detect NOSA, including ANA, ASMA, AMA, LKM-1, and anti-liver cytosol antibody type 1 (LC-1), using the multiple-substrate panel of HEp-2 cells, rat liver, rat kidney and rat stomach (EUROIMMUN AG, Lübeck, Germany). Titers of 1:100 or higher were regarded as a positive result. Five staining patterns of ANA positive were characterized: homogenous, nuclear dots, speckled, centromere, and nucleolar.

Serological and virological detection

Serum samples were collected within 1 week prior to liver biopsy. Serum biochemistry tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), direct bilirubin (DBIL), γ-glutamyltransferase (γ-GGT) and alkaline phosphatase (ALP) were determined using an automated analyzers with standard techniques. The normal upper limit (NUL) of ALT and AST level was set as 40 IU/L. Serum HBeAg were measured by ELISA assay according to the manufacturer's instructions. HBV DNA load was determined by real-time RT-PCR using an ABI7500 quantitative PCR instrument (Applied Biosystems, USA) with a low detection limit of 500 copies/mL.

Histological assessment

Liver biopsies were obtained, fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) and Masson's trichrome. Inflammatory activity (G) and fibrosis staging (S) were scored from 0 to 4 according to the Scheuer's scoring system. Significant histological abnormality was defined as necroinflammation grade ≥G2 and/or fibrosis stage ≥S2.

Flow cytometry analysis

Peripheral blood samples (100 μL) from patients were collected in EDTA pretreated tubes. Cells were stained with appropriate dilu-

Table 2. Comparison of biochemistry and virological features between NOSA-positive and NOSA-negative CHB patients

	All patients (n=312)	Positive (n=45)	Negative (n=267)	X^2	*P*-value
ALT, n (%)					
≤ULN*	87	12 (26.7%)	75 (28.1%)	0.127	0.939
>ULN, ≤2ULN	97	15 (33.3%)	82 (30.7%)		
>2ULN	128	18 (40.0%)	110 (41.2%)		
AST, n (%)					
≤ULN*	144	19 (42.2%)	125 (46.8%)	0.344	0.842
>ULN, ≤2ULN	108	17 (37.8%)	91 (34.1%)		
>2ULN	60	9 (20.0%)	51 (19.1%)		
TBIL (μmol/L), n (%)					
≤20	246	39 (86.7%)	207 (77.5%)	1.928	0.165
>20	66	6 (13.3%)	60 (22.5%)		
DBIL (μmol/L), n (%)					
≤5.6	179	29 (64.4%)	150 (56.2%)	1.076	0.300
>5.6	133	16 (35.6%)	117 (43.8%)		
ALP (U/L), n (%)					
<42	11	1 (2.2%)	10 (3.7%)	1.043	0.678
42≤ALP≤128	292	44 (97.8%)	248 (92.9%)		
>128	9	0 (0.0%)	9 (3.4%)		
γ-GGT (U/L), n (%)					
<8	2	0 (0.0%)	2 (0.7%)	2.966	0.237
8≤γ-GGT≤50	200	34 (75.6%)	166 (62.2%)		
>50	110	11 (24.4%)	99 (37.1%)		
HBeAg, n (%)					
Positive	249	37 (82.2%)	212 (79.4%)	0.190	0.663
Negative	63	8 (17.8%)	55 (20.6%)		
HBV DNA (log_{10} copies/mL), n (%)					
≤4	23	2 (4.4%)	21 (7.9%)	0.254	0.614
>4	289	43 (95.6%)	246 (92.1%)		

*ULN, upper limit of normal. Normal range ALT: <40 U/L.

tions of PerCP-conjugated anti-CD3, FITC-conjugated anti-CD4, and PE-conjugated anti-CD8 mAbs (BD Biosciences, San Diego, CA) for 15-20 min. A volume of 2 mL of Red Blood Cell Lysis Buffer was added (10 min, at room temperature). Then, samples were centrifuged, and pellets were resuspended in 300 μl of phosphate buffered saline. The stained samples were analysed by flow cytometry (FACSCanto II, Becton Dickinson).

Statistical analysis

All statistical analyses were conducted using SPSS software package version 19.0. Continuous variables were presented as mean ± SD, while non-normal continuous variables were shown as medians and interquartile range, and categorical variables were expressed as frequency and percentage. Independent student *t*-test was used to compare group differences for normal parameters, and Mann-Whitney U test was utilized for non-normal parameters. The comparison of categorical variables was performed using Chi-square test or Fisher's exact test. A two-sided *P* value<0.05 was considered as statistically significant.

Results

Prevalence of NOSA

Of the 312 enrolled patients, 204 (65.4%) were males and 108 (34.6%) were females, their mean age was 33.5 (range 18 to 65) years. The detailed information was shown in **Table 1**. In all CHB patients, 14.4% (45/312) were positive for one or more NOSA. Of which, ANA was present in 40 (12.8%), ASMA in 4 (1.3%), and AMA in 1 (0.3%) patients. None of the patients were positive for LKM-I and LC-1. For the IIF patterns of ANA positivity, the results showed that the most majority pattern was speckled (72.5%), follow by homogeneous (22.5%), while nucleolar (2.5%) and nuclear dots (2.5%) were very fewer, and no centromere was shown. The range of ANA titers was from 1:100 to 1:640, with a geometric mean titer of 1:174 (**Table 2**).

The association between NOSA and age and gender

The age- and gender-associated alterations in the percentage of NOSA positivity were investigated (**Figure 1**). The results showed that females had a significantly higher percentage of NOSA positivity than males, however, there were no difference among different age groups. Further analysis found that a decreasing trend in the percentage of NOSA positivity was associated with increasing age in the female

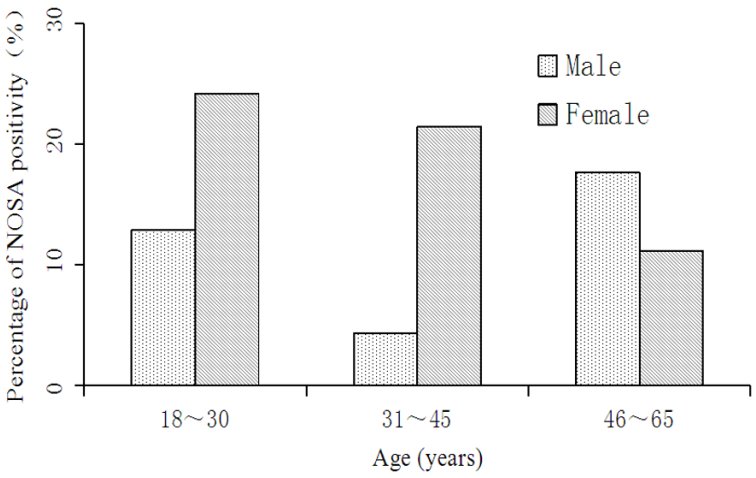

Figure 1. The percentage of NOSA positivity in different age and gender patients with chronic HBV infection. Serum samples were collected, and NOSA were determined by indirect immunofluorescence (IIF).

Table 3. The titers of positive ANA in CHB patients (n=45)

ANA titers	n	%
1:100	21	52.5%
1:160	2	5.0%
1:320	15	37.5%
1:640	2	5.0%

groups. Among the male patients, no significant difference was observed in all three age groups, though males aged 31-45 years group exhibited a small decrease.

The association between NOSA and clinical and virological parameters

The clinical and virological data were collected and the percentage of NOSA-positive patients was evaluated (**Table 3**). The results revealed that there were no significant differences in liver function tests (ALT, AST, TBIL, DBIL, ALP and γ-GGT) in the NOSA-positive group compared with the NOSA-negative group. In HBeAg-positive patients, the percentage of NOSA positivity was 17%, which slightly lower than HBeAg-negative patients, but no significant statistical difference was observed. Likely, no correlations were observed between NOSA positivity and HBV DNA levels.

The association between NOSA and liver histology

The association between NOSA and liver inflammation and fibrosis was also investigated (**Table 4**). Unexpectedly, the results showed that a lower percentage of NOSA positivity was present in CHB patients with higher hepatic fibrosis scores compared to those with lower fibrosis scores. Similarly, a trend of negative association was found between percentage of NOSA positivity and inflammatory score in these patients.

The association between NOSA and T cell subsets

T lymphocytes play an important role in activating B cells, and subsequent production of antibodies. Therefore, we investigated whether an association existed between NOSA and T cell subsets. FACS analysis revealed that no difference was found in the frequency of $CD3^+$ T cells between NOSA-positive and NOSA-negative group. There were also no differences in frequency of T cells subsets, including $CD4^+$ and $CD8^+$ cells, between two groups.

Discussion

Increasing evidences have shown that CHB is a highly prevalent disease, in which immune dysfunction is the major cause of liver necroinflammatory damage. The release of autoantigens due to hepatocytes apoptosis and the generation of pathogenic B cells can result in autoimmune reaction, subsequently lead to the production of autoantibodies [17]. Although many studies have investigated the prevalence of NOSA among patients with chronic HCV infection [9-15], relatively a few studies have examined the frequency and the titers of NOSA in CHB patients, and the results were conflicting. In the present paper, we found that the overall prevalence of NOSA in our patients with chronic HBV infection was 14.4%. This result is consistent with two previous studies [18, 19], which reported the percentage of NOSA positivity in chronic HBV patients was about 15% and 19.4%, respectively. However, in contrast to this result, a relative lower prevalence (7%) was found in subjects positive for HBsAg by Lenzi *et al*. [20], and a much higher seropositivity (58.2%) was observed by Li *et al.* [21] in CHB cases. Therefore, it is essential to further inves-

Table 4. Comparison of histopathological change between NOSA-positive and NOSA-negative CHB patients

	All patients (n=312)	Positive (n=45)	Negative (n=267)	X^2	*P*-value
Inflammatory activity, n (%)					
<G2	161	27 (60.0%)	134 (50.2%)	1.485	0.223
≥G2	151	18 (40.0%)	133 (49.8%)		
Fibrosis stage, n (%)					
<S2	180	32 (71.1%)	148 (55.4%)	3.879	0.049
≥S2	132	13 (28.9%)	119 (44.6%)		

tigate the prevalence of NOSA in chronic HBV patients.

Several subtypes of autoantibodies have been observed in our patients, and shown that the most commonly found is ANA, follow by ASMA, in agreement with study by Li *et al*. [20], but in contrast with a previous report by Cacoub *et al*. [19]. However, the percentage of ANA is significantly lower compared with the data reported by Li *et al*. (12.8% vs 23.4%). Neither anti-LKM-1 or anti-LC-1, two disease-specific serological markers for autoimmune hepatitis [22], were found in our patients. These results indicate that the occurrence of anti-LKM-1 and anti-LC-1 is much lower in patients with liver diseases caused by HBV than those by HCV [23]. We further investigated the titers of ANA and found that low to medium titers of NOSA were frequently detected in CHB patients. This result is in agreement with previous studies, which found that virus infection, such as HBV and HCV, induced NOSA with low titers, while patients with autoimmune hepatitis had high titers of NOSA [24]. Considering these findings, we consider that the existence of autoantibodies is probably an epiphenomenon of tissue destruction and an indication of alteration in immune function caused by HBV infection, but not development of autoimmune diseases.

Another main finding of this study was the clear relationship between the positive NOSA and the degree of fibrosis in CHB patients. A significant lower frequency of NOSA positive was found in CHB patients with higher fibrosis stage, and slightly lower in patients with higher liver inflammation grade, indicating that NOSA are negatively associated with the severity of liver damage. This result was different from whose from patients with chronic HCV infection in which a significant correlation between NOSA positivity and the degree of inflammation and fibrosis, even though the results are variable [15, 25-27]. Although the mechanism involved is unclear, immune dysfunction may be one of the important factors. Many studies have shown that HBV infection can lead to functional impairment of B cells, an important effectors for producing autoantibodies [28, 29], and T cells, which are required for B cell-mediated humoural immune responses [30]. The lower NOSA in patients with severity liver lesions may reflect that they have an lower immune function, which results in the decreased production of autoantibodies. We found that there are no any association between the presence of NOSA and liver biochemistry. Additionally, neither HBeAg status nor HBV DNA levels appeared to have relationship with the presence of NOSA. This finding is consistent with previous reports [20], suggesting that NOSA production is independent with HBV replication.

There is limited knowledge about the relationship between the present of NOSA in CHB patients and peripheral T lymphocyte subsets, even though it is well known that chronic HBV infection is an immune-mediated disease and T cells are important player in the pathogenesis [4]. We investigated the frequency of $CD3^+$, $CD4^+$ and $CD8^+$ T cells in the peripheral blood. The results showed that there were no differences between NOSA-positive and NOSA-negative chronic CHB patients, suggesting that NOSA production might not associate with T lymphocyte subsets. Recently, a number of studies have been investigated the role of receptor and signaling physiology in T cells as well as T cell subpopulation on the production of antibodies. Inducible costimulator (ICOS) is a costimulatory molecule expressed in activated T cells, which interacts with ICOSL markedly enhances the production of total IgG and pathogenic anti-dsDNA antibodies by B cells [31]. Treg cells are capacity of suppress antibody-producing B cells which leads to the reduction of anti-DNA antibody [32]. However, $CD4^+CXCR5^+$ follicular helper T cells, which have been reported to be increased in CHB patients and positively correlated with the levels of ALT and AST [33], can promote B-

cell maturation and autoantibody production. Therefore, further studies will be required to determine the role of receptors of T cells and subpopulation of $CD4^+$ T cells on the production of autoantibodies.

In conclusion, the present study shows that NOSA are present in patients with CHB, with a low to medium titer of antibodies. The most crucial result is that there is a negative association between the presence of NOSA and the severity of liver fibrosis. Further understanding of mechanisms involved in increased NOSA and the effect of these NOSA on the disease progress may be helpful for clinical management of chronic HBV infection.

Disclosure of conflict of interest

None.

Abbreviations

NOSA, non-organ specific autoantibodies; ANA, antinuclear antibody; SMA, smooth muscle antibody; AMA, antimitochondrial antibody; LKM-1, liver kidney microsome type-1; LC-1, liver cytosol antibody type 1; HBV, hepatitis B virus; HCV, hepatitis C virus; CHB, chronic HBV infection; IIFs, indirect immunofluorescence assay; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; γ-GGT, γ-glutamyltransferase; ALP, alkaline phosphatase; NUL, the normal upper limit.

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