

Quantitative Serum HBsAg and M2BPGi Levels in Patients with Chronic Hepatitis D

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Objectives: Quantification of serum HBsAg has several clinical significances such as acting as a biomarker for the hepatitis D virus (HDV)-RNA level and necroinflammatory activity in HDV infection. The WFA+-M2BP is a new glyco-biomarker for liver fibrosis. The aim of this study was to compare the quantitative HBsAg and M2BPGi levels between groups of HBV infected patients with or without HDV infection. **Methods:** Totally, 112 consecutive HBsAg carriers with or without HDV infection (median age 36 vs 38) were enrolled in the study. Serum HBsAg and M2BPGi levels were measured using commercial tests. **Results:** Mean qHBsAg concentrations were 3.8 ± 0.6 log IU/mL and 3.5 ± 0.8 log IU/mL in the HDV positive and HDV negative HBsAg carriers, respectively ($p = 0.02$). Yet 45 patients out of 56 HDV positive patients have HBsAg level >2000 IU/mL whereas only 33 patients in the HBV mono infected group had concentrations this high. The mean M2BPGi was a 1.2 cut-off index (COI) in the HDV negative group vs. 2.1 COI in the HDV positive group ($p < 0.001$). **Conclusion:** The qHBsAg level was higher in HDV positive patients compared with HDV negative subjects. But qHBsAg levels were higher in the HBeAg positive subjects despite the presence of HDV infection. The mean M2BPGi value in the HDV positive group was higher than in HDV negative subjects.

Keywords: Hepatitis B, Chronic; Hepatitis D, Chronic; Liver Fibrosis; Mac-2 Binding Protein, Human; Hepatitis B Surface Antigens

Introduction

Mongolia has the highest rate of hepatocellular carcinoma (HCC)-related mortality due to its high prevalence of viral hepatitis caused by hepatitis B virus (HBV), hepatitis C virus

(HCV) and hepatitis Delta virus (HDV) [1]. In 2014, the HCC-related mortality rate was 47.4 per 100,000 population, which was 5-fold higher than the world average [2].

HDV infection was an endemic worldwide in the 1980s with varying prevalence in different areas [3]. Following the

widely-introduced HBV vaccination program, the incidence of HDV infection declined rapidly worldwide [4]. The estimated prevalence of HDV infection is 5% of HBsAg carriers, thus 15-20 million people have been exposed to HDV worldwide [5]. Recent data suggests that HDV infection is endemic in the Mediterranean Basin, Turkey, the Middle East, Mongolia and some parts of South America [6]. Currently, this virus is divided into eight genotypes, with different clinical patterns. Genotype I is the most frequent worldwide and causes varying disease courses, while genotype II and III were isolated from East Asia and associated with a milder disease. Genotype III is reported as causing fulminant hepatitis in South America [7].

Previous studies have shown that the prevalence of HBV and HCV infection in Mongolia was 9.6 and 11%. One third of HBsAg carriers were co-infected with HDV [8-10]. The HDV infection rate remains higher among HBsAg carriers in Mongolia regardless of the implementation of a nationwide HBV vaccination program, which was introduced in 1992 [10].

HDV, the smallest RNA virus among mammals, is known as a mutant virus that requires Hepatitis B surface Antigen (HBsAg) for its replication and assembly [5]. HDV infection can be acquired by either co-infection (exposed HBV and HDV at same time) or super-infection (to people who have chronic infection with HBV) [11]. HDV infection causes a more aggressive form of viral hepatitis with rapid progression of liver disease than HBV mono-infection does. It leads to liver cirrhosis within 5-10 years and slightly increases the risk of HCC and early hepatic decompensation [12-13]. The diagnosis of HDV infection is based on antibodies of the immunoglobulin M (IgM) and immunoglobulin G (IgG) classes against the hepatitis Delta antigen (HDAG). But the detection of HDV ribonucleic acid (HDV-RNA) by polymerase chain reaction (PCR) is the most accurate diagnostic tool of active HDV. Recently, the HDV-RNA quantitative assay, which received certification as the first World Health Organization Standard for HDV-RNA, became available.

Quantification of serum HBsAg (qHBsAg) has clinical significance in predicting sustained virological responses during antiviral treatment and determining HCC risk among HBsAg carriers [14, 15]. Higher qHBsAg level is correlated with not only HDV-RNA levels but also necroinflammatory activity of hepatocytes in HDV infection [16].

The *Wisteria floribunda* agglutinin-positive Mac-2 binding protein (WFA⁺-M2BP) is a new glyco-biomarker for

liver fibrosis founded by Japanese researchers. They reported that serum WFA⁺-M2BP level represents a reliable non-invasive marker for liver fibrosis in patients with chronic hepatitis B, C, non-alcoholic fatty liver disease and primary biliary cirrhosis [17-20]. Therefore, the aim of this study was to compare qHBsAg and M2BPGi levels between groups of HBsAg carriers with or without HDV infection.

Materials and Methods

1. Patients

Totally, 112 consecutive HBsAg carriers with or without HDV infection were enrolled in this study. They visited the gastroenterology clinic at the United Family Intermed Hospital and University Hospital of the Mongolian National University of Medical Sciences between June 2015 and March 2016. All patients had detectable HBsAg for more than 6 months, no evidence of concurrent HCV infection, alcoholic liver disease and autoimmune hepatitis. HDV positive patients were positive for anti-HDV Ig (IgM and/or IgG) and had detectable HDV-RNA.

2. Laboratory testing

Collected samples were tested for complete blood count and prothrombin time (PT) by standard laboratory methods. Liver function tests included aspartate aminotransferase (AST) and alanine aminotransferase (ALT), γ -glutamyl transpeptidase (GGT), alkaline phosphatase (ALP), albumin, total protein, and total bilirubin. HBsAg, antibody to hepatitis B surface antigen (anti-HBs), antibody to hepatitis B core antigen (anti-HBc), hepatitis B envelope antigen (HBeAg), antibody to HBe (anti-HBe), antibody to HCV (anti-HCV) and α -1 fetoprotein AFP level were detected by commercially available immunoassays (Sysmex Corporation, Japan). Anti-HDV IgM and IgG levels were determined by assays from DRG Instruments GmbH (Germany) and Fortress Diagnostics (UK), respectively. Serum HDV-RNA levels were detected by RoboGene HDV-RNA Quantification Kit 2.0 (Analytic Jena AG, Germany). Quantitative determination of HBsAg titer was performed by HISCL HBsAg assay (Sysmex Corporation) with a diagnostic range from 0.03 IU/mL to 2500 IU/mL. Samples with HBsAg level >2500 IU/mL were retested at a higher dilution. Serum Mac-2 binding protein glycosylation isomer (M2BPGi) level was measured by an immune assay based on a chemiluminescent enzyme immune-assay technique with a

commercially available kit (HISCL M2BPGi, Sysmex Corporation). M2BPGi level was expressed by a cut-off index (COI). We used non-invasive fibrosis markers such as the AST to platelet ratio index (APRI) and Fibrosis-4 (FIB-4) index. The APRI and FIB-4 indices were calculated using equations 1 and 2, respectively:

$$\text{APRI} = \frac{\text{AST/ULN}}{\text{PLT}} * 100 \quad \text{Equation 1}$$

$$\text{FIB-4} = \frac{\text{Age} * \text{AST}}{\text{PLT} * \sqrt{\text{ALT}}} \quad \text{Equation 2}$$

In which AST is the aspartate aminotransferase concentration, ULN is the upper limit of normal AST, PLT is the platelet count ($\times 10^9/\text{L}$), ALT is the alanine aminotransferase concentration, and age is the age in years of the patient.

3. Ethical statement

The Ethical Review Committee of the Mongolian National University of Medical Sciences approved the current study protocol and the written consent forms were obtained from all subjects prior to blood sampling.

Table 1. Demographic and clinical characteristics of patients with and without HDV infection

Variables	HDV positive patients	HDV negative patients	p-value
	(n = 56) Mean \pm SD	(n = 56) Mean \pm SD	
Males ^a	25 (44.6)	33 (58.9)	0.09
Median age (range) (years)	38 (22-66)	36 (18-65)	0.14
WBC ($\times 10^6/\text{L}$)	5.2 \pm 1.5	6.2 \pm 1.7	0.001
WBC $< 4.5 \times 10^6/\text{L}$ ^a	10 (17.9)	6 (10.9)	0.21
PLT ($\times 10^9/\text{L}$)	193 \pm 63	234 \pm 67	0.001
PLT $< 150 \times 10^9/\text{L}$ ^a	15 (26.8)	4 (7.3)	0.006
AST (U/L)	57.8 \pm 35.9	41.3 \pm 45.5	< 0.001
ALT (U/L)	72.1 \pm 58.6	50.8 \pm 59.7	0.003
ALP (U/L)	315 \pm 104	299 \pm 102	0.41
GGT (U/L)	73.7 \pm 94.1	52.8 \pm 75.2	0.08
Serum albumin (g/L)	44.7 \pm 3.9	46.8 \pm 4.0	0.008
Total bilirubin ($\mu\text{mol/L}$)	18.3 \pm 7.6	17.4 \pm 6.9	0.47
HBeAg positivity ^a	9 (16.1)	13 (23.2)	0.23
qHBsAg (\log_{10} IU/mL)	3.8 \pm 0.6	3.5 \pm 0.8	0.02
qHBsAg > 2000 IU/mL ^a	45 (80.3)	33 (58.9)	0.01
AFP (ng/mL)	7.0 \pm 11	5.6 \pm 14	0.002
M2BPGi (COI)	2.1 \pm 1.8	1.2 \pm 1.2	< 0.001
APRI	0.8 \pm 0.7	0.5 \pm 0.9	< 0.001
FIB-4	1.7 \pm 1.3	1.0 \pm 0.9	< 0.001

^aValues are n (%)

Table 2. Correlation with M2BPGi and other variables in patients with and without HDV infection

Variables	HDV negative (n = 56)		HDV positive (n = 56)	
	r _s	p-value	r _s	p-value
Age (years)	0.46	0.001	0.29	0.03
PLT (*10 ⁹ /L)	-0.11	0.42	-0.45	0.001
AST (U/L)	0.31	0.02	0.39	0.003
ALT (U/L)	0.29	0.03	0.23	0.08
ALP (U/L)	0.35	0.01	0.43	0.001
GGT (U/L)	0.35	0.01	0.50	<0.001
Serum albumin (g/L)	-0.08	0.56	-0.35	0.009
Total bilirubin (µmol/L)	0.01	0.9	0.22	0.09
AFP (ng/mL)	0.44	0.001	0.54	<0.001
APRI	0.24	0.08	0.24	0.01
FIB-4	0.36	0.008	0.36	0.008

4. Statistical analysis

Statistical analyses were performed with the SPSS 16.0 software package (SPSS Inc. Chicago, IL, USA). The mean and standard deviation were calculated for numerical data. The HBsAg titers were logarithmically transformed for analysis. A nonparametric approach was used to examine variables showing an absence of normal distribution as verified by the Kolmogorov-Smirnov test. Differences between groups were analyzed using the Mann-Whitney U test and the Kruskal-Wallis test where appropriate. The Spearman’s correlation test was used to determine the correlation between M2BPGi level and other continuous variables. P-values of <0.05 were considered statistically significant.

Results

1. Baseline characteristics

All 112 HBsAg carriers were divided into two groups comparing HDV positive (n = 56) and HDV negative (n = 56) subjects. The baseline characteristics are shown in Table 1. There were 33 men and 23 women in the HDV negative group (median age 36; range 18-65). Thirteen patients out of fifty-six HDV negative

HBsAg carriers were positive for HBeAg. In the HDV positive group, there were 25 men and 31 women with median age 38; range 22-66. Age and sex distribution within the groups of patients were similar. Nine patients out of fifty-six HDV positive HBsAg carriers were positive for HBeAg. HDV positive and negative patients did not differ in gender distribution (percent male was 44.6% vs. 58.9%, p = 0.09).

The mean WBC and PLT levels were significantly lower in HDV positive patients than the HDV negative group (5.2 ±1.5 x 10⁶/L vs. 6.2 ±1.7 x 10⁶/L, p = 0.001; 193 ±63 x 10⁹/L vs. 234 ±67 x 10⁹/L, p <0.001). The number of patients with lower WBC level (<4 x 10⁶/L) was 10 (17.9%) in the HDV positive group while it was 6 (10.9%) in the HDV negative patients. Patients with thrombocytopenia (PLT level <150 x 10⁹/L) were frequently observed in the HDV positive group (26.8% vs. 7.3%, p = 0.006). Higher transaminase level was observed more in HDV positive patients than HDV negative HBsAg carriers. Mean ALT levels were 72.1 ±58.6 U/L and 50.8 ±59.7 U/L in HDV positive and HDV negative groups, respectively (p = 0.003). A similar difference was observed of AST level (57.8 ±35.9 U/L vs. 41.3 ±45.5 U/L, p<0.001). But there were not significant differences of GGT and ALP levels between the two groups. In

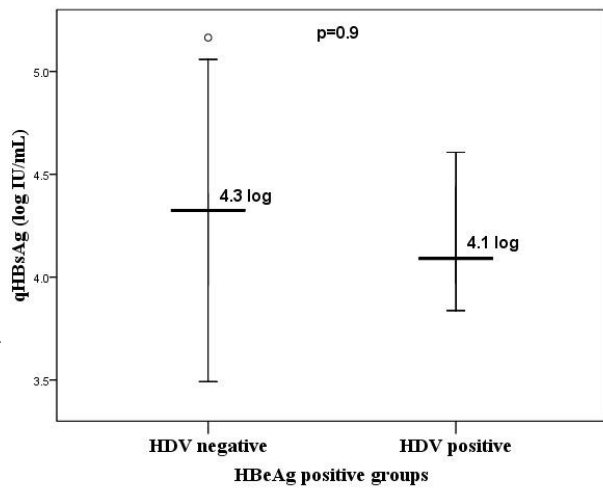


Figure 1. Mean levels of quantitative HBsAg production in HBeAg positive groups with and without HDV infection. The mean is indicated with a horizontal line and standard deviations are indicated with error bars.

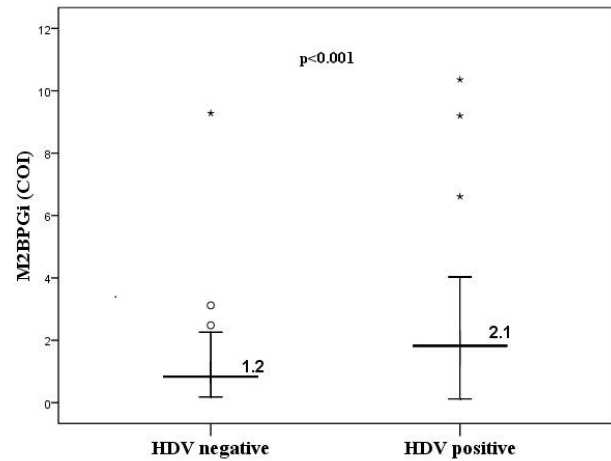


Figure 3. Mac-2 binding protein glycosylation isomer (M2BPGi) levels in HDV negative and positive patients. The mean is indicated with a horizontal line and standard deviations are indicated with error bars.

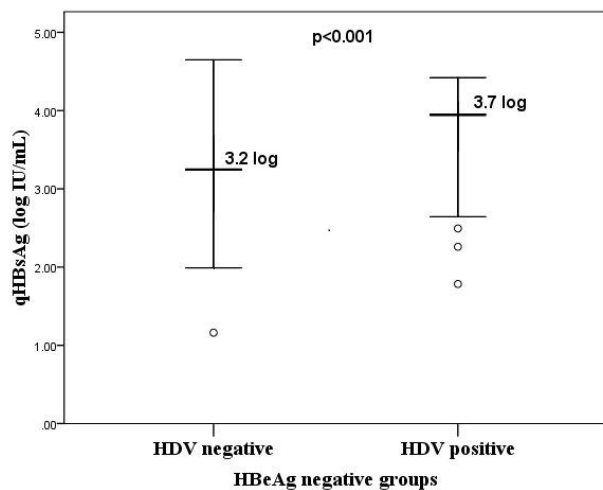


Figure 2. Mean levels of quantitative HBsAg production in HBeAg negative groups with and without HDV infection. The mean is indicated with a horizontal line and standard deviations are indicated with error bars.

addition, albumin level was lower in the HDV positive group with statistical significance (44.7 ± 3.9 g/L vs. 46.8 ± 4.0 g/L, $p = 0.008$). HDV positive patients had higher AFP levels than HDV negative patients (7.0 ± 11 ng/mL vs. 5.6 ± 14 ng/mL $p = 0.002$).

2. Levels of quantitative HBsAg and correlation with HBeAg positivity

Mean qHBsAg were 3.8 ± 0.6 log IU/mL and 3.5 ± 0.8 log IU/mL

in HDV positive and HDV negative HBsAg carriers, respectively ($p = 0.02$). Also, the number of patients with qHBsAg >2000 IU/mL was significantly higher in the HDV positive group (80.3%) than the HDV negative group (58.9%, $p = 0.01$).

Both groups were divided into four subgroups depending on the HBeAg positivity: (1) HBeAg positive and HDV negative, (2) HBeAg negative and HDV negative, (3) HBeAg positive and HDV positive, (4) HBeAg negative and HDV positive. Mean qHBsAg levels in HBeAg positive subjects were comparable between HDV negative and positive groups (4.3 ± 0.5 log IU/mL vs. 4.1 ± 0.3 log IU/mL, respectively, $p = 0.9$, Figure 1). On the other hand, qHBsAg levels in HBeAg negative/HDV positive patients were 3.7 ± 0.6 log IU/mL and were 3.2 ± 0.7 log IU/mL in HBeAg negative/HDV negative subjects ($p < 0.001$, Figure 2).

3. M2BPGi level and correlation with other variables

The mean M2BPGi was 1.2 COI in the HDV negative group vs. 2.1 COI in the HDV positive group ($p < 0.001$, Figure 3). The HDV positive group has a higher mean compared to the HDV negative HBsAg carriers for APRI (0.8 ± 0.7 vs. 0.5 ± 0.9 , respectively, $p < 0.001$) and for FIB-4 (1.7 ± 1.3 vs. 1.0 ± 0.9 , respectively, $p < 0.001$).

Demographic (age), clinical (PLT, AST, ALT, ALP, GGT, serum albumin, total bilirubin) and non-invasive fibrosis markers were examined for their correlation with M2BPGi level in HBsAg carriers (Table 2). Age, AST, ALP, GGT and AFP were significantly

correlated with serum M2BPGi level in HDV negative patients. Among these, age had highest r_s values (0.46) followed by AFP (0.44). On the other hand, PLT, AST, ALP, GGT and AFP were significantly correlated with serum M2BPGi level in HDV positive patients. AFP had the highest r_s value (0.54) in the HDV positive group.

Discussion

Study on qHBsAg showed that this biomarker has a positive association with intrahepatic closely covalent circular DNA and HBV-DNA levels [21]. The serum HBsAg quantification assay is less expensive than HBV-DNA quantification, which is a standard tool for selecting candidates for antiviral therapy and monitoring treatment response. Higher qHBsAg level is correlated with both HDV-RNA levels and necroinflammatory activity of hepatocytes in HDV infection [16]. WFA+-M2BP has been reported to be a non-invasive, rapid, useful marker for predicting liver fibrosis in several chronic liver diseases [17-20]. Yet whether M2BPGi level can be useful for determining liver fibrosis in patients with chronic hepatitis D remains unknown. Thus, we conducted this study to compare M2BPGi level and qHBsAg level between two groups of HBsAg carriers with or without HDV infection.

Chronic HDV infection has been known as an HBeAg negative disease from the studies conducted in Mediterranean countries. Because most patients with HBV in this region were HBeAg negative, mainly HBeAg negative HDV infection was studied [22]. HBeAg is known as a marker for HBV replication and associated with increased risk of liver cirrhosis and HCC in HBV mono infected patients [23, 24]. Recently, HBeAg positive HDV infections were reported by German researchers from the large European cohort which compared 71 HBeAg positive HDV patients with 450 HBeAg negative HDV patients [25]. They revealed that the positivity of HBeAg does not influence either HBV-DNA level or the long-term clinical outcome of patients with chronic HDV [25]. Also, HDV-RNA and qHBsAg levels in HBeAg positive or negative groups were not significantly different [25]. In our study, 9 out of 56 (16.1%) HDV positive HBsAg carriers were detectable for HBeAg vs. 13 out of 56 (23.2%) HDV negative patients. This finding suggests that HBeAg positive HDV infection is frequent in Mongolia. It may be associated with the fact that 1/5 of HBV mono-infected patients were detectable for HBeAg in this country [26].

HDV positive patients had significantly higher qHBsAg levels compared with HDV negative patients. This finding is consistent with data reported by Heidrich et al. [25]. Our study also demonstrated that qHBsAg levels were higher in HBeAg positive patients despite the presence of HDV infection. On the other hand, qHBsAg levels in HBeAg negative/HDV positive patients were significantly higher than HBeAg negative/HDV negative patients. Pollicino et al. reported that serum qHBsAg levels were comparable between HDV positive and negative patients and there were decreased HBV-DNA and increased pre-S/S RNAs [27]. The number of patients who had a qHBsAg level >2000 IU/mL was greater in the HDV positive group than those with HBV mono infection, which suggests that HDV positive patients have a higher risk for HCC development.

This study clearly showed that M2BPGi levels in HDV positive patients were significantly higher than in HDV negative patients. Also, M2BPGi level was associated with other non-invasive markers of liver fibrosis and transaminase level in both groups. The mean M2BPGi level in the HDV negative group was 1.2 COI. This finding is well in line with recent reports from Japan. Ishii et al. found that the median serum WFA+-M2BP level was 1.2 COI in treatment of naive chronic hepatitis B patients [28]. Ichikawa et al. reported that serum WFA+-M2BP level was 0.97 COI in 112 treatment naive patients with HBV-related chronic hepatitis and liver cirrhosis [17]. They later noticed WFA+-M2BP level was also independently associated with HCC development in HBsAg carriers [17]. Serological tests in this study showed that HDV-positive patients have lower albumin level, higher transaminase and higher fibrosis scores. These results support the fact that HDV co-infection causes more aggressive disease.

To the best of our knowledge, this is the first study investigating M2BPGi level in HDV positive patients. But several limitations of this study need to be considered. First of all, the serum HBV-DNA levels were not included in analysis due to lack of data. Second, we could not use liver biopsy to determine liver fibrosis in all patients for the reason that patients mainly hesitated to undergo a biopsy procedure. Further studies comparing M2BPGi with liver biopsy and/or FibroScan are necessary to confirm the efficacy of serum M2BPGi levels for assessing the stage of fibrosis among patients with HDV infection.

In conclusion, qHBsAg level was higher in HDV positive patients compared with HDV negative subjects. But qHBsAg levels were higher in HBeAg positive subjects despite the

presence of HDV infection. Mean M2BPGi value in HDV positive group was higher than in the HDV negative group.

Conflict of Interest

The authors state no conflict of interest.

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