Article

Discovery Medicine 2023; 35(179): 982–987
https://doi.org/10.24976/Discov.Med.202335179.94

Haptoglobin 2-2 Genotype is Related to the Severity of Liver Damage in Hepatitis B Patients with Liver Steatosis

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Published: 1 December 2023

Background: The Haptoglobin (*Hp*) genotypes have been linked to immune diseases and play a significant role in metabolic diseases. This study aimed to analyze the correlation between *Hp* gene polymorphism and the severity of hepatitis B accompanied by liver steatosis.

Methods: A total of 182 with Hepatitis B and concurrent hepatic steatosis were included in the study. Clinical biochemical indices for each participant were recorded. DNA was extracted from peripheral blood leukocytes for globin genotyping. Of these participants, 128 underwent biopsy from which histological data were collected.

Results: Subjects with hepatitis B and hepatic steatosis carrying the *Hp* 2-2 genotype exhibited elevated alanine transaminase (ALT), c-glutamyl transferase (GGT), and aspartate amino transferase (AST) levels. In contrast, high-density lipoprotein (HDL) levels and the copy number of Hepatitis B Virus (HBV)-DNA were significantly reduced in those with the *Hp* 2-2 genotype ($p < 0.05$). Furthermore, individuals processing the *Hp* 2-2 genotype demonstrated a heightened hepatitis score and advanced fibrosis stage ($p < 0.05$). Notably, the *Hp* 2-2 genotype was independently associated with increased inflammation (odds ratio (OR) = 7.059, $p < 0.001$) and progressive fibrosis (OR = 3.05, $p < 0.022$).

Conclusions: The *Hp* 2-2 genotype is significantly associated with increased severity in cases of hepatitis B with coexisting hepatic steatosis.

Keywords: hepatitis B; haptoglobin genotype; nonalcoholic fatty liver disease

Introduction

The concurrent presence of Hepatitis B Virus (HBV) infection and fatty liver in the liver is common. Research has shown that the prevalence of liver steatosis in individuals with HBV infection varies from 14.0% to 70.0% [1]. The prevalence of nonalcoholic fatty liver disease (NAFLD) in those with HBV is believed to be influenced by host factors, primarily components of Metabolic Syndrome (MS) such as diabetes mellitus, dyslipidemia, abdominal obesity, and arterial hypertension [2–4]. Whether hepatic steatosis affects viral load and treatment response in HBV patients remains debatable [5,6]. However, several studies have provided evidence that in patients with chronic hepatitis B, components of Metabolic Syndrome (MS) correlate with the onset of NAFLD and can lead to liver fibrosis [2]. Furthermore, the MS, which encompasses NAFLD, is also considered pivotal in the progression of hepatic cirrhosis in patients with chronic hepatitis B [7].

Haptoglobin (*Hp*) is an alpha-2 globulin primarily synthesized by the hepatocytes in the liver [8]. Functioning as a hemoglobin-binding protein, it exhibits three main genetic polymorphisms: 1-1, 2-1, and 2-2 [9]. *Hp* plays a crucial role in safeguarding against kidney damage and preventing iron loss during hemolysis. Additionally, as a positive acute-phase protein, its synthesis increases considerably in response to various inflammatory stimuli [10]. *Hp* protein displays antioxidant properties, with the relative antioxidant efficacy being: *Hp* 1-1 > *Hp* 2-1 > *Hp* 2-2 [11]. The haemoglobin-binding capacity is notably higher in *Hp* 1-1, while the antibody-like characteristics are attributed to *Hp* 2-1 and *Hp* 2-2. Notably, Haptoglobin has been found to bind the CD22 receptor on B-lymphocytes [12]. Compared to individuals with the *Hp* 1-1 phenotype, those with the *Hp* 2-2 phenotype typically exhibit stronger immune responses to various stimuli. Moreover, autoimmune diseases are more prevalent among *Hp* 2-2 individuals [13,14]. Beyond its role in diabetes and its association with an elevated risk for diabetic cardiovascular disease [7,9,10], the *Hp* 2 allele is considered a potential risk factor for NAFLD. The *Hp* 2-2 genotype has a significant correlation with the onset of inflammation and liver fibrosis in NAFLD patients [15,16].

In the study, we aim to discern the influence of *Hp* gene polymorphism in cases of hepatitis B coexisting with hepatic steatosis and to investigate its implications for disease progression.

Table 1. Clinical characteristics of the subjects—HBV+NAFLD.

Variables	Total	*Hp* 2-2 genotype	Non-*Hp* 2-2 genotype	T/Z/chi-square value	*p* value
N (male/female)	182 (155/27)	109 (97/12)	73 (58/15)	3.15	0.076
Age (years)	41 (32–48)	41.1 ± 9.0	37 (30–45)	1.48	0.191
BMI (kg/m^2)	25.04 ± 3.05	25.1 ± 2.9	24.8 ± 3.3	0.65	0.553
TG (mg/dL)	1.37 (1.04–1.84)	1.36 (0.96–1.81)	1.35 (1.09–1.86)	0.07	0.902
TC (mg/dL)	4.71 (4.15–5.26)	4.68 (4.13–5.15)	4.9 ± 1.0	–1.55	0.274
LDL (mg/dL)	2.89 ± 0.78	2.90 ± 0.77	2.90 ± 0.75	0.001	0.816
HDL (mg/dL)	1.16 (0.99–1.33)	1.12 (0.96–1.30)	1.22 (1.07–1.39)	–1.75	0.015
ALT (U/L)	56 (30–84.0)	69 (36.0–87.0)	49 (27–73.5)	1.68	0.07
AST (U/L)	35 (26–51.25)	40 (27–70)	33 (24–44)	1.63	0.08
GGT (U/L)	43.63 ± 24.50	50.34 ± 25.99	38.7 ± 22.3	3.23	0.013
FPG (mg/dL)	5.38 (4.93–5.95)	5.41 (5.02–5.81)	5.30 (4.85–6.04)	0.61	0.551
HBsAg (IU/mL)	1365 (250–4199.79)	1697.5 (250–3817.09)	1370 (189.09–10,175.93)	0.36	0.795
HBeAg positive, n (%)	71 (41.52%)	43 (41.57%)	28 (43.08%)	0.24	0.746
HBV DNA positive, n (%)	130 (74.29%)	69 (65.71%)	61 (87.14%)	8.79	0.001
HBN DNA (log10 copies/mL)	4.08 ± 2.72	3.58 ± 3.55	4.87 ± 2.74	–2.7	0.001

HBV, Hepatitis B Virus; NAFLD, nonalcoholic fatty liver disease; BMI, body mass index; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; LDL, low density lipoprotein; ALT, alanine transaminase; AST, aspartate amino transferase; GGT, c-glutamyl transferas; HBeAg, hepatitis Be antigen; HBsAg, hepatitis B surface antigen; HBN, hexagonal boron nitride; *Hp*, Haptoglobin.

Methods

Subjects and Study Design

One hundred eighty-two individuals newly diagnosed with hepatitis B, further complicated by liver steatosis, were successively enrolled in the Department of Infectious Diseases at Hangzhou Normal University Affiliated Hospital between June 2017 and October 2018. All individuals have signed informed consent forms. This study followed the principles of the Helsinki Declaration and obtained the permission of the Ethics Committee of the Hospital Affiliated to Hangzhou Normal University (2015-HS-0010). All participants were aged 18 years and above. Inclusion criteria defined a positive hepatitis B surface antigen as confirmed by serological testing. Additionally, either abdominal ultrasound or liver puncture had to indicate the presence of lipids in the liver. All participants tested negative for anti-HIV and anti-Hepatitis C virus (HCV) antibodies. Those consuming more than 20 g of alcohol daily or taking medication known to cause liver steatosis, which includes amiodarone, methotrexate, tamoxifen, corticosteroids, or tetracycline, were excluded from the study.

Clinical and Laboratory Evaluations

All participants provided their medical history and were evaluated by serum biochemical test and abdominal ultrasound (Siemens S2000, Siemens Medical Solutions, Erlangen, Germany). Liver biopsies were performed on 128 subjects, from whom histopathological information was acquired from these individuals. Liver sections were stained by H&E and Masson trichrome, and then two professional pathologists who did not know the patient's clinical data participated in the histological score. Ishak *et al*'s [17] modified histological activity index (MHAI) and Metavir [18] scoring system were used to evaluate the inflammatory activity (grade) and fibrosis stage of hepatitis B. Every liver biopsy sample was reviewed according to the NAFLD pathologic protocol [19]. The liver steatosis evaluated in this protocol is divided into four grades: none, 6–32%, 33–66%, and 67–100%.

Hp Genotyping

We conducted PCR using the following procedure: Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Kit (Qiagen, Valencia, CA, USA). The first PCR reaction included 15 µL of DNA, 1.5 µL of primer A (5-GAGGGGAGCTTGCCTTTCCATTG-3) and primer B (5-GAGATTTTTGAGCCCTGGCTGGT-3), 25 µL of Taq MIX polymerase and add 7 µL of ddH2O; The subsequent PCR reaction included 10 µL of DNA, 25 µL of Taq MIX polymerase, 1.5 µL of the primer C (5-CCTGCCTCGTATTAACTGCACCAT-3) and primer D (5-CCGAGTGCTCCACATAGCCATGT-3), finally add 12 µL of ddH2O. Start with initial denaturation at 94 °C step for 3 minutes. Then proceed with the three-step thermal cycle: denature at 94 °C for 30 seconds, anneal for 30 seconds, and extend at 72 °C for 4 minutes when using primer A\B, or for 1 minute for C\D, repeated for 35 cycles. Conclude with the final extension at 72 °C for 5 minutes. The *Hp* genotype was then identified using agarose gel electrophoresis [20].

Statistical Analysis

Data are presented as either 'n', or the mean ± standard deviation, or median with interquartile range. Pearson Chi-Square Test and Student's *t*-test were used to as-

Table 2. Clinical characteristics of the subjects who underwent biopsy.

Variables	*Hp* 2-2 genotype	Non-*Hp* 2-2 genotype	T/Z/chi-square value	*p* value
N (Male/Female)	75 (67/8)	53 (42/11)	2.50	0.114
Age (years)	40.6 ± 9.11	36.5 (30.2–44.75)	–1.37	0.213
BMI (kg/m^2)	25.3 ± 3.00	24.7 ± 3.37	–1.82	0.281
TC (mg/dL)	4.69 ± 0.78	4.93 ± 1.04	1.42	0.159
TG (mg/dL)	1.36 (0.94–1.30)	1.32 (1.07–1.86)	0.74	0.971
LDL (mg/dL)	2.86 ± 0.70	2.98 ± 0.78	0.85	0.396
HDL (mg/dL)	1.10 (0.94–1.30)	1.21 (1.08–1.36)	1.25	0.039
ALT (U/L)	69.26 ± 32.04	43 (26.0–67.75)	–3.24	0.002
AST (U/L)	40 (30.0–51.0)	31 (22.0–43.0)	–3.03	0.002
GGT (U/L)	49.85 ± 23.31	35.42 ± 17.97	–3.54	0.001
FPG (mg/dL)	5.38 (4.85–5.81)	5.17 (4.81–5.71)	–1.05	0.296
HBsAg	1295.14 (250–3474.15)	2895 (233.68–14,225.93)	1.276	0.175
HBeAg positive, n (%)	33 (54.79%/45.21%)	21 (45.65%)	0.244	0.962
HBV DNA positive, n (%)	55 (78.87%)	47 (92.16%)	4.518	0.031
HBN DNA (log10 opies/mL)	4.09 ± 2.55	5.30 ± 2.53	2.06	0.009
Liver Histology scores				
Steatosis, n (%)				
(0–1)	60 (80.0%)	45 (84.9%)	0.51	0.476
(2–3)	15 (20.0%)	8 (15.1%)	0.51	0.476
Inflammatory grade, n (%)				
(0–1)	18 (24.0%)	31 (58.5%)	15.64	<0.001
(2–4)	57 (76.0%)	22 (41.5%)	15.64	<0.001
Fibrosis stage, n (%)				
0–1	32 (43.7%)	38 (71.7%)	10.56	0.001
2–4	43 (56.3%)	15 (18.3%)	10.56	0.001

sess inter-group differences for categorical variables and for quantitative with normal distribution, respectively. Correlations between the *Hp* genotype, inflammation, and Fibrosis were examined using multiple linear regression. Odds ratio (OR) values are provided with a 95% confidence interval (CI). Statistical analyses were executed through SPSS software version 23 (SPSS, Chicago, IL, USA). $p < 0.05$ was statistically significant.

Results

Comparison of Anthropological Parameters, Blood Parameters, and Virological Parameters between Non-Hp 2-2 and Hp 2-2 in Hepatitis B Complicated with Hepatic Steatosis Patients

One hundred eighty-two subjects diagnosed with hepatitis B complicated by hepatic steatosis were enrolled in the study. The gender distribution was 155 males to 27 females, with an average of 41 years (range: 32–48 years). The mean body mass index (BMI) was 25.04 ± 3.05 kg/m^2 (Table 1). Of these subjects, 109 were categorized as *Hp* 2-2 and 73 as non-*Hp* 2-2. The non-*Hp* 2-2 consisted of *Hp* 1-1 (n = 4), *Hp* 2-1 (n = 69) genotypes. Patients with the *Hp* 2-2 genotype exhibited significantly higher c-glutamyl transferase (GGT) levels ($p < 0.05$) compared to those with the non-*Hp* 2-2 group. Conversely, high-density lipoprotein (HDL) levels were notably lower in the *Hp* 2-2 group ($p < 0.05$). There was no significant difference between the two groups in the quantity of hepatitis B surface antigen (HBsAg) and quality of hepatitis Be antigen (HBeAg). However, the copy number of HBV DNA was significantly higher in the non-*Hp* 2-2 patients ($p = 0.001$).

The Differences in Virological Indices and Severity of Liver Histology between Different Hp Genotypes in Biopsied Hepatitis B Complicated Hepatic Steatosis Patients

No difference was seen in gender ratio, age, and BMI between non-*Hp* 2-2 and *Hp* 2-2 genotypes in hepatitis B who underwent biopsy and complicated hepatic steatosis patients (Table 2). The alanine transaminase (ALT), aspartate amino transferase (AST), and GGT were significantly higher in cohorts with the *Hp* 2-2 genotype ($p < 0.05$), while the HDL level was considerably lower in *Hp* 2-2 genotype patients ($p < 0.05$). Otherwise, the difference in quantitative HBsAg and qualitative HBeAg between the groups shows no difference, while the copy number of HBV DNA was notably higher in non-*Hp* 2-2 genotype patients ($p = 0.009$). A detailed comparison was made between non-*Hp* 2-2 and *Hp* 2-2 genotypes regarding liver histological severity, including steatosis, inflammatory activity (grade), and fibrosis stage. The proportion of higher inflammatory activity scores (2–4) was significantly higher in *Hp*

Table 3. Associations of *Hp* 2-2 genotype with GRADE (≥2) and Fibrosis (Fibrosis stage ≥2).

Variables	Odds ratio	95% CI	*p* value
Inflammatory grade ≥2			
ALT (U/L)	1.009	0.990–1.029	0.348
GGT (U/L)	1.029	0.997–1.061	0.073
HDL (mg/dL)	8.108	0.962–68.332	0.054
TC (mg/dL)	0.511	0.260–1.007	0.052
HBV-DNA (log10 copies/mL)	1.238	0.988–1.550	0.063
Hp genotype (*Hp* 2-2)	7.059	2.746–18.143	0.000
Fibrosis stage ≥2			
ALT (U/L)	1.003	0.986–1.021	0.709
GGT (U/L)	1.030	1.005–1.055	0.018
HDL (mg/dL)	1.838	0.365–9.258	0.461
TC (mg/dL)	1.057	0.588–1.898	0.854
HBV-DNA (log10 copies/mL)	0.902	0.743–1.094	0.295
Hp genotype (*Hp* 2-2)	3.05	1.178–7.927	0.022

ALT, alanine transaminase; GGT, c-glutamyl transferase; HDL, high-density lipoprotein; TC, total cholesterol.

2-2 genotype patients ($p < 0.001$). Similarly, we found the proportion of fibrosis stage ≥2 was also markedly higher in the *Hp* 2-2 genotype than in other genotypes ($p = 0.001$).

Hp 2-2 Genotype is Related to Significant Liver Inflammatory and Fibrosis

For the goal of identifying the risk factors associated with the inflammatory activity (grade) of hepatitis B complicated with hepatic steatosis, we incorporated ALT, GGT, HDL, total cholesterol (TC), HBV-DNA (log10 copies/mL), and *Hp* 2-2 genotype into the Regression model. Besides fibrosis stage ≥2 as the dependent variable, ALT, GGT, HDL, TC, HBV-DNA (log10 copies/mL), and *Hp* 2-2 genotype were included in the regression model. We found that the *Hp* 2-2 genotype was independently related to the significant inflammatory, with an OR of 7.059 ($p < 0.05$) (Table 3). Then, ALT, GGT, HDL, TC, HBV-DNA (log10 copies/mL), and *Hp* 2-2 genotype were also included in the Regression model to find independent risk factors for fibrosis stage ≥2. Our findings showed that the *Hp* 2-2 genotype was independently related to fibrosis (stage), with an OR of 3.05 ($p < 0.05$), as shown in Table 3.

Discussion

Our study represents the preliminary investigation to determine the association between hepatitis B complicated with hepatic steatosis and *Hp* gene polymorphism. The results suggested that the *Hp* 2-2 genotype is crucial in hepatitis B complicated with hepatic steatosis.

We have recruited 182 individuals in this study, categorizing them into two groups based on the *Hp* genotype. When compared with the non-*Hp* 2-2 genotype, we noted that the *Hp* 2-2 genotype showed more significant liver injury, mainly reflected in higher ALT, AST, and GGT, and the metabolic indicators include low density lipoprotei (LDL), TC, triglyceride (TG), and fasting plasma glucose (FPG) showed no difference between the groups except the HDL, which have the function of anti-inflammatory [21] and anti-oxidant [22], is much lower in *Hp* 2-2 genotype subjects; Furthermore, in terms of virology indices, the HBV-DNA (log10 copies/mL) is significantly higher in the non-*Hp* 2-2 genotype. However, the quantitative of HBsAg and the Positive rate of HBeAg remained comparable between the two groups. Additionally, among individuals who underwent biopsy, the *Hp* 2-2 genotype has been identified as an independent risk factor for significant inflammation and fibrosis. However, there was no significant association with hepatic steatosis.

The atherosclerotic protective effect of high-density lipoprotein is related to their ability to promote cholesterol removal from peripheral cells, transport cholesterol to the liver, and excrete it through bile, which is called cholesterol reverse transport [23]. Besides, the function of anti-inflammatory [21] and anti-oxidant [22] activities of HDL has also been learned. *Hp* is an HDL-related protein, and its binding site is located on the helix 6 of apolipoprotein A1 [24,25]. *Hp* could combine hemoglobin with HDL, which may promote atherosclerosis and inflammation [26,27]. This combination of *Hp* 2-hemoglobin complex with HDL increases with its damaged clearance, resulting in increased plasma concentration and a significant decrease in HDL level of the *Hp* 2-2 genotype [28,29]. Our findings are consistent with the observation that the patients with the *Hp* 2-2 genotype have notably lower HDL levels compared to those with the non-*Hp* 2-2 genotype. Such modifications do not occur when the relatively redox-inert *Hp* 1-1–hemoglobin complex [30] is combined with HDL [31].

The virus is non-cytopathic, so the host immune system is generally believed to mediate virus control and liver pathology [32,33]. Adaptive immunity is usually considered the critical factor in eliminating HBV infection; It includes a complex effector cell network. CD4 T cells are potent producers of cytokines and are necessary to develop effective CD8 CTLs. CD8 T cells eliminate HBV-infected hepatocytes through cytolysis and non-cytolysis mechanisms and reduce the level of circulating virus [34,35]. Haptoglobin (*Hp*) affects T-lymphocyte functions, explicitly interacting with CD4+ and CD8+ T cells, strongly suppressing induced T-cell proliferation. Furthermore, *Hp* exhibits a vigorous *in vitro* inhibitory influence on the release of Th2 cytokine and the production of interleukin-2 (IL-2) and interferon-gamma (IFN-gamma), which plays a modulating role on the Th1/Th2 balance [36,37]. This effect is much lower in the *Hp* 2-2 genotype [38], which may explain why the copy number of HBV DNA in the *Hp* 2-2 genotype was lower than that of the non-*Hp* 2-2 genotype.

Hepatitis B, complicated with hepatic steatosis, results from the combined action of metabolic and immune factors in the liver. After the accumulation of fat in hepatocytes, the

liver's uptake of free fatty acids (FFA) increases, resulting in oxidative stress of lipid peroxidation, the release of pro-inflammatory molecules, and mitochondrial damage [39], causing progress from steatosis to steatohepatitis [40,41], and even fibrosis. The *Hp* 2-2 genotype exhibited stronger oxidative stress than the non-*Hp* 2-2 genotype and has been regarded as an independent risk factor of steatohepatitis and fibrosis [42]. Additionally, following HBV infection, the *Hp* 2-2 genotype shows a stronger ability to clear the virus than the non-*Hp* 2-2 genotype, while the accentuation of immunologic status caused more severe liver damage [34,35], including inflammation and fibrosis. Above all, the liver injury induced by *Hp*2-2 in hepatitis B complicated with hepatic steatosis is based on the two aspects of oxidative stress and immune clearance and embodied in the rise of ALT, AST, GGT, and higher G, S scores.

Our research indicates that the *Hp* 2-2 genotype significantly influences hepatitis B when complicated by hepatic steatosis, and it is associated with the progression of the disease.

Conclusions

This study demonstrated that the *Hp* 2-2 genotype is associated with the severity of hepatitis B complicated with liver steatosis. This study offers fresh perspectives for future clinical research on hepatitis B complicated by hepatic steatosis.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions

NY and YL designed experiments; BG carried out experiments; ZJ and XY analyzed experimental results. NY and YL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Hangzhou Normal University Affiliated Hospital (2015-HS-0010), and written informed consent was obtained from each enrolled subject.

Acknowledgment

Not applicable.

Funding

Taizhou Science and Technology Plan A (NO.1901ky18).

Conflict of Interest

The authors declare no conflict of interest.

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