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Association Between *IL-28B* Polymorphism and Clinical Laboratory Features in Patients with Chronic Hepatitis D

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Objectives: We aimed to determine an association between *IL-28B* rs12989760 single nucleotide polymorphism (SNP) and clinical laboratory parameters in patients with chronic hepatitis D. **Methods:** A total of 59 patients who were chronically infected with HDV were had their serum tested for the presence of qHBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc, anti-HCV, anti-HDV IgM, anti-HDV IgG, HDV RNA, HBV DNA and AFP. Liver function tests, coagulation tests and CBC were also performed. *IL-28B* polymorphism was performed using a TaqMan 5' allelic discrimination assay by real time PCR. **Results:** Of these 59 patients, 40 patients (67.8 %) carried the rs12979860CC homozygote genotype, 11 patients (18.6%) were rs12979860CC homozygote and 8 patients (13.6%) were rs12979860CT heterozygote. Alkaline phosphatase and direct bilirubin values were higher ($p<0.05$) and total protein levels were lower ($p<0.05$) in patients who were homozygotes for the T allele as opposed to the carriers of the C allele. AFP levels were significantly higher ($p=0.02$) in the rs12979860 TT genotype group. Liver enzymes including AST, ALT and GGT were significantly not higher in patients who carry C allele ($p>.05$). **Conclusion:** The most frequent genotype was *IL-28B* rs12979860CC among patients with chronic hepatitis D. The *IL-28B* rs12979860 polymorphism was associated with some abnormal liver function test parameters and AFP levels. Additional studies are necessary to understand the immune mechanisms of *IL-28B* polymorphism in HDV infection.

Keywords: Hepatitis Delta Virus, Interleukin 28B, Single Nucleotide Polymorphism

Introduction

Dual infection with hepatitis Delta virus (HDV) and hepatitis B virus (HBV) dual infection is a major cause of chronic liver disease in Mongolia. Most studies suggest that HDV and HBV co-infected patients tend to have more serious liver disease, progressive cirrhosis, and have a higher risk of developing hepatocellular carcinoma (HCC) and a higher mortality rate. Worldwide there

are more 350 million individuals infected with the chronic hepatitis B virus; from them, approximately 20 million are co-infected with hepatitis delta virus (HDV) [1, 2]. It is estimated that 6-7% of the Mongolian population (approximately 50-60% of all HBV carriers, 56% of patients with chronic liver disease) are chronically co-infected with HDV [3-5]. Since the discovery of HDV, even though huge progress has made in the understanding of HDV infection, the precise mechanism for these co-infections remain poorly described. Furthermore, the treatment for chronic HDV continues to be difficult, with limited treatment options that are not very effective [6].

Interferon-lambda 3 (IFN-λ3) belongs to the type III IFNs family involving IFN-λ1, IFN-λ2 and IFN-λ3 which is encoded by *IL-28B* gene. *IL-28B* (IFN-λ3) generates an antiviral state by activating the JAK-STAT cascade that regulates the IFN-stimulated genes (ISGs) [7]. This cytokine has been distinguished as a crucial regulator of the immune response to the hepatitis C virus (HCV). *IL-28B* polymorphism largely determines the outcome of spontaneous and interferon-α treated HCV infection [8-10]. Patients who carry rs12979860CC genotype have a higher likelihood of experiencing spontaneous clearance of the virus after acute infection and are more likely to respond to treatment with interferon alpha. Some studies have shown a relationship between unfavorable *IL-28B* genotypes and fibrosis, cirrhosis and hepatocellular carcinoma [11-14].

Similar with HCV, IFN-α and ISGs are considered to be significant in the immune response to HDV and pegylated IFN-α is only one treatment to chronic hepatitis D infection. Therefore, *IL-28B* polymorphism may affect the natural history, clinical features and treatment response of the other chronic hepatitis virus infection, including HBV and HDV. In this study, we aimed to determine an association between *IL-28B* rs12989760SNP and clinical laboratory parameters in patients with chronic hepatitis D.

Materials and Methods

Patients. From June 2015 to June 2016 at the University General Hospital of the Mongolian National University of Medical Sciences, a total of 441 patients who were positive for HBsAg for more than 6 months and were willing to participate in this study were recruited. All subjects were native Mongolians and provided written consent for participation in this study and for the use of their genetic materials. Peripheral blood samples were obtained from all patients for serological, virological, clinical laboratory tests and *IL-28B* genotype analyses. For confirmation of HBV/HDV co-infection and exclusion of HCV co-infection, patients were assessed for the presence of HBsAg, anti-HCV, anti-HDV IgG and anti-HDV IgM. Based on these exclusion criteria, a total of 142 participants were excluded from this study. From 299 patients who were positive for qHBsAg and anti-HDV antibody, we have randomly chosen 66 treatment naive patients for the current study. Quantitative detection of HDV RNA and HBV DNA in serum was performed. Seven patients who were negative for HDV RNA were considered to have no current HDV infection and were excluded from the study. *IL-28B* rs12979860 SNP genotyping, virological, serological tests, complete blood count (CBC), biochemical and coagulation tests were performed for the remaining 59 patients.

Laboratory tests. The serum samples were tested for the presence of qHBsAg, anti-HBs, HBeAg, anti-HBc, anti-HBe, anti-HCV and AFP using chemiluminescence enzyme immunoassay with HISCL-5000 Automated Immunoassay System from Sysmex according to the manufacturer's protocols. Hepatitis delta virus antibodies anti-HDV IgM and anti-HDV IgG were determined by enzyme-linked immunoassay (ELISA) using commercially available kits (Fortress Diagnostics, UK). Evaluating liver function, the levels of total protein, albumin, alkaline phosphatase, total bilirubin, direct bilirubin ALT, AST and GGT were measured by biochemistry analyzer using standard methods (BX-4000, Sysmex, Japan). CBC, coagulation tests were also performed (CA-104, XN1000; Sysmex, Japan). Serum HBVDNA quantification was carried out by the real time polymerase chain reaction (RT-PCR) (COBAS AmpliPrep/COBAS TaqMan [RocheMolecular Diagnostics]) which has a lower limit of quantification of 12 IU/mL. Quantitative analysis of HDVRNA was performed with LightCycler480 (Roche Molecular Diagnostics) following the manufacturer's protocol. All samples were analyzed for the IL-28B rs12979860 genotype. The detection of SNP was performed by using quantitative RT-PCR method (CFX-96, Bio-Rad, USA) with allele-specific TaqMan probes (Primer Design, UK). The PCR conditions were: enzyme activation at 95°C for 2 minutes, followed with 50 cycles of 95°C for 10 seconds, 60°C for 60 seconds and fluorescence was measured at the end of every cycle per the manufacturer's protocol.

Statistical analyses. Statistical calculations were carried

out with STATA version 12.0 software (StataCorp. 2011, USA). Data were expressed as the median (range), counts, proportions, means, and standard deviations, as appropriate. Pearson chi-square analysis or Fisher exact test was used to compare categorical variables, while the Student T-test or Mann-Whitney U test was used to compare continuous variables. One-way ANOVA and Kruskal–Wallis tests were used where applicable. A p-value below 0.05 was regarded significant.

Results

This study recruited 59 adult Mongolian patients infected with chronic hepatitis D (CHD), including 27 women (45.7%) and 32 men (54.3%), with a mean age of 41.5±10.7 years. The base line characteristics of the patients are presented in Table 1.

Forty patients (67.8%) carried the rs12979860CC homozygote genotype, 11 patients (18.6%) were rs12979860TT homozygote and 8 patients (13.6%) were rs12979860CT heterozygote. The frequency of allele C was 74.6 % while frequency of allele T was 25.4% in the study population. The genotype frequencies were in Hardy–Weinberg equilibrium.

Although there were no statistically significant differences in the results of the immunological tests according to *IL-28B* genotypes, 2 (3.4%) of all patients were positive for anti-HBs and 9 (15.2%) of all patients were positive for HBeAg.

Serological testing revealed that the frequency of anti-HDV IgM antibody positivity was higher in patients carrying T allele (Table 2). qHBsAg levels were >1000IU/ml in 86% of the patients. *IL-28B* SNP had no association to the quantity of HDV RNA. However, a significant difference was observed in the quantity of HBV DNA between the genotypes (p=0.02).

Biochemical baseline data are presented in Table 3. There were no significant differences among groups in the median value of the analyzed liver function tests. To determine the possible association between the *IL-28B* SNP and the abnormality of the liver function, altered liver function variables were evaluated among *IL-28B* genotype groups with chi-square test (Figure 1). Liver function abnormality results that revealed differences at p<0.05 were total protein (p=0.04), total bilirubin (p= 0.05), and direct bilirubin (p=0.005) (Figure 1).

TP-total protein, TB-total bilirubin, DB-direct bilirubin, ALP-alkaline phosphatase, ALT-alanine aminotransferase, AST-aspartate aminotransferase, GGT-gamma-glutamyl transferase

Despite the lack of statistical significance, ALT, AST and GGT activity were higher in patients carrying C allele than patients carrying T allele. In Table 4 the results of complete blood count and coagulation tests were shown. No significant differences were found in CBC and coagulation test results between the genotypes.

We also explored the association between serum AFP level and *IL-28B* genotype. In serological tests AFP levels were significantly higher (p=0.02) in the rs12979860TT genotype group (Table 5).

Table 1. Baseline characteristic of the patients

Characteristics	All patients (n=59)
Age (years) (mean±SD)	41.5±10.7
Gender	
Male, no (%)	32 (54.3)
Female, no (%)	27 (45.7)
IL28-B rs12979860 genotypes	
CC, no (%)	40 (67.8)
CT, no (%)	8 (13.6)
TT, no (%)	11 (18.6)
Allele frequency	
C (%)	74.6
T (%)	25.4

Table 2. Analysis of immunological and virological variables in relation with the genotypes

Variables	*IL-28B* rs12979860 genotypes			p-value
	CC (n=40)	CT (n=8)	TT (n=11)	
qHBsAg (IU/mL) median (range)	4472.6 (152.5-30470.3)	10987.6 (55.0-23375.3)	5375.5 (179.4-27627.1)	0.1
anti-HBs n (%)	2 (5.0)	0 (0.0)	0 (0.0)	1.0
HBeAgn (%)	4 (10.0)	2 (25.0)	3 (27.3)	0.2
anti-HBen (%)	34 (85.0)	8 (100.0)	9 (81.8)	0.7
anti-HBcn (%)	40 (100.0)	8 (100.0)	11 (100.0)	-
anti-HDV IgG n (%)	40 (100.0)	8 (100.0)	11 (100.0)	-
anti-HDV IgM n (%)	22 (55.0)	6 (85.7)	9 (81.8)	0.1
HBV DNA (IU/mL) median (range)	20.0 (0-164.0)	29.5 (20.0-5664.0)	25.0 (20.0-1326.0)	0.02
HDV RNA (IU/mL) median (range)	256500 (256-45800000)	428500 (255-5100000)	282000 (157-7000000)	0.1

Discussion

In this study, we determined that *IL-28B* rs12979860 polymorphism is related to some clinical features in patients with hepatitis B, delta dual infection. The role of *IL-28B* polymorphism in hepatitis C is clear but yet remains undefined in hepatitis delta. There are contradictory reports about CHD and *IL-28B* polymorphism in the literature.

The *IL-28B* rs12979860CC genotype was the most frequent in our study population. This high prevalence of the major *IL-28B* polymorphism alleles has been reported in several previous studies which included the Asian and American ethnicities [8, 15]. In a study among Chinese patients who had HBV chronic infection, the *IL-28B* rs12979860TT, CT and CC genotypes

Table 3. Analysis of biochemical variables in relation with the genotypes

Variables	*IL-28B* rs12979860 genotypes			p-value
	CC (n=40) median (range)	CT (n=8) median (range)	TT (n=11) median(range)	
Total protein (g/L)	73.0 (65.0-83.0)	71.5 (63.0-84.0)	69.0 (63.0-74.0)	0.05
Albumin (g/L)	40.0 (33.0-49.0)	38.5 (31.0-47.0)	38.0 (30.0-50.0)	0.5
Total bilirubin (μmol/L)	17.5 (8.0-37.0)	18.0 (14.0-37.0)	22.0 (8.0-49.0)	0.5
Direct bilirubin (μmol/L)	2.0 (1.0-4.0)	2.0 (0-10.0)	2.0 (1.0-20.0)	0.4
Alkaline phosphatase (U/L)	316.0 (205.0-616.0)	434.5 (169.0-736.0)	342.0 (161.0-489.0)	0.5
AST (U/L)	56.0 (12.0-228.0)	44.5 (20.0-425.0)	37.0 (17.0-182.0)	0.6
ALT (U/L)	46.0 (21.0-172.0)	49.5 (17.0-192.0)	37.0 (17.0-139.0)	0.4
GGT (U/L)	40.0 (10.0-475.0)	30.0 (13.0-573.0)	27.0 (13.0-200.0)	0.2

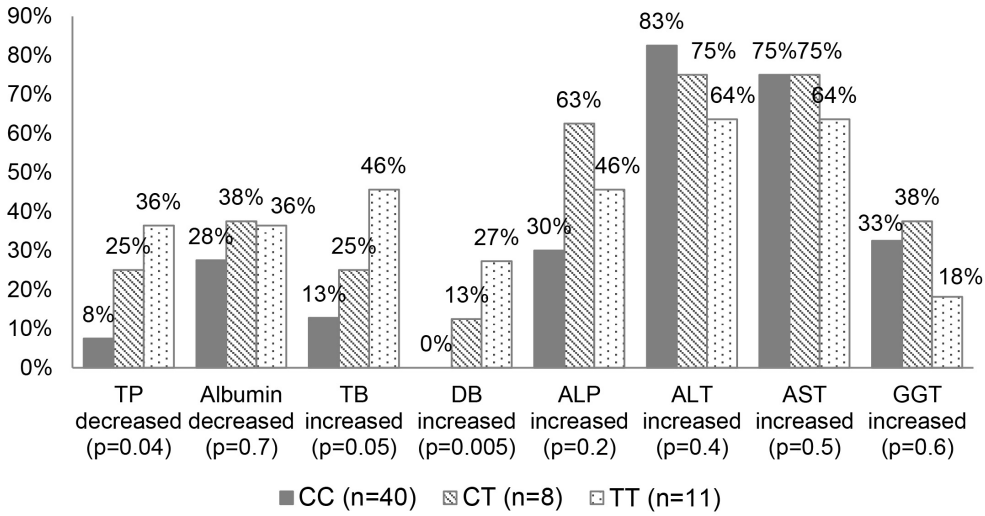

Figure 1. Liver function tests abnormalities among *IL-28B* genotype groups

Table 4. Analysis of the complete blood count and coagulation test variables in relation with the genotypes

Variables	*IL-28B* rs12979860 genotypes			p-value
	CC (n=33) m±SD	CT (n=8) m±SD	TT (n=9) m±SD	
WBC (*10^9/l)	5.01±1.2	4.6±08	5.29±1.9	0.55
RBC(*10^{12}/l)	4.8±0.4	4.5±0.4	4.6±0.8	0.30
HGB (g/dl)	14.5±1.3	13.7±1.7	14.1±2.0	0.37
HCT (%)	41.4±3.7	39.9±3.2	40.0±5.5	0.58
PLT (*10^9/l)	185.5±59.4	201.3±70.8	206.3±92	0.65
Prothrombin time (sec)	12.1±1.6	12.2±1.6	12.2±1.6	0.99
INR median (range)	0.97 (0.8-1.8)	0.8 (0.8-1.3)	0.88 (0.8-1.3)	0.99

Table 5. Analysis of serum AFP concentration in relation with the genotypes

Variables	*IL-28B* rs12979860 genotype			p-value
	CC (n=40)	CT (n=8)	TT (n=11)	
AFP ng/ml median (range)	2.75 (0.9-16.3)	4.0 (0.6-31.0)	4.4 (1.2-77.2)	0.5
Normal	35 (87.5)	6 (75.0)	8 (72.7)	0.02
10-200ng/ml	5 (12.5)	2 (25.0)	3 (27.3)	

prevalence was 0.7%, 10.4% and 88.9% , respectively [16].

In 2009, several SNPs close to the gene encoding *IL-28B* were determined to be significantly impact the response to treatment of patients chronically infected with HCV, with rs12979860 having the strongest association with response rate. The C allele for SNP rs12979860 is the "favorable allele" and is associated with higher rates of treatment response than the T allele [8, 9, 15]. High prevalence of the CC genotype in our study population could be predicting higher response rates for α-interferon treatment against HDV infection.

Furthermore, our study found that the rs12979860C allele was related to higher serum ALT and AST compared to the T allele. This finding corresponds with the result of a Genome Wide Association study that demonstrated that the rs12979860CC was significantly associated with ALT levels which is regarded as a necro inflammatory marker of the liver cells. Another study showed that the carriers of the rs12979860CC genotype had higher values for ALT comparing with carriers of the rs12979860CT/TT genotypes among Spanish individuals, which is a similar result to our study [17]. As several previous studies have reported, *IL-28B* CC genotype is connected to a strong immune response against hepatitis viruses, which on a positive side leads to higher viral clearance, yet negatively, to a more advanced cell injury which explains the result of higher levels of ALT and AST in CC genotype group, and supporting an idea that the *IL-28B* genotypes are directly linked to the pathogenesis of liver damage in delta hepatitis [17-19]. Although, it is unclear if this is due to direct viral effects or an altered immune response against HDV.

A further finding of this study was that patients carrying the rs29789860TT genotype had decidedly higher serum total bilirubin and direct bilirubin levels compared to those carrying the CC genotypes. This indicates that glucoronyl conjugation of bilirubin and the transport mechanism of hepatocytes are impaired in patients carrying the T allele. One interpretation of this phenomenon could be liver cell failure resulting from higher rates of liver fibrosis or cirrhosis in those patients [20]. A study conducted in Egypt which focused on the association between liver fibrosis and *IL-28B* clearly showed the elevated concentrations of total and direct bilirubin and the advanced stages of fibrosis in non-CC IL28B patients [21].

The tendency of HBV replication by HDV in HBV/HDV dual infection is a common phenomenon observed through different studies [25-27]. All the subjects of our study had high HDV RNA levels combined with low HBV DNA levels consistent with previous studies. Additionally, we observed a significant difference in the quantity of HBV DNA in different genotypes, but not in HDV RNA level. A median value of HBV DNA was higher in patient with CT genotype. Similar to our findings, Ispirologlu et al. reported no difference in HDVRNA level between *IL-28B* rs12979860 genotypes in Turkish patients with CHD and mean HBV DNA levels observed to be significantly higher in patients with the CT genotype [28].

This study has also shown that AFP concentrations were lower in patients with CHD who carry the rs12979860CC genotype than in rs12979860CT or TT genotype. Eurich et al. in a study reported that the C allele acts as a protective factor against the development of HCC. Ren et al. discovered that the T allele was related to the susceptibility of HCC in Chinese patients [12, 13]. With AFP being the main serum marker of the development of HCC, our outcome could promote the notion of the C allele having a protective role against HCC.

Furthermore, in our study qHBsAg levels were>1000IU/ml in 86% of the patients suggesting a higher risk of HCC development in those patients [14]. However, conflict continues about the potential link between the risk of development of HCC and *IL-28B* gene polymorphism.

Due to an insufficient number of patients, our study may have inadequate statistical power to detect some clinically important differences, so the results of this study should be treated with caution. It is, therefore, paramount that additional studies be done by other investigators be done to verify the role of *IL-28B* polymorphism in HDV infected patients. Another drawback was the absence of histopathological examination, which is regarded as the gold standard for evaluating necro inflammation, liver fibrosis and cirrhosis. However, a recent study has not detected a parallel between *IL-28B* polymorphism and the necro inflammatory grade and the stage of fibrosis through liver biopsy in CHC patients [17]. Moreover, liver biopsy for chronic Hepatitis B infection before antiviral therapy in chronic hepatitis B (CHB) patients is not routinely part of contemporary practice, especially for those with obviously active CHB (ALT above 2 times ULN and HBV DNA>2000 IU/mL) [29, 30]. The different pathogenesis of those viruses set aside, we believe that researching the correlation between *IL-28B* polymorphism and the histopathological progress of the disease in CHD is vital in the future to understand the true association between *IL-28B* genetic polymorphism and chronic HDV infection.

In conclusion, the most frequent genotype was *IL-28B* rs12979860CC among patients with chronic hepatitis D. The *IL-28B* rs12979860 polymorphism was associated with some liver function test parameters and AFP levels. Additional studies are necessary to understand the immune mechanisms of *IL-28B* polymorphism in HDV infection.

Conflict of Interest

The authors state no conflict of interest.

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