Association of interleukin-28B polymorphisms with platelet count and liver function recovery after liver transplant

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Abstract

The present genome-wide association study investigated the relationship of interleukin 28B (IL-28B) genetic variants with HBV susceptibility and prognosis of HBV-infected patients. This study aims to examine the role of IL-28B polymorphisms on transplant etiologies and the liver function recovery in Chinese liver transplant recipients.

A total of 231 liver transplant recipients were enrolled in the study. The transplant etiologies included progressive HBV hepatitis, HBV-related liver cirrhosis (LC), HBV-related hepatocellular carcinoma (HCC), and non-HBV-related disease. All recipients were in stable condition before transplantation. Three single nucleotide polymorphisms (SNPs) of IL-28B (rs12979860, rs12980275, rs8099917) of recipients were analyzed by high-resolution melting (HRM) curve analysis. Liver function, blood cell count, and coagulation function were regularly tested before and for next 5 years after transplantation.

No significant association was found between IL-28B gene polymorphisms and transplant etiologies. Peripheral platelet count in the third and fourth days after transplantation were significantly higher in recipients carrying IL-28B rs12979860 T allele, or rs8099917 C allele ($P < 0.016666667$), while there were no significant differences between these variants and International Normalized Ratio (INR) levels. In addition, gamma-glutamyltransferase (GGT) levels in recipients with rs12980275 G allele were higher than those in the wide-type recipients before transplantation ($P < 0.016666667$, respectively); nevertheless, no influence of these variants on GGT recovery was observed after transplantation.

Genetic variations of IL-28B might impact on liver function recovery by influencing peripheral platelet counts and reducing liver inflammation, but have weak association with transplant etiologies.

Abbreviations: GGT = gamma-glutamyl transpeptidase, HCC = hepatocellular carcinoma, IFN-$\lambda$3 = interferon-lambda, LC = liver cirrhosis, LT = liver transplantation, SNPs = single nucleotide polymorphisms.

Keywords: coagulation function, gamma-glutamyltransferase, hepatitis B virus, IL-28B, liver transplantation, platelet

1. Introduction

Hepatitis B is one of the most common liver diseases worldwide, especially in China. It is often complicated by the development of cirrhosis and hepatocellular carcinoma (HCC). If HBV infection is not effectively treated, hepatocellular function may deteriorate progressively. For patients with end-stage liver diseases, liver transplantation (LT) is a highly effective treatment.\textsuperscript{[1–16]} However, liver function recovery after LT varies between individuals and the recurrent HBV infection is one of the most important graft diseases that can occur after LT due to using the immunosuppressant therapy. The course of hepatitis B in a graft is usually more severe than that in nontransplant patients. In addition to viral, environmental, and behavioral factors, host genetic diversity is thought to contribute to the spectrum of the disease.\textsuperscript{[4]}

Interleukin 28B (IL-28B), a member of type III interferons which termed interferon-lambda (IFN-$\lambda$3), plays an important role in antiviral immunity, especially in the IL-28B-mediated antiviral defense against hepatotropic viruses, such as HBV and HCV.\textsuperscript{[5,6]} The gene for IL-28B is located on the long arm of chromosome 19 at position 19q13.13.\textsuperscript{[17,18]} It has been reported that IL-28B SNPs (rs1297860, rs12980275, and rs8099917) were associated with HCV spontaneous clearance as well as different outcomes of treatment with IFN/ribavirin therapy for chronic HCV infection.\textsuperscript{[5–16]} In our previous studies, we found that genotypes of IL-28B SNPs (rs8099917, rs12979860, and rs12980275) were associated with alanine aminotransferase (ALT) levels and aspartate aminotransferase (AST) levels in HBV-related LT recipients. Recipients with risk genes on IL-28B (the TT genotype of rs12979860, the GG
genotype of rs12980275, and the CC genotype of rs8099917) had a significantly higher AST concentration. However, we have not found any association between IL-28B gene polymorphisms with the HBV recurrence in LT recipients. The observations above suggest an important role of IL-28B SNPs against HBV infection. They also indicate that IL-28B SNPs might be associated with the recovery of HBV-related LT recipients.

As the IL-28B plays an important role in antiviral immunity and IL-28B SNPs can affect the outcomes of HCV or HBV infection, we analyzed whether IL-28B polymorphisms were associated with transplant etiologies and liver function recovery in liver transplant recipients.

2. Materials and methods

2.1. Patients

A total of 231 LT recipients, who underwent LT in West China Hospital from September 2000 to December 2011, were enrolled in this retrospective study. The HBV-related recipients were diagnosed by hepatitis B virus surface antigen (HBsAg) positive or high copies of serum HBV DNA (>1 × 10^7 copies/mL) before transplantation. The differentate of LC, HCC, and progressive hepatitis B was by histologic analysis of liver biopsy specimens during the liver transplant. All of these recipients included were in stable condition. Before transplantation, recipients were evaluated by Model for End-Stage Liver Disease (MELD) score and Child–Pugh score. Besides, those who had acute rejection, chronic rejection or platelet transfusion after LT were excluded from this study. After transplantation, all recipients received a calcineurin inhibitor (CN)-based regimen (tacrolimus + mycophenolate mofetil + steroids). Steroids were discontinued in 3 to 6 months after transplantation. Liver function and coagulation function were monitored routinely in the next 5 years after LT. This study was approved by the ethics committee of West China Hospital and consistent with the guidelines of the Helsinki Declaration. All of the LT recipients signed an informed consent before inclusion in this study.

2.2. IL-28B gene polymorphisms

DNA was extracted from an EDTA anticoagulated peripheral whole blood using the whole blood DNA kit (Biotake Corporation, Beijing, China), diluted to 10 ng/mL by the AE buffer provided by the manufacturer and stored at −80°C until analyzed. The IL-28B gene polymorphisms in the promoter region (rs1297860, rs12980275, and rs8099917) were assessed. Some samples were previously genotyped by sequencing as controls for the 3 SNPs. The polymerase chain reaction (PCR) and melting curve analyses were performed under the same conditions in a 96-well plate on the Light Cycler 480 (Roche Diagnostics, Penzberg, Bavaria, Germany). Primers for the 3 SNPs were as follows: rs1297860: 5’-ATTCTGGAGCTGTGTGGTAAC-3’ (forward); 5’-AGCCGG GAAGTCAATTTCA-3’ (reverse); rs8099917: 5’-TTGTCACTGGAGAATG-3’ (forward); 5’-TGAGAATG CAAATGAGATACCA-3’ (reverse); rs12980275: 5’-GCCAGTCT CAAAAGAA CAAATGC-3’ (forward); 5’-CTACCCCGGCAA ATATTAGACA-3’ (reverse). SNP genotyping was performed in a 10 µL reaction system contained 3 µL Roche Master Mix (Roche Applied Science, Mannheim, Germany) which comprises FastStart Tag DNA Polymerase and the High Resolution Melting Dye in a reaction buffer, 1.2 µL 25 mM MgCl2, 0.1 µL 10 µmol/L forward primer and 0.1 µL 10 µmol/L reverse primer, 2.6 µL deionized water and finally 1 µL DNA sample. Real-time PCR was performed under the following conditions: a predenaturation step at 95°C for 15 minutes, continued with 30 cycles of 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 20 seconds. After the amplification phase, a melting curve analysis was performed at 95°C for 1 minute, 40°C for 1 minute, 65°C for 1 seconds, followed by slow heating at 0.01°C/second to 95°C. The results were analyzed by the Light Cycler 480 Gene Scanning software v1.2 (Roche Diagnostics). The genotype of subset was defined according to known genotypes of controls.

2.3. Laboratory tests measurement

HBV serological markers for HBsAg, HBeAg, anti-HBs, anti-HBc, and anti-HBe were conducted with ELISAs (Dade Behring, Marburg, Germany). All these recipients were divided into 4 groups by etiologies including liver cirrhosis (LC), HCC, progressive HBV hepatitis and non-HBV-related disease. Liver function, blood cell count, and coagulation function were regularly tested before LT, at the day of LT, in the 1st to 7th day, 14th day, 1st month, 3rd month, 6th month, 9th month, and the 1st to 5th year after LT. We tested alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (A1b), gamma glutamyl transpeptidase (GGT), total bilirubin (TB), direct bilirubin (DB), platelet count (PLT), prothrombin time (PT), international standardization ratio (INR), fibrinogen (FIB), activated partial thromboplastin time (APTT), and tacrolimus concentration.

2.4. Statistical analysis

Concordance of genotype distribution with Hardy–Weinberg equilibrium was assessed by Pearson Chi-square test. Clinical data were expressed as mean ± SD or median (interquartile range). Statistical analysis was performed by SPSS 19.0 (SPSS, Inc., Chicago, IL.). Continuous variables with the normal distribution were analyzed using Student t test. Mann–Whitney U test or Wilcoxon rank sum test was used to compare the continuous variables that follow the skewed distribution. Pearson Chi-square test or Fisher exact test were used to analyze the categorical variables. A 2-sided P-value <.05 was considered statistically significant. We corrected the results with the Bonferroni method accounting for the number of SNPs (P < 0.016666667). When comparing the clinical indices, recipients were divided into 2 groups by genotypes: AA versus AB+BB (allele A as the major allele, B as the minor allele). Because the minor alleles of the IL-28B SNPs are dominant and the number of recipients with BB genotype on rs12979860 was limited (<3). Haplotype analysis, which was performed to explore whether the selected SNPs were in strong linkage disequilibrium (LD) or they independently contribute to the transplant etiologies of liver transplant recipients, was evaluated by SHEsis online software (http://analysis.bio-x.cn/myAnalysis.php).

3. Results

3.1. Demographic characteristics

Total recipients (n = 231) were divided into different groups by their genotypes in IL-28B SNPs (rs12979860, rs12980275, and rs8099917). The detailed clinical characteristics of liver transplant recipients were depicted according to the different genotype groups in Table 1. There was no significant difference in age nor
3.2. Association analysis of IL-28B polymorphisms with transplant etiologies

Three genotypes of IL-28B gene polymorphisms (rs12979860, rs12980275, and rs8099917) were distinguished by the melting profiles from the normalized melting curves. All the genotypes distribution of recipients were in Hardy–Weinberg equilibrium ($P > .05$). Distribution of IL-28B SNPs’ genotypes and alleles in recipients are shown in Table 2. No significant association between IL-28B polymorphisms and different transplant etiologies was observed among LT recipients (rs12979860, $P = .406$ by genotype, $P = .280$ by allele; rs12980275, $P = .295$ by genotype, $P = .306$ by allele; rs8099917, $P = .985$ by genotype, $P = .986$ by allele) ($P > .00833333$ for multiple testing).

IL-28B haplotype was constructed from rs12979860 (minor allele T), rs12980275 (minor allele G), and rs8099917 (minor allele C). We set up the threshold frequency no. <.03 to avoid some extremely rare haplotypes. LD value ($D'$) indicated the 3 SNPs were in high linkage disequilibrium (Fig. 1). Meanwhile, in liver transplant recipients, IL-28B haplotype block CAA and TGC showed no correlation with transplant etiologies between HBV-related recipients and non-HBV-related recipients (CAA: OR = .028, 95% CI = .009–.075; TGC: OR = 4.206, 95% CI = .778–22.739) (Table 3).

To identify whether IL-28B polymorphisms were associated with HBV infection in the recipients, we divided the patients into HBV-related diseases group and non-HBV diseases group. No association was found in IL-28B polymorphism alleles between HBV-related diseases and non-HBV diseases (rs12979860, adjusted OR = 3.06, 95% CI = 0.69–13.49 by genotype, adjusted OR = 3.07, 95% CI = 0.71–13.25 by allele; rs12980275, adjusted OR = 5.30, 95% CI = 0.69–40.63 by genotype, adjusted OR =
3.3. Association of IL-28B gene with platelet counts and coagulation function

Platelet counts and coagulation function are directly associated with liver function, since liver products thrombopoietin and some clotting factors (Ⅰ, Ⅱ, Ⅶ, Ⅸ, Ⅹ) promote platelet production.[23–25] Therefore, we tested the PLT counts and INR to evaluate the recovery of the patients’ liver function after LT. Platelets are a component of blood whose function (along with the coagulation factors) is to stop bleeding by clumping and clotting blood vessel injuries, so the lower serum platelet counts may increase the risk of bleeding. PLT concentration was compared among HBV-related LT recipients with different genotypes. Patients who had different genotypes on rs12979860 were divided into CC and (CT+TT) group. For IL-28B gene rs12980275, the 2 genotypes groups were AA and (AG+GG). For rs8099917 the 2 groups were AA and (AC+CC). The numbers of the recipients with different genotypes in different time after LT are shown in Table 4. After Bonferroni correction (P < .016666667) we detected a significant difference for rs12979860 on 3rd and 4th day and rs8099917 on the day of LT, 3rd and 4th day. HBV-related recipients with IL-28B genetic variants, rs12979860 T allele and rs8099917 C allele, had significantly lower serum platelet counts (P < .016666667, Table 4, Figs. 2–4). A week after LT, there seemed to be no difference between each 2 groups of recipients (Figs. 2–4). INR is derived from prothrombin time (PT) and widely used to evaluate the extrinsic pathway of coagulation. There was no difference in INR ratio among LT recipients with different IL-28B SNPs genotypes (Table 5). It seemed that HBV-related recipients with favorable IL-28B genetic genotypes on rs12979860 and rs8099917 SNPs (CC on rs12979860, AA on rs8099917) had lower serum platelet counts in the third and fourth days after LT but had similar coagulation function.

3.4. Association of IL-28B gene with liver function recovery

For all of the liver transplant recipients, routine liver function tests were done to evaluate the recovery of the patients after LT. We gathered the data before LT, on the day of LT, and 1st to 7th day, 14th day, 1st month, 3rd month, 6th month, 9th month, and the 1st to 5th year after LT. We evaluated the association between IL-28B SNPs and liver function tests. The GGT concentration of HBV-related LT recipients with genotype of IL-28B gene rs12980275 were AA: (AG+GG). The numbers of the recipients with different genotypes in different time are shown in Table 6. After Bonferroni correction (P < .016666667) we detected a significant association of rs12980275 with GGT concentration before transplant (Table 6). We observed that a lower GGT concentration was significantly associated with the AA genotype of rs12980275 in HBV-related recipients before LT (Table 6). It meant that HBV-related recipients with protective genotypes on IL-28B SNPs rs12980275 had lower serum GGT concentration and better liver function before LT. After LT, there seemed to be no differences between recipients with different genotypes. Other indices of liver function did not show significant difference before and after LT (P > .016666667).

4. Discussion

In this study, the relationship between IL-28B polymorphisms and transplant etiologies of patients who went through LT were analyzed. In addition, we analyzed the relationship between IL-28B polymorphisms and liver function recovery of HBV-related recipients.
recipients. It seemed that there was no significant difference in the frequencies of distribution among LT recipients with different transplant etiologies ($P > 0.0083333$). In the third and fourth days after LT, HBV-related recipients who had IL-28B protective genotypes on rs12979860 and rs8099917 SNPs (CC on rs12979860, AA on rs8099917) were significantly had lower platelet levels ($P < .01666667$) (Table 4, Figs. 2 and 4). However, the INR of recipients with different IL-28B polymorphisms had no difference (Table 4). Meanwhile, recipients who had protective genotypes on rs12980275 (AA on rs12980275) had lower GGT level before LT ($P < .01666667$) (Table 6).

It is well-known that blood platelets play an important role in hemostasis, with growing evidence showing that platelets could also affect tissue repair,[23] inflammation,[24] angiogenesis,[25] and ischemia/reperfusion injury.[26] In a previous study, it was found that platelets contribute to liver damage by promoting the intrahepatic accumulation of virus-specific CD8+ T cells and virus-nonspecific inflammatory cells.[27] Some studies indicated that antiplatelet therapy could prevent HBV-associated HCC without increasing bleeding risk.[28,29] In our study, recipients with protective genetic variants in IL-28B had lower serum platelet levels but the coagulation function measured by INR had no difference in the first 4 days after LT. This indicated that the lower platelet counts might be beneficial to the HBV-related recipients. Recent studies found that platelets played a critical role in liver injury and regeneration, which could also predict outcomes after LT.[30–34] Low perioperative platelet count (<60 × 10^9/L) was an independent factor associated with severe complications and early graft and patient survival after LT.[33] These findings made low platelet count a risk factor for recipients. However, there was no definite conclusion on whether the low platelet counts was beneficial or risky to the HBV-related LT recipients.[34] More researches were needed to investigate this problem.

In addition, there was no direct evidence showing the relationship between IL-28B genetic variants and peripheral platelet counts in previous studies. Homoncik et al[35] found that single-dose interferon-α could decrease platelet counts in patients with chronic hepatitis C. Platelet counts were related with thrombopoietin produced by liver. We could guess that maybe the IL-28B polymorphisms could also influence the platelet counts by regulating the concentration of IFN-α or thrombopoietin in patients with hepatitis B. Whether the IFN-α could affect the

| Table 5 | IL-28B genotypes and International Normalized Ratio (INR) of HBV-related recipients. |
|---------|---------------------------------|
| SNP     | rs12979860 | rs8099917 |
| Time    | CC | n | CT+TT | CC | n | CT+TT | CC | n | CT+TT | CC | n | CT+TT | CC | n | CT+TT | CC | n | CT+TT |
| Before LT | 1.46 (1.13-1.92) | 16 | 1.39 (1.29-1.53) | 0.9 | 131 | 1.43 (1.12-1.91) | 13 | 1.43 (1.10-2.47) | 0.54 | 131 | 1.43 (1.12-1.91) | 13 | 1.40 (1.30-2.37) | 0.53 |
| The day of LT | 1.59 (1.35-1.92) | 13 | 1.61 (1.28-2.77) | >0.9 | 119 | 1.59 (1.33-1.91) | 12 | 1.80 (1.34-2.96) | 0.63 | 120 | 1.59 (1.35-1.93) | 11 | 1.86 (1.29-2.38) | 0.96 |
| 1st day after LT | 1.56 (1.37-1.82) | 15 | 1.52 (1.38-2.05) | 0.92 | 135 | 1.57 (1.36-1.82) | 14 | 1.49 (1.39-1.87) | 0.85 | 135 | 1.54 (1.37-1.81) | 14 | 1.63 (1.37-2.14) | 0.70 |
| 2nd day after LT | 1.39 (1.02-1.58) | 16 | 1.43 (1.24-1.83) | 0.61 | 132 | 1.39 (1.18-1.66) | 14 | 1.43 (1.22-1.72) | 0.57 | 132 | 1.39 (1.18-1.66) | 14 | 1.54 (1.33-1.96) | 0.12 |
| 3rd day after LT | 1.22 (1.12-1.48) | 15 | 1.23 (1.19-1.63) | 0.13 | 128 | 1.23 (1.12-1.45) | 7 | 1.32 (1.17-1.67) | 0.28 | 128 | 1.22 (1.12-1.45) | 14 | 1.34 (1.22-1.67) | 0.08 |
| 4th day after LT | 1.21 (1.08-1.38) | 9 | 1.24 (1.15-1.58) | 0.56 | 107 | 1.21 (1.09-1.38) | 9 | 1.24 (1.12-1.66) | 0.66 | 104 | 1.21 (1.08-1.37) | 10 | 1.31 (1.15-1.36) | 0.32 |
| 5th day after LT | 1.21 (1.06-1.35) | 10 | 1.21 (1.08-1.43) | 0.87 | 93 | 1.21 (1.07-1.35) | 8 | 1.23 (1.05-1.44) | 0.99 | 93 | 1.21 (1.07-1.35) | 9 | 1.36 (1.05-1.45) | 0.69 |
| 6th day after LT | 1.14 (1.03-1.30) | 10 | 1.29 (1.06-1.45) | 0.67 | 84 | 1.14 (1.03-1.29) | 11 | 1.38 (1.03-1.55) | 0.50 | 82 | 1.12 (1.03-1.28) | 10 | 1.37 (1.11-1.45) | 0.12 |
| 7th day after LT | 1.10 (1.01-1.23) | 12 | 1.17 (1.07-1.47) | 0.20 | 111 | 1.10 (1.01-1.24) | 10 | 1.14 (1.06-1.28) | 0.59 | 109 | 1.10 (1.01-1.23) | 13 | 1.20 (1.07-1.45) | 0.10 |
| 14th day after LT | 1.08 (0.99-1.17) | 11 | 1.10 (1.02-1.23) | 0.70 | 101 | 1.08 (0.99-1.17) | 5 | 1.10 (1.02-1.26) | 0.89 | 101 | 1.07 (0.99-1.17) | 10 | 1.12 (1.02-1.40) | 0.46 |
| 1st month after LT | 1.09 (1.01-1.16) | 7 | 1.12 (1.02-1.28) | 0.74 | 78 | 1.09 (1.01-1.15) | 9 | 1.18 (1.07-1.84) | 0.38 | 78 | 1.06 (1.01-1.23) | 7 | 1.18 (1.12-1.28) | 0.15 |

Continuous variables are expressed as median (interquartile range). The P-value cutoff after multiple test correction is: $P < .01666667$. LT = liver transplantation, n = number of patients, SNP = single nucleotide polymorphism.
The unit of platelet quantity is (10^9/L). Continuous variables are expressed as median (interquartile range).

|           | 1st year after LT | 2nd year after LT | 4th year after LT | 7th day after LT | 15th day after LT | 30th day after LT | 1st month after LT | 3rd month after LT | 6th month after LT | 9th month after LT | 1st year after LT | 2nd year after LT | 3rd year after LT | 4th year after LT | 5th year after LT |
|-----------|-------------------|-------------------|-------------------|------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| n         | 127               | 135               | 133               | 114              | 126              | 131              | 138              | 100              | 113              | 80                | 131              | 129              | 103              | 73                | 52                |
| CC (×10^9/L) | 55 (28–100)       | 46 (32–87)        | 65 (46–94)        | 43 (27–73)       | 51 (29–85)       | 51 (25–119)      | 65 (73–108)      | 77 (71–117)      | 111              | 98                | 72              | 105              | 104              | 97                | 63                |
| CT+TT (×10^9/L) | 124 (72–238)     | 21 (29–95)        | 177 (25–142)      | 145 (72–189)     | 177 (25–142)     | 117 (21–139)     | 177 (73–310)     | 145 (36–297)     | 98                | 72 (24–297)      | 70 (8–190)       | 63 (8–190)       | 66 (21–100)      | 51 (18–199)      | 50 (23–199)      |
| P          | .12               | .12               | .12               | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              |
| NA (×10^9/L) | 124 (72–238)     | 21 (29–95)        | 177 (25–142)      | 145 (72–189)     | 177 (25–142)     | 117 (21–139)     | 177 (73–310)     | 145 (36–297)     | 98                | 72 (24–297)      | 70 (8–190)       | 63 (8–190)       | 66 (21–100)      | 51 (18–199)      | 50 (23–199)      |
| P          | .12               | .12               | .12               | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              | .12              |

It is difficult to explain this kind of difference concentrated in such a short time. Secondly, we did not test the relationship between IL-28B genetic variants, IFN-α, thrombopoietin, and peripheral platelet by doing molecular biology experiment.

In conclusion, recipients with protective genotypes on IL-28B polymorphisms and liver transplant etiologies. This finding may provide a valuable gene therapy tool to regulate the liver function of recipients before and after LT.

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