Original Article

Association of gene polymorphisms in toll-like receptors 4 with bacterial infection after orthotopic liver transplantation in Han Chinese patients

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Abstract: Bacterial infections represent the most common complications after liver transplantation (LT). Toll-like receptor 4 (TLR4) plays a pivotal role in recognizing pathogens. The study aimed to determine the association between TLR4 single nucleotide polymorphisms (SNPs) with susceptibility to bacterial infection within 6 months after orthotopic liver transplantation. This was a prospective cohort study of 113 consecutive LT recipients (n=44 with infections; n=69 without infection as controls) at the Shanghai Jiaotong University Affiliated First People Hospital (China) between January 2007 and January 2011. The association between TLR4 SNPs (rs1927907, rs1927914, rs11536889, rs1927906, and rs2149356) in recipients with the susceptibility to bacterial infection after orthotopic liver transplantation within 6 months was analyzed. A total of 44 transplant recipients (38.9%) developed bacterial infections within 6 months of LT, including 19 and 25 patients with Gram-positive and negative bacteria, respectively. Pulmonary infection (n=21), cholangitis (n=9), sepsis (n=9), and other bacterial infections (n=5) were observed. Recipient TLR4 rs1927907, rs1927914, rs11536889, and rs2149356 SNPs were associated with infections within 6 months after LT. Multivariate analysis showed that endotracheal intubation time ≥72 h (P=0.040, OR=2.84, 95% CI 1.05-7.70) and rs2149356 (AA vs. AC/CC, P=0.003, OR=4.24, 95% CI 1.65-10.93) were independently associated with bacterial infection within 6 months after LT. Kaplan-Meier analysis indicated that patients with the AA rs2149356 genotype could be at higher risk of developing a bacterial infection within 6 months after LT. Prolonged duration of endotracheal intubation and the TLR4 rs2149356 SNP were independently associated with infections after LT within 6 months.

Keywords: Toll-like receptor 4, single nucleotide polymorphism, bacterial infection, liver transplantation

Introduction

Liver transplantation (LT) is an effective and cost-efficient option for the treatment of end-stage liver diseases [1]. With the development of surgical techniques and new immunosuppressive regimens, patient survival after LT has increased in the past decade [1]. However, bacterial infections are the most frequent complications post-LT as well as being the leading cause of morbidity and mortality in LT recipients [1-4]. Hence, identifying patients at high risk of developing infections is of vital importance for improving long-term prognosis after LT. Most bacterial infections occur within 8 weeks after LT. In addition, the spectrum of infection after liver transplantation changes with time. Early infections occur in the first month after transplant, and 90% of them are nosocomial bacterial and fungal infections; mid-term infections occur within the first 1-6 months after LT, and are mainly viral infections. Late infections occur after six months, are relatively rare, and most of them are opportunistic infections [1-4].

So far, studies have suggested that clinical parameters significantly associated with infection include age, MELD scores, Child-Pugh scores, empirical antibiotic administration, large volume of blood loss and packed red cell transfusion, operation time, ICU stay, reoperation,
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and acute renal insufficiency [1, 2, 5-7]. In addition, we previously showed that recovery diet, prolonged endotracheal intubation, and biliary complications were risk factors for infection [8]. Interestingly, some patients with no definitive clinical risk factors are still susceptible to infections [7, 9]. There is growing evidence that susceptibility and response to infectious diseases is, in part, inheritable. Particularly, single-nucleotide polymorphisms (SNPs) in innate immune response genes may play a key role in the development of post-LT infections [10-12].

As pattern recognition receptors, toll-like receptors (TLRs) play important roles in activating innate immunity and developing adaptive immunity by inducing the expression of specific cytokines and chemokines [12]. They can recognize various components of bacteria, viruses, fungi, and parasites [12]. Among the TLR family members, TLR4 recognizes the lipopolysaccharides (LPS) of Gram-negative bacteria [12]. Patients with TLR4 polymorphisms are more likely to suffer from intestinal infections, and are highly susceptible to bowel inflammation diseases like Crohn's disease and ulcerative colitis [13]. In addition, an independent association was reported between TLR4 polymorphisms and infections in renal transplant recipients [14]. It has been shown that some polymorphisms such as Asp299Gly decreased the affinity of TLR4 protein to LPS [15]. A study of 238 renal transplant recipients showed that TLR4 polymorphisms (Asp299Gly and Thr399Ile) were associated with a higher risk of severe infections [16]. Other common TLR4 polymorphisms were also associated with infections in different contexts: the rs1927914 SNP has been associated with the development of diabetic foot [17], the rs1927907 SNP has been associated with the response to tacrolimus after LT [18], the rs11536889 SNP has been associated with worst outcomes of sepsis [19], the rs1927906 SNP has been associated with susceptibility to tuberculosis [20], and the rs2149356 SNP has been associated with invasive bacterial infections [21]. Therefore, TLR4 polymorphisms might play an important role in the susceptibility to infections after LT.

Nevertheless, the genetic basis of susceptibility to infection after LT is still poorly understood. In this context, the aim of our study was to investigate the association between TLR4 gene polymorphisms and bacterial infection in Han Chinese patients within 6 months after LT. Results of this study could help identifying patients at higher risk of infection after LT. More aggressive prophylactic measures could be taken, improving their prognosis.

Materials and methods

Study population

This was a prospective cohort study of 113 consecutive LT recipients from the Shanghai Jiaotong University Affiliated First Peoples Hospital (China) between July 2007 and January 2011. Inclusion criteria were: 1) aged 18 years or older; 2) compatible blood and tissue types with donors; and 3) Han Chinese. The exclusion criterion was combined liver/kidney transplantation. In patients who underwent two LT surgeries, only the data from the first LT were included for analysis.

All patients were evaluated using the United Network for Organ Sharing (UNOS) Model for End-Stage Liver Disease (MELD) scoring system [22]. Among patients with hepatocellular carcinoma, UNOS TNM stage, histological grade, Milan criteria, tumor size, and multinodular tumor frequency were assessed. All LT were performed using cadaveric livers and orthotopic LT (OLT), with end-to-end biliary anastomosis without T-tube drainage. All livers were from willing donors.

Clinical and demographic data including perioperative demographics and clinical characteristics (age, gender, Child-Pugh score, encephalopathy grade, diabetes, dialysis, use of antibiotics, MELD score, and indication for LT), operative variables (operation time, anhepatic phase, blood loss, and transfusion), and clinical events within 6 months post-transplantation (use of prednisone, rejection, duration of initial intubation, tracheotomy, biliary complication, transfusion, intensive care unit [ICU] stay, reoperation, recovery diet time, renal function, and dialysis) were collected. In our study, there were 19 females and 94 males, with a median age of 48 years. The main reasons for LT were hepatocellular carcinoma (53.1%, n=60), followed by hepatitis B-related cirrhosis (29.2%, n=33). Among these patients, bacterial infection occurred in 44 recipients (38.9%) within 6 months after LT (Table 1): 19 of these cases (43.2%) were due to Gram-positive bacteria.
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and 25 (56.8%) to Gram-negative bacteria. The most frequent bacterial infections were pneumonia (n=21, 47.7%), followed by sepsis (n=9, 20.5%), cholangitis (n=9, 20.5%), and other infections (n=5, 11.3%) [23].

The study was approved by the Ethics Committee of the First People's Hospital affiliated to Shanghai Jiaotong University (2013KY046), and performed in accordance with the Declaration of Helsinki. Each patient provided a written informed consent, as well as the legal representative of the liver donor.

**Infection definitions and infectious prophylaxis protocol**

The presence of an infectious episode was determined by the presence of any one of the following criteria [14]: 1) a positive culture of a pathogenic microorganism in any sample for two consecutive tests (e.g., lung, common bile duct, blood, and others); 2) isolation of any microorganism from a sample obtained under sterile conditions; or 3) isolation of a potentially pathogenic microorganism in a sample from any location, accompanied with compatible symptoms of infection (e.g., chills, fever, hypotension, or characteristic CT/chest X-ray properties) within 6 months after LT. We included only those infectious events considered to be major bacterial infections: bloodstream infections, pneumonia, cholangitis, and other infections.

All patients received the same presurgical antibiotic prophylaxis one hour before surgery: 1) antianaerobic drug (metronidazole) + third-
generation cephalosporins or cephalosporins/enzyme inhibitors or penicillin/enzyme inhibitors (cefoperazone sodium/sulbactam sodium or piperacillin/tazobactam sodium); 2) preoperative bath of chlorhexidine; 3) oral wash of compound chlorhexidine gargle; and 4) ultrasonic atomization of ambroxol hydrochloride + terbutaline sulfate or ipratropium bromide aerosol. If secondary infections occurred, sensitive antibiotics were given according to the results of bacterial culture and susceptibility testing. If extended-spectrum beta-lactamase (ESBL) infection occurred, metronidazole + cefoperazone sodium/sulbactam sodium or piperacillin/tazobactam sodium or carbapenem antibiotics (imipenem-cilastin) were given. If Gram-positive bacterial infections (such as MRSA and MRSE), glycopeptide antibiotics (vancomycin or teicoplanin) were used. In the presence of fungal infections, echinocandins (caspofungin or micafungin)/triazoles (voriconazole or posaconazole) were used.

Immunosuppressive and rejection therapy

All LT patients received primary standard immunosuppressive therapy, including tacrolimus (FK506) or cyclosporine and low-dose prednisone. Acute rejection episodes were diagnosed by patients' clinical presentations, serum biochemical results, and liver biopsy. Rejection episodes were mainly treated with methylprednisolone and increasing FK506 blood concentrations. Persistent or steroid-resistant rejection was treated with antithymocyte globulin (ATG) (2.5 mg/kg/d for 7-10 days) in the absence of thrombocytopenia and leukopenia.

Polymorphisms and genotyping

Genomic DNA was extracted from EDTA-anticoagulated whole blood of the recipients using the QIAamp DNA Blood mini kit (QIAGEN, Valencia, CA, USA). Genotyping of polymorphisms was performed using a Sequenom Mass ARRAY platform according to the manufacturer's protocols (Sequenom, San Diego, CA, USA) and as previously described [24]. Five TLR4 SNPs (rs1927907, rs1927914, rs11536889, rs1927906, and rs2149356) gene were genotyped.

Follow-up

The follow-up included visits at the Outpatient Department, telephone inquiries, and hospital review. For the first 3 months, the patients were seen every weeks. For months 4-6, the patient was seen monthly. For months 7-12, the patient was seen every 2 months. The patient was seen every 2-3 months for the second year, and then lasted for years 3-5. Each visit included blood routine examination, liver and kidney function, blood drug concentration, and HBV virological indicators.

Recipients with primary malignancy disease underwent chest and abdomen CT and whole body bone scan. Immunosuppressant therapy was adjusted according to the test results.

Statistical analysis

If normally distributed, continuous data are expressed as means ± standard deviation (SD) and were compared using independent samples t-test. If non-normally distributed, data are expressed as median (Q1, Q3) and were compared using the Mann-Whitney U test for independent samples. Categorical variables are expressed as absolute count and frequencies, and were compared using the chi-square test or Fisher's exact test, as appropriate. Genotypes were analyzed for deviations from the Hardy-Weinberg equilibrium. SHEsis Online Version (http://analysis.bio-x.cn/myAnalysis.php) was used to analyze linkage disequilibrium. Risk factors for bacterial infection were evaluated using multivariate logistic regression analysis with the stepwise forward method. First, univariate analysis of all variables was performed. Variables with P<0.05 were subsequently used in the multivariate analysis. Cumulative hazard for infection incidence after liver transplantation within 6 months was analyzed using the Kaplan-Meier method and the log-rank test. The Bonferroni method was used to correct for multiple comparisons when applicable. SPSS 19.0 (IBM, Armonk, NY, USA) was used for analysis. Two-tailed P-values <0.05 were considered statistically significant.

Results

Association analyses of TLR4 polymorphisms with susceptibility to bacterial infection

Five TLR4 SNPs (rs1927907, rs1927914, rs11536889, rs1927906, and rs2149356)
Table 2. Single-nucleotide polymorphisms

<table>
<thead>
<tr>
<th>SNP</th>
<th>SNP type</th>
<th>Major/minor allele</th>
<th>MAF</th>
<th>HWE P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1927907</td>
<td>Intron</td>
<td>G/A</td>
<td>0.30</td>
<td>0.70</td>
</tr>
<tr>
<td>rs1927914</td>
<td>Upstream variant 2 KB</td>
<td>T/C</td>
<td>0.46</td>
<td>0.54</td>
</tr>
<tr>
<td>rs11536889</td>
<td>3'utr</td>
<td>G/C</td>
<td>0.20</td>
<td>0.80</td>
</tr>
<tr>
<td>rs1927906</td>
<td>Downstream variant 500</td>
<td>A/G</td>
<td>0.08</td>
<td>0.92</td>
</tr>
<tr>
<td>rs2149356</td>
<td>Intron</td>
<td>C/A</td>
<td>0.45</td>
<td>0.55</td>
</tr>
</tbody>
</table>

MAF: minor allele frequency; HWE: Hardy-Weinberg equilibrium.

Table 3. Genotype and allele distributions of the five single-nucleotide polymorphisms in TLR4 between the infection and control groups

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype and Allele</th>
<th>Infection (n=44)</th>
<th>Control (n=69)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1927907</td>
<td>A/A</td>
<td>9 (22.0)</td>
<td>2 (3.1)</td>
<td>0.008**</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>14 (34.1)</td>
<td>27 (41.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>18 (43.9)</td>
<td>36 (55.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>32 (39.0)</td>
<td>31 (23.8)</td>
<td>0.019*</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>50 (61.0)</td>
<td>99 (76.2)</td>
<td></td>
</tr>
<tr>
<td>rs1927914</td>
<td>C/C</td>
<td>16 (37.2)</td>
<td>9 (13.0)</td>
<td>0.009**</td>
</tr>
<tr>
<td></td>
<td>C/T</td>
<td>18 (41.9)</td>
<td>35 (50.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T/T</td>
<td>9 (20.9)</td>
<td>25 (36.2)</td>
<td>0.004**</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>50 (58.1)</td>
<td>53 (38.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>36 (41.9)</td>
<td>85 (61.6)</td>
<td></td>
</tr>
<tr>
<td>rs11536889</td>
<td>C/C</td>
<td>2 (4.7)</td>
<td>2 (2.9)</td>
<td>0.032*</td>
</tr>
<tr>
<td></td>
<td>C/G</td>
<td>8 (18.6)</td>
<td>28 (41.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>33 (76.7)</td>
<td>38 (55.9)</td>
<td>0.063</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>12 (14.0)</td>
<td>33 (24.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>74 (86.0)</td>
<td>103 (75.7)</td>
<td></td>
</tr>
<tr>
<td>rs1927906</td>
<td>A/A</td>
<td>35 (81.4)</td>
<td>56 (87.5)</td>
<td>0.469</td>
</tr>
<tr>
<td></td>
<td>A/G</td>
<td>7 (16.3)</td>
<td>8 (12.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>G/G</td>
<td>1 (2.3)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>77 (89.5)</td>
<td>120 (93.8)</td>
<td>0.264</td>
</tr>
<tr>
<td></td>
<td>G</td>
<td>9 (10.5)</td>
<td>8 (6.2)</td>
<td></td>
</tr>
<tr>
<td>rs2149356</td>
<td>A/A</td>
<td>16 (37.2)</td>
<td>9 (13.8)</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td></td>
<td>A/C</td>
<td>17 (39.5)</td>
<td>30 (46.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C/C</td>
<td>10 (23.3)</td>
<td>26 (40.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>49 (57.0)</td>
<td>47 (36.2)</td>
<td>0.003**</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>37 (43.0)</td>
<td>83 (63.8)</td>
<td></td>
</tr>
</tbody>
</table>

Data are shown as number (percentage) of subjects. The P value was determined using the Chi-square test or Fisher exact test, as appropriate. OR: odds ratio; CI: confidence interval. *P<0.05; **P<0.01; ***P<0.001, infection group vs. control group.

weren't identified. All of the genotype distributions were consistent with the Hardy-Weinberg equilibrium (P>0.05) (Table 2).

Table 3 presents the distribution of the genotypes and alleles between the two groups.

Compared with controls, the infection group had a higher frequency of the A allele of rs1927907 (39.0% vs. 23.8%, P=0.019), as well as a higher frequency of the A/A genotype (22.0% vs. 3.1%, P=0.008). The infection group had a higher frequency of the C allele of rs1927914 (58.1% vs. 38.4%, P=0.004), as well as a higher frequency of the C/C genotype (37.2% vs. 13.0%, P=0.009). The infection group had a higher frequency of the G/G genotype of rs11536889 (76.7% vs. 55.9%, P=0.032). The infection group had a higher frequency of the A allele of rs2149356 (57.0% vs. 36.2%, P=0.003), as well as a higher frequency of the A/A genotype (37.2% vs. 13.8%, P<0.001). There was no difference in the allele distribution or genotype of rs1927906 between the two groups.

Risk factors of bacterial infection: multivariate logistic regression analysis

Several clinical variables and genetic factors were considered to be potential risk factors of bacterial infection by univariate analysis. In the multivariate logistic regression model, bacterial infection was significantly associated with two factors: post-operation endotracheal intubation time ≥72 h (P=0.040, OR=2.84, 95% CI 1.05-7.70) and rs2149356 (AA vs. AC/CC, P=0.003, OR=4.24, 95% CI 1.65-10.93) (Table 4).

Rs2149356 and bacterial infection: Kaplan-Meier survival curves analysis

The genotype distribution of rs2149356 for the development of bacterial infection was evaluated using Kaplan-Meier estimates and the log-rank test. Infection occurred significantly earlier among patients carrying the genotype AA compared with those carrying the AC and CC.
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Table 4. Multivariate logistic regression analysis of risk factors associated with bacterial infections within 6 months after LT

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endotracheal intubation time ≥72 h (1= endotracheal intubation time ≥72h, 0= endotracheal intubation time &lt;72h)</td>
<td>2.84 (1.05-7.70)</td>
<td>0.040</td>
</tr>
<tr>
<td>rs2149356 (1=AA, 0=AC/CC)</td>
<td>4.24 (1.65-10.93)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Variables with P=0.05 were subsequently used in the multivariate analysis. LT, liver transplant; OR: Odds ratio; CI: Confidence interval.

Figure 1. Kaplan-Meier curves of the infection incidence after liver transplantation within 6 months according to TLR4 rs2149356 genotypes (A), log-rank: P=0.024. The Bonferroni method was used to correct for multiple comparisons. AA vs. CC, P=0.011 (<0.05/3=0.017); AA vs. AC, P=0.012 (<0.017); CC vs. AC, P=0.059 (>0.017). (B) Polymorphism CC + AC and AA, log-rank: P=0.002.

Discussion

Infectious diseases are associated with morbidity and mortality after LT. The risk of bacterial infection after transplantation of a solid organ is the result of many factors [1, 2, 5-7]. Moreover, polymorphisms of recipient and/or donor organ may affect the innate immune system, which likely plays a crucial role in increasing the risk of infections [9-11].

In the present study, the association between recipient’s genotype polymorphisms of TLR4, a critical component of the innate immune system, and susceptibility to infection in patients after LT was assessed. Among the patients, 44 patients (38.9%) had bacterial infections within 6 months after LT. Of these, 19 and 25 patients were infected with Gram-positive and Gram-negative bacteria, respectively. Multivariate analysis revealed that the duration of endotracheal intubation and the rs2149356 SNP were independent risk factors for bacterial infection after LT, in agreement with previous reports [21, 23, 25]. Patients harboring the TLR4 rs2149356 variant also showed a higher incidence of infection within 6 months after LT.

Multiple studies showed an association between TLRs gene polymorphisms and susceptibility to infectious diseases [9-11, 26]. Recently, studies have reported that TLRs SNPs can increase susceptibility to infection in severely injured trauma patients [27] and Gram-positive infection in sepsis [28]. In addition to binding to endogenous ligands released from damaged tissues and exogenous ligands such as LPS, TLR4 also induces the proinflammatory response to Gram-negative bacteria [12]. There is evidence that TLR4 SNPs increase the risk of Gram-negative bacterial infections in patients.
Indeed, studies have demonstrated that TLR4 D299G and T399I SNPs confer increased risk to infection, as assessed by plasma LPS-binding protein, C-reactive protein, and white blood cell count [15]. The present study revealed that the TLR4 rs1927907, rs1927914, and rs2149356 SNPs had an influence on bacterial infection after LT; furthermore, the rs2149356 SNP was shown to be an independent risk factor for infection after LT. Previous studies have reported that the TLR4 rs2149356 SNP is associated with higher risk for sepsis in preterm infants, prostate cancer, normal tension glaucoma, and gouty arthritis [29]. In addition, the TLR4 rs2149356 SNP plays an important role in susceptibility to some inflammatory diseases, such as rheumatoid arthritis, Crohn’s disease, and ulcerative colitis [13].

Nevertheless, few studies have investigated the association between the TLR4 rs2149356 polymorphism and susceptibility to infection after transplantation, especially in LT. In this study, despite an association with the incidence of infection, there was no difference in long-term survival. In contrast, Lee et al. [30] showed that there was no significant association between the TLR4 SNPs D299G and T399I, and the risk and outcomes of Gram-negative bacterial infections, while a previous study of 50 genetic variants showed no association between TLR4 polymorphisms and susceptibility to bacterial and fungal infections after LT [31]. Nevertheless, previous studies also showed that TLR4 polymorphisms are associated with susceptibility to LPS in animals and humans [32-34]. It should be mentioned that other TLR4 polymorphisms have been widely implicated in infections and other diseases, but not in the context of LT; these include Asp299-Gly (rs4986790) and Thr399Ile (rs4986791), which are associated with viral and Gram-negative bacterial infections [35, 36].

The multivariate analysis showed that the duration of endotracheal intubation and the rs2149356 SNP were independent risk factors for bacterial infection after LT. Indeed, endotracheal intubation is a well-known risk factor for pneumonia, especially in patients with a compromised immune system [25, 37]. On the other hand, the role of the rs2149356 SNP in infections is less well known. A previous study showed that the rs2149356 SNP was associated with the development of invasive bacte-

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