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Association of TLR4 polymorphisms and polyomavirus BK infection in liver transplant patients

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Abstract. Toll-like receptors (TLRs) may have a role in orchestrating the immune responses against polyomavirus BKV and also may influence liver transplant outcomes. However, the clinical relevance of this experimental observation has not been examined. Improving knowledge regarding details of genetic source of TLR polymorphisms can promote new therapeutic strategies to inhibit virus related clinical disorders in post-liver transplantation. Therefore, the Asp299Gly and Thr399Ile TLR4 polymorphisms were evaluated in liver transplanted patients with and without polyomavirus BK infection. In a cross sectional study, 144 liver transplant patients received allograft at Transplant Center of Namazi Hospital affiliated to Shiraz University of Medical Sciences who were recruited between years: 2014-2015. Patients were followed for the graft outcome and acute rejection episode(s) and divided into two groups based on experiencing acute rejection or not. The genomic DNA of polyomavirus BK was diagnosed in studied patient using qualitative nested- PCR technique. Analysis of TLR4 gene polymorphisms were analyzed using PCR-RFLP protocols. The polyomavirus BK infection was found in 15 of 144 (10.4%) liver transplanted patients. A total of 14 of 15 (93.3%) and all of polyomavirus BK infected patients have been shown to be homozygous wild type AA genotype of TLR4-Asp299Gly (A896G) and CC genotype of TLR4-Thr399Ile (C1196T) polymorphisms. Homozygous mutant GG genotype of Asp299Gly (A896G) was found in 3 (2.1%) of the studied patients. Homozygous mutated TT genotype of Thr399Ile (C1196T) was found in only 5 (3.5%) of the liver recipients. There were no significant differences between homozygous wild type genotypes of studied TLR4 SNPs for liver transplant patients with or without polyomavirus BK infections. Significant association was also not found between homozygous mutated genotype of TLR4 SNPs for patients experiencing rejection episodes. However further completed studies on larger population and with longer follow-up are needed to confirm these results.

INTRODUCTION

Human polyomavirus BK is a viral infection ubiquitous in the general population and nearly 90.0% of adults are infected with the latent form of this virus (Bruggeman, 2007). The recurrence of polyomavirus BK infection is observed during immunosuppression early and late post-transplantation (Enache *et al*., 2012). Reactivation of polyomavirus BK may occur in 1–10.0% of the transplanted patients especially kidney recipients (Pahari & Rees, 2003). The clinical importance of polyomavirus BK was also not completely clear in liver transplant patients. Improving knowledge about the key parameters of innate immunity may have role on polyomavirus BK pathogenesis and reactivation is interesting. Innate immunity plays critical role in suppression and eradication of viruses. Cellular receptors in innate immunity participates significantly in defense against viral infections (Karimi-Googheri & Arababadi, 2014). Toll-like receptors (TLRs) are class of proteins of innate immunity system that play a determinative role in

detection of viral pathogens and liver transplant outcomes by their ability to bind pathogen-associated molecular patterns (PAMP) (Lechner *et al.*, 2000; Beutler, 2004; Mahla *et al.*, 2013). TLRs consist of 13 subclasses though the latter two are not found in humans (Mahla *et al.*, 2013). TLRs are expressed on the membrane and mark the key molecular events leading to innate immune responses and the development of antigen-specific acquired immunity (Medzhitov *et al.*, 1997; Takeda & Akira, 2005; Delneste *et al.*, 2007). It is believed that TLRs participate in orchestrating the innate and also adaptive immune responses against polyomavirus BKV although this is not well understood. However, the clinical relevance of this observation has not been examined in substantial studies.

Previous investigation have evaluated the roles of TLR3 against polyomavirus BK reactivation and involved in BK virus associated nephropathy (BKVAN) (Heutinck *et al.*, 2012a; 2012b; Ribeiro *et al.*, 2012). Activation of TLR3 up-regulate NF-kappaB and AP-1 (Karimi-Googheri & Arababadi, 2014). Polyomavirus BK also has binding motifs for NF-kappaB and Activator protein-1 (AP-1) in the promoter/enhancer region, which is induced by virus early genes to start lytic infection (Chakraborty & Das, 1989; 1991; Gorrill & Khalili, 2005).

Differences in genetic background of TLRs may play role in the determination of polyomavirus BK outcome in transplant patients. Therefore, improving knowledge regarding the details of genetic source TLR polymorphisms can promote new therapeutic strategies to inhibit virus related clinical disorders post-transplantation (Karimi-Googheri & Arababadi, 2014). On the other hand, Single nucleotide polymorphisms (SNPs) in TLRs can damage immune responses to respective ligands. These genetic variations in the TLR system may affect the incidence of acute rejection after liver transplantation (Dhillon *et al.*, 2010; Citores *et al.*, 2011).

Among the TLRs, TLR4 that is located on chromosome 9q32-q33, contains 4 exons, and is highly expressed in lymphocytes, monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, and splenocytes (Anders *et al.*, 2004; Ducloux *et al.*, 2005). TLR4 may be involved in multiple etiologies of liver injury, inflammation, and fibrogenesis and allograft rejection (Dhillon *et al.*, 2010; Citores *et al.*, 2011). TLR4 polymorphisms at Asp299Gly and Thr399Ile loci may affect TLR4 function by impairing transport of the receptor to the cell membrane. Earlier reports that studied TLR4 gene polymorphisms in kidney transplant patients (Ducloux *et al.*, 2005; Nogueira *et al.*, 2007; Mutlubas *et al.*, 2009; Kruger *et al.*, 2010; Krichen *et al.*, 2013; Dessing *et al.*, 2015) and liver (Dhillon *et al.*, 2010; Citores *et al.*, 2011; Oetting *et al.*, 2012; Citores *et al.*, 2016) emphasized on controversial role of mutant versus wildtype SNPs of TLR4 on allograft outcomes and patient surveillance. Based on this not well defined evidence of a role for TLR4 genetic variation in acute liver outcomes, allograft surveillance study was conducted by evaluating the relationship between for the Asp299Gly and Thr399Ile TLR4 polymorphisms in liver transplanted patients with and without polyomavirus BK infections.

PATIENTS AND METHODS

A total of 144 liver transplant patients received allograft at Transplant Center of Namazi Hospital affiliated to Shiraz University of Medical Sciences were recruited between years: 2014-2015. EDTA treated blood samples were collected from each studied liver recipients. Patients were followed up for the liver graft outcomes in two groups based on whether or not they have experienced acute rejection (Karimi *et al.*, 2014). Ethical approval was given by Shiraz University of Medical Sciences in accordance with Helsinki Declaration guidelines and informed consent signed with all studied patients. The immunosuppressive conditioning regimen included tacrolimus or cyclosporine with mycophenolate mofetil and steroids were used in liver transplanted recipients. Human leukocyte antigen (HLA) matching is not done for liver transplanted recipients in this center (Karimi *et al.*, 2014;

Zare *et al.*, 2016). The prevalence of underlying diseases of liver transplant patients was shown in Table 1. The presence of polyomavirus BK, genomic DNA was evaluated using qualitative nested- PCR technique. TLR4 gene polymorphisms were also analyzed using PCR-RFLP protocols.

This study was suffered from limitation about low prevalence of polyomavirus Bk infection in liver transplant patients that we only found 15 patients with history of BK infection during two years.

TLR4 gene polymorphisms

Total genomic DNA was extracted from Buffy coat isolated from blood samples using DNP Kit (CinnaGene, Iran) according to the manufacturer's instruction. The TLR4 gene polymorphisms included: Asp299Gly (A896G) with rs4986790 and Thr399Ile (C1196T) with rs4986791 were evaluated after optimization of PCR-RFLP protocols (Table 2). The 25 µL reaction mixture containing: 1 µL genomic DNA solution, GeneAmp Gold Buffer (15 mmol/L Tris-HCl, pH 8.0, 50 mmol/L KCl; PE Applied Biosystems), (4.0 mmol/L $MgCl_2$ for Asp299Gly and 3 mmol/L for Thr399Ile), 50 mol/L each of the dGTP, dATP, dTTP, and dCTP (Promega, Germany), 25 pmol each forward and reverse primers and 1.0 U AmpliTaq Gold polymerase. The PCR programs consist of: Hot start at 95°C for 5 minutes. Followed by 35 amplification cycles at 95°C for 60 seconds; 56°C for 60 seconds (Asp299Gly) or 60°C (Thr399Ile) and 72°C for 60 seconds, followed by one elongation step at 72°C for 5 minute were rune. Then a mix of 4 µL PCR product, 1 µL 10 × restriction enzyme, and buffer No. 2 were incubated overnight at 37°C and performing electrophoresis in a 3% agarose gel.

Polyomavirus BK analysis

Polyomavirus BK genomic DNA was extracted from plasma samples using Invisorb Spin Virus DNA Blood Mini Kit, (STRATEC Biomedical AG, Birkenfeld, Germany) according to the manufacturer instruction. Presentation of polyomavirus BK genomic DNA was analyzed in liver transplant patients using a nested-PCR protocol, as previously described (Emami *et al.*, 2017).

Other viral infections

Patients with a history of other viral infections including: human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) were

Table 1. The prevalence of the underlying diseases of liver transplanted patients

Underlying disease	Number	Percent
Cryptogenic + Alcoholic & NASH	37	25.7
Viral hepatitis (HBV + HCV)	34	23.6
Autoimmune hepatitis	15	10.4
PBS+PSC	25	17.4
Congenital (Tyrosinemia + Biliary atresia + Hypercholesterolemia + Hyperoxaluria + Wilson)	33	22.9

Table 2. The Primers, product size, thermo cycling condition, and PCR and restriction enzyme mixes

Locus	Primers	Restriction Enzymes	Product size
Asp299Gly (A896G) rs4986790	F:GATTAGCATACTTAGACTACTACCTCGA R:GATCAACTTCTGAAAAAGCATTCCCACC	NcoI	wild-type: 249 bp Mutant type: 23 bp
Thr399Ile (C1196T) rs4986791 (C1196T)	F:GGTTGCTGTTCTCAAAGTGATTTTGGGAA R:ACCTGAAGACTGGAGAGTGAGTTAAATGCT	Hinf I	wild-type: 406 bp Mutant type: 29 bp

excluded. Patients with active cytomegalovirus infection detected at post-transplantation using the CMV Brite Turbo kit (IQ Products, Groningen, The Netherlands) as described previously (Zare *et al.*, 2016; Emami *et al.*, 2017).

Statistical analysis

Allele and genotype frequencies for TLR4 genetic polymorphisms were calculated in the studied patients using direct gene counting protocol. Statistical Package for the Social Sciences (SPSS), version 16 was used for analysis of frequencies and comparison of the TLR4 alleles and genotypes between two studied liver transplant groups. Chi-square test and Fisher's exact test. Odds ratios and 95% confidence intervals (CIs) for relative risks were calculated. A $P<0.05$ was considered as statistically significant.

RESULTS

Patient profile

The 90 of 144 (62.5%) liver transplant patients were male and rest of them (54 of 144; 37.5%) were female. Male to female ratio (M/F) of patients was 1.6 (90/54). Each patient group was consisting of 72 reject and 72 non-rejected liver transplant recipients. The age range of patients was 2–65 with mean of 36.6±17.43 years old. The most prevalent age group in transplanted patients were 20–30 (20%) and 50–60 (21%) years old. The mean age of the patients who experienced and not experienced rejection were 35.4±2.1 and 37.8±2.09, respectively. The mean age of the patients was 36.66±1.4. The most frequent blood group in liver transplanted recipients was O^+.

Polyomavirus Bk infection and TLR4 polymorphism

The polyomavirus Bk infection was found in 15 of 144 (10.4%) total liver transplanted patients. 9 out of the 15 (60%) patients were male. The 9 of 15 (60%) patients with viral infections experienced at list one episode of rejection. The frequency of Thr399Ile (C1196T) and Asp299Gly (A896G) TLR4 genotype polymorphisms in polyomavirus BK infected versus non-infected liver transplanted patients is presented in Table 3.

The 14 of 15 (93.3%) polyomavirus BK infected patients have been shown homozygous wild type AA genotype of TLR4-Asp299Gly (A896G) gene. Only one polyomavirus BK infected liver transplant recipient who happened to be female and experiencing an allograft rejection had a mutation in the GG genotype. All 15 polyomavirus BK infected patients have been shown to be homozygous with wild type CC genotype of TLR4-Thr399Ile (C1196T) polymorphisms. Co-segregation of wild type

Table 3. Genotype distribution of TLR-4 (Thr399Ile and Asp299Gly) polymorphisms in polyomavirus BK infected versus non-infected liver transplanted patients

TLR Gene Polymorphisms	Polyomavirus BK Infection				
	GG	AG	AA	A allele	G allele
Asp299GlyA896G	F1=1(6.7) F2=2(1.6) p=0.18, OR=4.54, 95% CI=0.0–7.9	F1=0.0 F2=19(14.7) p=0.11, OR=0.0, 95% CI=0.0–2.14	F1=14 (93.3) F2=108(83.7) p=0.32, OR=2.72, 95% CI=0.34–58.3	F1=28 F2=235 p=0.67, OR=0.73, 95% CI=0.11–3.46	F1=2 F2=23 p=0.67, OR=0.73, 95% CI=0.11–3.46
	TT	CT	CC	T allele	C allele
Thr399IleC1196T	F1=0.0 F2=5(3.9) p=0.43, OR=0.0, 95% CI=0.0–10.58	F1=0.0 F2=12(9.3) p=0.21, OR=0.0, 95% CI=0.0–0.75	F1=15(100) F2=112(86.8) p=0.13, un	F1=0 F2=22 p=0.09, un	F1=30 F2=236 p=0.09, un

F1 = BK positive; F2 = BK negative.

homozygous CC and AA genotypes of Thr399Ile (C1196T) and Asp299Gly (A896G) were found to be predominant in 14 out of 15 (93.3%) polyomavirus BK infected liver transplant patients.

TLR4 genotype distribution and importance

The frequency of different genotypes of Asp299Gly (A896G) was also as follow: homozygous wild type AA (122 of 144; 84.2%), AG (19 of 144; 13.2%), and mutant GG (3 of 144; 2.1%). The 1 of 3 studied patients have been shown mutated GG genotype experienced graft rejection and 1 of these patients was male. The 10 of 19 (52.6%) patients who showed heterozygous AG genotype experienced graft rejection and 13 (68.4%) of these patients were male.

The frequency of Thr399Ile (C1196T) different genotypes was as follow: homozygous wild type CC (127 of 144; 88.2%), heterozygous CT (12 of 144; 8.3%), and homozygous mutated TT (5 of 144; 3.5%). The 2 of 5 liver recipients have been shown mutant TT genotype experienced acute rejection that 2 of them were male. The 5 of 12 (41.6%) patients with heterozygous CT genotype was experienced graft rejection that 8 (66.7%) of them were male.

Significant association was only found between age and Thr399Ile (C1196T) and Asp299Gly (A896G) TLR4 genotype polymorphisms (p=0.013 and p=0.001, respectively). But no significant associations were found between other risk factors with studied TLR4 gene polymorphisms. No significant association was found between TLR4 gene polymorphisms and experiencing or not-experiencing rejection.

DISCUSSION

TLRs can induce inflammatory responses as pattern recognition receptor (PRR) activated by PAMPs and damage-associated molecular patterns (DAMPs) (Gluba *et al.*, 2010; Leemans *et al.*, 2014). TLRs are expressed by different liver cells including: kupffer cells, hepatocytes, hepatic stellate cells, biliary epithelial cells, sinusoidal endothelial cells, and dendritic cells (Schwabe *et al.*, 2006; Yohe *et al.*, 2006). Form TLRs, TLR4 is sentinel immune sensors throw viral infections and liver disorders especially ischemic reperfusion injury and fibrosis (Lechner *et al.*, 2000; Schwabe *et al.*, 2006). The expression level of TLR4 is low in normal liver, but after liver disease or cirrhosis, expression of TLR4 increases leading to acute allograft rejection in liver allografts (Zarember & Godowski, 2002; Deng *et al.*, 2007; Ellett *et al.*, 2009; Pimentel-Nunes *et al.*, 2010; Hei *et al.*, 2010; Dhillon *et al.*, 2010; Soares *et al.*, 2010; Testro *et al.*, 2011; Berzsenyi *et al.*, 2011). Polymorphisms in TLRs impair intracellular signaling pathways and associated with blunted immune responses to microbial pathogens (Ducloux *et al.*, 2005; Mutlubas *et al.*, 2009). There are conflicting data on the role of TLR4 and other TLR signaling sequence variants on post liver transplant outcomes that might be explained by the variety in the patient databases. In addition, studies vary in their definition of study endpoints or studies use only one single endpoint liver (Oetting *et al.*, 2012; Citores *et al.*, 2016). Relatively rare studies have also investigated the contribution of TLR regulatory outcomes on polyomavirus BK infection (Eid *et al.*, 2007). No report also exists about the role of polymorphisms in TLR-related genes and their association with polyomavirus Bk infection in transplant patients. Therefore, in this study the impact of TLR4 gene polymorphisms in the context of polyomavirus BK infection and liver transplant outcomes was evaluated.

The Thr399Ile (C1196T) and Asp299Gly (A896G) TLR4 SNPs that have been shown to attenuate TNF-α secretion had determinative role in liver transplant outcomes and more occurrences of opportunistic infections (Dhillon *et al.*, 2010; Oetting *et al.*, 2012). TLR4 progresses hepatic fibrosis by enhancement of TGF beta production in hepatic stellate cells (Citores *et al.*, 2011). Reconstitution of hepatic stellate cells with Thr399Ile (C1196T) and/or Asp299Gly (A896G) cDNA showed reduction in TLR4-mediated inflammation and fibrogenesis.

Therefore, lower frequency of TLR4 Asp299Gly mutated genotype was found in recipients compared with donors (Dhillon *et al*., 2010). Recipients with liver disease had a lower frequency of mutated variant of TLR4 Asp299Gly genotype compared to controls. TLR4 mutations in the donor liver conveyed a higher rate of immediate graft function. TLR4 mutated genotype of Asp299Gly (A896G) in the donor of livers also reducing the rate of post-ischemic acute liver injury in transplanted patients (Oetting *et al*., 2012). However, earlier reports on functional variants of TLR4 SNPs (Thr399Ile and Asp299Gly) showed no association with graft failure in liver transplantation. This result may relate to minimal contribution of these SNPs to graft failure or no association with the donor genotype. On the other hand, minor allele of other TLR4 SNPs including; rs11536856, rs5030717, and rs913930, were shown in deceased donors that they were significantly associated with liver failure. This association may be related to an increase TLR4 expression or mRNA instability resulting in organ damage and eventual graft loss.

Similar to earlier reports, the homozygous wild type AA genotype was found in 84.2% in studied liver transplant patients. Only one of 3 studied patients with mutated GG genotype experienced graft rejection.

Reports also exist about contraction between viruses with TLR4 polymorphisms. Co-segregation of Thr399Ile (C1196T) and Asp299Gly (A896G) in chronic HCV infected liver transplant patients to be protective against fibrosis progression after reducing TLR4-mediated inflammatory and fibrogenic signaling. However, Eid *et al.* (2007) found no association between TLR4 polymorphisms and adverse outcomes in HCV infected transplant patients with liver cirrhosis. HCV infected liver recipients and TLR4 mutated recipients also had worse graft survival. The TLR4 SNP (rs10759930) was associated with HCV infection in liver donors with unknown importance (Wu *et al*., 2004; Eid *et al*., 2007). TLRs also involved in CMV recognition. TLR ligand induced activation of dendritic cells and enhanced CMV antigen presentation. Patients with decreased TLR4 function might have an impaired cellular response to CMV (Ducloux *et al*., 2005). In this study, homozygous wild type AA genotype of Asp299Gly and CC genotype of Thr399Ile TLR4 gene polymorphisms were found in 93.3% and all of polyomavirus BK infected patients, respectively. The polyomavirus Bk infection was found in 15 of 144 (10.4%) total liver transplanted patients. The 60% of viral infected patients experienced at list one episode of rejection. The 14 of 15 (93.3%) polyomavirus BK infected patients have been shown homozygous wild type AA genotype of TLR4-Asp299Gly (A896G) gene. Only one polyomavirus BK infected liver transplant recipient was showed mutated GG genotype that was female and experienced allograft rejection. All 15 polyomavirus BK infected patients have been shown homozygous wild type CC genotype of TLR4-Thr399Ile (C1196T) polymorphisms. No significant difference was also found between experiencing or not polyomavirus BK infection and distribution of Asp299Gly and Thr399Ile TLR4 SNPs.

The findings suggest that homozygous wild type genotypes of both Asp299Gly and Thr399Ile of TLR4 SNPs prevalent in liver transplant patients. However, no significant differences were observed in patients with or without polyomavirus BK infections. Significant associations were also not found between homozygous mutated genotype of Asp299Gly and Thr399Ile TLR4 SNPs with liver transplant rejection episodes. These results may relate to the use of an intensive immunosuppressive conditioning regimen that limits the potential regulatory role of TLR4 on liver graft outcome. However further studies are needed on larger population groups and with longer follow-up to confirm these results.

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