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Clinical Significance of the Single Nucleotide Polymorphism TLR2 R753Q in Heart Transplant Recipients at Risk for Cytomegalovirus Disease

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Abstract

Background—The Toll-like receptor 2 (TLR2) is a significant component of innate immunity against cytomegalovirus (CMV) infection but information on the clinical significance of the most common single nucleotide polymorphism (SNP) (R753Q) is conflicting.

Objectives—The inconsistent observations of the immunological and clinical significance of the TLR2 R753Q polymorphism for CMV infection indicates the influence of confounders.

Study design—The presence of the TLR2 polymorphism was determined by a genotyping assay of 175 HTX patients and 281 healthy blood donors and evaluated in relation to selected virological and clinical parameters.

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All authors declare no conflict of interest.

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The study was performed in accordance with the Declaration of Helsinki and good clinical practice guidelines and was approved by the local ethics committee (EK2179/2013).

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Results—Relative frequency of TLR2 polymorphism was similar in HTX patients and blood donors (homozygous wild-type, 94.3% vs. 94.0%; heterozygous, 5.1% vs. 5.7%; homozygous mutated, <1%). CMV viremia was detectable in 108 (61.7%) of HTX patients. The TLR2 polymorphism was neither associated with occurrence or level of CMV infection nor with survival, graft failure or rejection, or CMV serostatus of patient before transplantation. Nevertheless, CMV viremia occurred in 83.1% of R+/D+, 77.1% of R+/D-, and 64.3% of R-/D+ patients. Time of first CMV viremia was in R-/D+ patients later than in CMV-seropositive patients (median, 182 days versus 23 days; $P<0.001$) corresponding to the duration of antiviral prophylaxis in R-/D+ patients.

Conclusions—The TLR2 R753Q polymorphism is extremely rare in the general population and HTX patients. Screening for this risk factor of CMV disease may not be cost-effective in contrast to testing for CMV viremia.

Keywords

TLR2 polymorphism; heart transplantation; CMV infection

Background

Innate immunity plays an important role in the prevention and control of opportunistic pathogens in solid-organ transplantation. Cytomegalovirus (CMV) is one of the most significant viral opportunistic pathogens in solid-organ and bone-marrow transplantation [1]. The innate immune response to CMV is initiated by recognition of the viral envelope glycoproteins B (gB) and H (gH) by TLR2. This TLR2-CMV interaction initiates a cascade of intracellular signaling events that lead to the production of antiviral peptides and cytokines and ultimately to the generation of protective adaptive immunity [2]. Single-nucleotide polymorphism (SNPs) in genes coding for TLRs may reduce the recognition capacity of receptors by changes to their structure. For example, the substitution of arginine for glutamine in position 753 of TLR2 (R753Q) (rs5743708) was associated in several studies with a predisposition to several infections and diseases [3–8].

Recently, homozygosity for the TLR2 R753Q polymorphism was identified as significant risk factor for CMV replication and disease in liver transplant recipients with chronic hepatitis C virus infection [9,10]. In contrast, the TLR R753Q polymorphism was not associated with an increased risk for CMV replication and disease in kidney transplant recipients [11]. Moreover, Jablonska et al. observed a significantly lower frequency of heterozygosity for the TLR2 R753Q polymorphism in immunocompetent patients with primary CMV infection when compared to non-infected individuals [12]. This observation may even suggest a protective effect of the TLR2 R753Q polymorphism against CMV infection.

Objectives

The conflicting observations of the immunological and clinical significance of the TLR2 R753Q polymorphism for CMV infection indicates the influence of confounders. To evaluate the significance of the TLR2 R753Q polymorphism together with other clinical and

laboratory characteristics of solid-organ transplant patients, we evaluated a well-characterized cohort of heart transplant recipients (HTX) in relation to occurrence of CMV infection.

Study design

Study population

The present study was based on an ongoing observational, cross-sectional study of HTX patients with stratification for CMV status of organ donor and recipient (Medical University of Vienna Biobank Study). So far, 320 patients have been included in this study from 2009 to 2014. For the present study, only consecutive patients with archived leukocyte preparations available for DNA isolation were included (n=175). Patients included in the present study received a heart transplant between 1991 and 2014 and were clinically followed for a mean period of 8.2 years (SD, 6.0 years). As controls, healthy blood donors were included at the Austrian Red Cross (n=281).

Clinical investigation

Key clinical data on patient characteristics were available through a prospectively maintained data-base which includes patient demographics, donor and recipient CMV serostatus, HLA status and episodes of organ rejection. To correlate genetic and virological data with clinical outcome, additional clinical information was assessed retrospectively by review of charts and internal documentation. As prophylaxis for CMV infection, patients received 100 mg CMV hyperimmunoglobulin intravenously per week for 4 weeks. High risk patients (Donor (D)+ /Recipient (R)-) received additionally Ganciclovir/Valganciclovir for 90 days. All patients received a triple-immunosuppressant therapy immediately after transplantation which included a proliferation-inhibitor, corticosteroids and a calcineurin-inhibitor. As prophylactic treatment against *Pneumocystis jiroveci* pneumonia, patients received sulfamethoxazole- trimethoprim combination treatment three times a week for 6 months. Tissue-invasive CMV disease was defined according international guidelines and as the presence of clinical signs and symptoms of tissue involvement [13].

Virological investigation

Assessment of the CMV serostatus was performed on the Diasorin Liaison system (Diasorin S.p.A., Saluggia (VC), Italy) using the Liaison CMV IgG II assay for the detection of CMV specific IgG antibodies and the Liaison CMV IgM II assay for the detection of CMV specific IgM antibodies. CMV DNA viral load in human plasma samples was screened in regular intervals in the first six months on the Abbott m2000sp/m2000rt real-time PCR platform (Abbott Laboratories, Illinois, U.S.A) using the CE/IVD approved Abbott Real-Time CMV assay for the quantification of CMV DNA. All assays were performed according to the manufacturer's manual.

Toll-like receptor polymorphism

DNA was isolated from leukocyte preparations of HTX patients with use of E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tech Inc., Norcross, GA, USA) and from healthy blood donors with the QIAamp 96 DNA Blood Kit (QIAGEN, Hilden, Germany) following

manufacturers' instructions. Detection of the single-nucleotide TLR2 R753Q polymorphism was performed with a TaqMan SNP Genotyping Assay that was based on two allele-specific TaqMan probes with distinct fluorescent dyes (FAM-AAGCTCGGAAGAT-NFQ-MGB for the wild-type genotype and VIC-AAGCTGCAGAAGATA-NFQ-MGB for the SNP-genotype). Upon amplification of the polymerase the probe that has annealed to the template is degraded. During the degradation the fluorophore is released and fluorescence can be detected by the cycler. Reaction mixes included 250nM of each TaqMan probe, 150nM of primers (TLR2 forward: 5'-CATCCCCAGCGCTTCTG-3' and TLR 2 reverse: 5'-TCCAGGTAGGTCTTGGTGTTCA-3'), iTaq Universal Probes Supermix (BioRAD, Hercules, USA). Cycles were run under standard conditions. Positive controls for wild type detection were created from extracted DNA from human embryonic kidney cells (HEK 293), which are known to be homozygote for the wild type genotype [10]. The region of interest, was cloned into a pcDNA3.1 vector (Invitrogen, Carlsbad, CA). The positive controls for SNP detection was based on the HEK_TLRwt_pcDNA3.1 vector which was modified with QuickChange XL Site-Directed Mutagenesis Kit following manufacturers' recommendations (Agilent technologies, Santa Clara, CA) to yield the SNP TLR2 R753Q.

Statistical analysis

Descriptive analysis was done by computing means and standard deviations (SD), or medians and interquartile ranges (IQR) as appropriate. To evaluate associations between the wild-type (G/G) (wt) in contrast to the mutated genotype among HTX-transplant recipients (A/G and A/A) (mut) or CMV viremia and clinical attributes cross-tabs were calculated and Fisher's exact test or Pearson's Chi squared test performed. Associations of the time to viremia/CMV viral load and CMV area under the curve in wt and mut patient group were estimated with the Mann-Whitney U test. Analysis of the CMV serostatus and CMV viremia were done by Cox regression model. All analyses were performed using SPSS software version 22 (IBM Corp. USA).

Results

The study population included a total of 175 patients who underwent allogeneic heart transplantation. Key demographic and clinical characteristics of the 175 HTX patients are summarized in Table 1. The mean age of the patients at the time of HTX surgery was 49 years (SD, 14 years) and the majority of patients (74.9%) were male. The most common reason for heart transplantation was dilated cardiomyopathy (57.1%) and the majority of patients were still alive (93.7%) after a mean observation period of 8.2 years (SD, 6.0 years). The HLA-mismatch was positive in 45 (25.7 %) cases. A majority of HTX patients was CMV-seropositive at the time of transplantation (57.1%) and patients at high risk for CMV infection (R-/D+) were a minority (24.0%).

Leukocyte preparations from all 175 transplant patients were tested for the TLR2 R753Q polymorphism with use of a TaqMan SNP genotyping assay. The wild-type genotype G/G (wt) was detected in 165 (94.3%) of the 175 HTX patients and the heterozygous mutated genotype A/G (mut) in 9 (5.1%) of the 175 patients. Only a single patient (0.6%) of the 175 HTX patients had a homozygous mutated genotype A/A (mut) (Figure 1 A). To correlate

this finding with the relative frequency distribution of these three genotypes in the general population of the same geographic region, leukocyte preparations from 281 healthy blood donors were screened also for the TLR2 R753Q polymorphism. The relative frequency of TLR2 R753Q genotypes was similar to that found in HTX patients - 94.0 % (264 of 281) of donors displayed the wild-type, 5.7% (16 of 281) the heterozygous mutated and only 0.4% (1 of 281) the homozygous mutated for the TLR2 R753Q genotype (Figure 1 B).

Next, we evaluated whether the TLR2 R753Q polymorphism is associated with outcome of HTX or infection status in the present cohort (Table 2). In this analysis, the TLR2 R753Q polymorphism was neither associated with transplant failure, death, or CMV-serostatus of the patients. A total of four patients suffered from *Pneumocystis jiroveci* pneumonia during the observation period. Interestingly, only the single homozygous mut patient suffered from this disease during chemoprophylaxis for *Pneumocystis jiroveci* infection (four months after transplantation). The other three patients with wt TLR2 genotype suffered from *Pneumocystis jiroveci* pneumonia a mean time of 16 months (SD, 18 months) after transplantation and 10 months after cessation of chemoprophylaxis.

To evaluate a possible association between TLR2 R753Q polymorphism and CMV replication, we analyzed the present patient cohort for timing and magnitude of CMV replication. Cases of tissue-invasive CMV disease were not observed in the present cohort. CMV viremia was detectable by PCR in a total of 108 (61.7%) of the 175 HTX patients. The mean time from transplantation to first CMV-positive PCR was 436 days (SD, 1348 days) in the wt group and 1230 days (SD, 2848 days) in the mut group ($P=0.295$ [Mann-Whitney-U test]). Maximum levels of CMV viremia were detected in all patients before antiviral treatment and did not differ significantly between the wt group (mean, 2.1×10^5 copies/ml; SD, 1.6×10^6 copies/ml) and the mut group of HTX patients (mean, 7.4×10^4 copies/ml; SD, 9.8×10^4 copies/ml; $P=0.102$ [Mann-Whitney-U test]). The area under the curve (AUC) of changes in viral load over time was not statistically different between wt and mut group (median, 2 versus 14; $P=0.167$ [Mann-Whitney-U test]) (Figure 2).

To find other risk factors for CMV viremia, we evaluated selected clinical and immunological characteristics in relation to occurrence of CMV viremia (Table 3). The only parameter that was associated with the occurrence of CMV viremia in this evaluation was the CMV-serostatus of solid-organ recipient and donor. Rates of CMV viremia were highest in R+/D+ (54/65, 83.1%) and lower in the R+/D- (27/35, 77.1%) and the R-/D+ (27/42, 64.3%) patients who are supposed to be at highest risk for CMV viremia and disease. Nevertheless, CMV viremia occurred in the R-/D+ patient group clearly later than in recipient seropositive- HTX patients (median, 182 days versus 23 days; $P<0.001$ [Mann-Whitney U]) (Figure 3 A), which corresponds with the duration of antiviral prophylaxis in R-/D+ patients. The high risk R-/D+ patient population had a lower risk for CMV viremia (Figure 3 B).

Discussion

Current evidence on the significance of the TLR2 R753Q polymorphism in CMV infection is conflicting. Two studies on the relevance of the TLR2 R753Q polymorphism in a total of

>800 liver transplant recipients found a significantly increased risk for severe CMV disease in patients with a mutated genotype [9,10]. Another study evaluated the polymorphism in 265 kidney-transplant patients and found no significant effect in relation to graft survival, renal function, or incidence of opportunistic infections, including CMV infection [11]. We were unable to identify a significant effect of the polymorphism on incidence of tissue-invasive CMV disease or viremia in 175 HTX patients. The present observations have to be interpreted with caution in view of the smaller cohort studied in comparison to the liver-transplant cohort and the small number of homozygotes of this polymorphism. A significant difference to the previous studies is that we did not observe a single case of tissue-invasive CMV disease. We hypothesize therefore that the high efficacy of prophylactic followed by pre-emptive antiviral treatment neutralizes a potentially increased risk of tissue-invasive CMV disease predicated upon the TLR2 genotype.

The high efficacy of antiviral strategies is demonstrated by the clear difference in first time of viremia between HTX patients who did or did not receive prophylaxis. Administration of antiviral prophylaxis for several months following transplantation is well established and was shown to reduce significantly rates of CMV disease, graft-rejection and mortality [14,15]. In the present cohort, patients at particularly high risk for CMV disease (R-/D+) received CMV prophylaxis for 3 months. CMV replication was noted in all of these patients after the end of prophylaxis at an interval that was comparable to that following transplantation in low-risk patients who did not receive prophylaxis. Hence, a prolongation of CMV prophylaxis to six months or even longer may delay late-onset CMV replication to even later times after transplantation, when immunosuppressive treatment is less intense [16,17]. Indeed, a study on CMV prophylaxis for 100 vs. 200 days found a clearly reduced risk of CMV disease and viremia 12 months after transplantation in high risk (D+/R-) kidney transplant recipients [18]. Nevertheless, adverse effects of the prolonged administration of antiviral drugs also have to be considered such as interactions with other medications, toxicities such as severe leukopenia, or development of antiviral resistance [19]. Preemptive treatment of CMV may be an equally effective strategy and associated with fewer adverse effects.

The TLR2 R753Q polymorphism was proposed to partially protect from CMV infection as the mutated genotype was associated with a lower CMV-seroprevalence [12]. We could not confirm that the present HTX patients had a lower rate of CMV-seropositivity associated with the TLR2 R753Q polymorphism before transplantation, despite studying a comparably large cohort of patients [12].

The low prevalence of the TLR2 R753Q polymorphism shades doubt on the clinical usefulness of screening for rare genetic markers as risk factor for opportunistic infectious diseases. The relative frequency of the heterozygous TLR2 R753Q polymorphism varies with geographic region and population studied – 0% in Asia [20,21], 9% in the US [10], and 1.3%-11.4% in Europe [22–27]. In concordance, we found a relative frequency of the heterozygous polymorphism of 5.7% and of the homozygous mutated polymorphism of 0.4% in the general Austrian population. The relative frequency distribution of these genotypes was almost identical in the HTX patient cohort. Accordingly, a high number-to-screen is required to detect a single transplant patient with a mutated genotype.

Remarkably, we found a different relative frequency of CMV infection in the present HTX patients than in other, previously studied cohorts. CMV-seronegative patients receiving an organ from a CMV-seropositive donor are considered to be at highest risk for CMV infection and disease [28]. In contrast, we found a higher rate of CMV viremia in HTX patients that were CMV-seropositive before transplantation. CMV viremia occurred in these patients also sooner after transplantation when immunosuppressive treatment was most intense because they did not receive antiviral prophylaxis. Nevertheless, periods of CMV viremia were treated preemptively and rigorously with antivirals and the absence of cases of tissue-invasive CMV disease may further underline the efficacy of preemptive treatment in HTX patients.

Conclusions

We evaluated the risk for CMV infection and tissue-invasive disease in relation to this polymorphism in a further cohort of patients – HTX recipients – to test for potential confounders that may explain the discrepant observations in previous studies. The most significant prognostic parameter for CMV infection was the CMV-specific serological match between recipient and donor in HTX patients receiving optimized antiviral prophylaxis. The TLR2 R753Q polymorphism may influence the natural course of CMV infection and reactivation but modern medicine, including CMV prophylaxis with use of antiviral compounds, reduces the associated risk more efficiently than screening for the TLR2 polymorphism. Limited resources may be better invested in screening for other, more common and clinically relevant predictors of CMV disease in immunosuppressed patients. In conclusion, the prevalence of the TLR2 R753Q polymorphism is too low in the general population to be a good predictive marker for CMV disease.

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Abbreviations

CMV	Cytomegalovirus
D	Donors
gB	glycoprotein B
gH	glycoprotein H
HTX	Heart transplant recipients
IQR	Interquartile ranges
MGB	Minor Groove Binder
Mut	mutated
R	Recipient

SD	Standard deviation
SNP	Single nucleotide polymorphism
TLR2	Toll-like receptor 2

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1**Highlights**

- Evaluation of TLR2 R753Q polymorphism in relation to CMV infection
- HTX were screened in relation to clinical parameters
- Targeted antiviral prophylaxis is more important than polymorphism

Figure 1.
Relative frequency distribution of the TLR2 R753Q SNP polymorphism in 175 heart transplant recipients and 281 healthy blood donors

Figure 2. CMV viremic load (area under the curve (copies/ml)*days) in 108 heart transplant recipients in relation to TLR2 R753Q polymorphism. wt: wild-type (genotype G/G); mut: mutated (genotype A/G or A/A); logarithmic scale

Figure 3.

Timing of CMV viremia in 108 heart transplant recipients. (A) Time interval between heart transplantation and first detectable CMV viremia in relation to CMV serostatus of the transplant recipient (R) before transplantation; dashed line indicates last day of antiviral prophylaxis. (B) Cox regression analysis of time of first detectable CMV viremia in relation to CMV serostatus of recipient and donor (D) before transplantation (n=108); None of the R-/D- patients became viremic during the observation period and these patients were therefore not included in the analysis

Table 1
Demographic and Clinical Characteristics of 175 Heart Transplant Recipients

Characteristic		
Age	Mean years (SD)	49.2 (13.5)
Range, years		9 - 72
Female, no. (% of total)		44 (25.1)
Indication for HTX, no. (% of total)		
Dilated Cardiomyopathy		100 (57.1)
Coronary artery disease		40 (22.9)
Others		35 (20.0)
Follow-up after transplantation		
Mean years (SD)		8.2 (6.0)
Range, years		0-23
Positive HLA-Mismatch, no. (% of total)		45 (25.7)
CMV serostatus of HTX recipient and donor[*], no. (% of total)		
R-/D-		30 (17.1)
R+/D-		35 (20.0)
R+/D+		65 (37.1)
R-/D+		42 (24.0)
Deceased, no. (% of total)		11 (6.3)

* in three patients the donor's CMV-serostatus was unknown

Abbreviations: SD, Standard Deviation; R, Recipient; D, Donor; +, CMV-seropositive; -, CMV-seronegative

Table 2
Clinical characteristics of 175 Heart Transplant Recipients in relation to TLR2 polymorphism

Characteristic	TLR2 polymorphism		
	wt	mut	p-value
	(n=165)	(n=10)	
No. of patients with second HTX because of transplant failure, no. (% of total)	10 (6.1)	0 (0)	1.000
Deceased, no. (% of total)	9 (5.5)	2 (20.0)	0.123
Graft versus host disease, no. (% of total)	70 (42.4)	7 (70.0)	0.088
Negative CMV-serostatus of HTX-patient [*] , no. (% of total)	68 (41.2)	4 (40.0)	1.000
Pneumocystis jiroveci pneumonia, no. (% of total)	3 (1.8)	1 (10.0)	0.211

^{*} in three patients the donor's CMV-serostatus was unknown

Abbreviations: SD, Standard Deviation; R, Recipient; D, Donor; +, CMV-seropositive; -, CMV-seronegative; wt, wild-type [G/G]; mut, mutated [G/A or A/A]

Table 3
Clinical and immunological characteristics of 175 Heart Transplant Recipients in relation to occurrence of post-transplant CMV viremia

Characteristic	CMV viremia		
	no	yes	p-value
	(n=67)	(n=108)	
Mean age (SD)	47.6 (14)	50.2 (13)	0.188
Female, no. (% of total)	13 (19.4)	31 (28.7)	0.210
Indication for HTX, no. (% of total)			
Dilated Cardiomyopathy	38 (56.7)	62 (57.4)	0.242
Coronary artery disease	19 (28.4)	21 (19.4)	
Second HTX, no. (% of total)	4 (6.0)	6 (5.6)	1.000
Wild-type TLR2, no. (% of total)	63 (94.0)	102 (94.4)	1.000
Positive HLA-Mismatch, no. (% of total)	14 (20.9)	31 (28.7)	0.464
Graft versus Host Disease, no. (% of total)	27 (40.3)	50 (46.3)	0.263
CMV serostatus of HTX recipient and donor [*] , no. (% of total)			
R-/D-	30 (44.8)	0 (0)	<0.001
R+/D-	8 (11.9)	27 (25.0)	
R+/D+	11 (16.4)	54 (50.0)	
R-/D+	15 (22.4)	27 (25.0)	
Deceased, no. (% of total)	2 (3.0)	9 (8.3)	0.208

^{*} in three patients the donor's CMV-serostatus was unknown

Abbreviations: SD, Standard Deviation; R, Recipient; D, Donor; +, CMV-seropositive; -, CMV-seronegative; wt, wild-type [G/G]; mut, mutated [G/A or A/A]