Prevalence and Genotype Distribution of Cytomegalovirus UL55 Sequence in Renal Transplant Recipients in North West of Iran

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Cytomegalovirus (CMV) is one of the most important infections in renal transplant recipients. Kidney transplant is the last hope for the patients with end stage renal diseases. Cytomegalovirus infection can threaten patients and graft survival after transplantation. Four hundred and thirty-four renal transplant recipients contributed to this study. PCR and RFLP analyses were performed in order to determine CMV viremia and its genotypes. CMV viremia was detected in 68 (15.9%) recipients. The mean post-transplantation time in our recipients was 50 months, ranging from 1 to 354 months. Viremia was detected in 31.2%, 30.7%, 17.5%, 10.2%, and 6.4% of the recipients in 0–3, 4–6, 7–12, 13–24, and more than 24 months post-transplantation, respectively. The distribution of gB1, gB2, gB3, and gB4 genotypes was detected as 26.5%, 20.5%, 17.6%, and 5.9%, respectively. Mixed genotype infection was observed in 29.4% of the recipients. Incidence of viremia was higher in the first 6 months after the transplantation compared with the later stages. Moreover, CMV gB1 and mixed genotype infection were more common in our recipients. J. Med. Virol. 88:1622–1627, 2016.

KEY WORDS: cytomegalovirus; transplantation; genetic variation; glycoproteins

BACKGROUND

Human cytomegalovirus (CMV) is the serious cause of mortality and morbidity in renal transplant recipients [Patel et al., 1996]. CMV genome is a double strand DNA molecule with approximately 230–240 kb in length, which encodes more than 200 polypeptides that play important roles in virus life cycle and interaction with the immune system during acute or latent infections [Varnum et al., 2004].

CMV infection occurs in 60–100% of renal transplant recipients, but only 20–60% develop symptomatic infection within 3 months post-transplantation [Aquino and Figueiredo, 2000]. CMV infection in renal transplant recipients occurs in three patterns: (i) when the recipient is CMV-seronegative and the donor is seropositive, which may lead to primary CMV infection; (ii) secondary infection or reactivation of latent infection may occur when the recipient is seropositive and the donor is seronegative; and (iii) superinfection or reinfection in the recipient occurs when both donor and recipient are seropositive [Sia and Patel, 2000]. In the latter case, the risk of simultaneous infection with more than one genotype or mixed genotype infection is much higher [Manuel et al., 2009].

Glycoprotein B (gB, UL55) is one of the major proteins of envelope, which has an undeniable role in viral attachment and cell transmission [Navarro et al., 1993]. It is also a major target for neutralizing antibodies [Chou and Dennison, 1991]. According to the sequence diversity of UL55 gene, four genotypes have been described [Chou and Dennison, 1991]. There are some studies which suggest the tendency of some genotypes toward congenitally infected infants or hematopoietic stem cell recipients or developing CMV-related complications in these patients [Jin et al., 2007; Banan et al., 2009].
Coincidence of any specific gB genotype and clinical manifestation in solid organ transplant still remains as a controversial topic [Manuel et al., 2009]. To our best knowledge, the distribution of CMV genotypes has not been studied in renal transplant recipients in Iran. In this study, the frequency of CMV viremia and its genotype distribution are examined in renal transplant recipients.

**METHODS**

**Patients and Sample Collection**

This study included blood samples from the patients who underwent renal transplantation at Emam Hospital, Urmia University of Medical Sciences, Iran. The blood samples were collected from 434 renal transplant recipients. Plasma was separated immediately using EDTA as anti-coagulant and aliquots of plasma samples were stored at −80°C in RNase DNase free micro-tubes. This study was approved by Ethics Committee, Urmia University of Medical Sciences. Informed consent was obtained from all the participants.

Main underlying diseases leading to kidney transplantation were hypertension, glomerulonephritis, diabetic nephropathy, polycystic kidney disease, and other causes in the 21%, 19%, 17.4%, 11%, and 31% of recipients, respectively.

**Immunosuppressive Regimen**

Induction therapy was started with cyclosporine 6 mg/kg or Tacrolimus 0.1–0.15 mg/kg and Mycophenolate Mofetil (MMF) 2 g/d at day before surgery and intravenous methylprednisolone pulses 10–15 mg/kg 1 hr before and daily up to 3 days after the operation (triple therapy). In highly sensitive recipients and when delayed graft function was suspected, we administered intravenous thymoglobulin 1 mg/kg daily up to 7–10 days accompanied with IV gancyclovir (1.25 mg/kg) for CMV infection prophylaxis. In high-risk recipients (antilymphocyte therapy; D+/R−), Valgancyclovir (900 mg) once daily adjusted based upon glomerular filtration rate (GFR) was prolonged to 3 months.

**DNA Extraction and PCR Amplification of gB Gene**

DNA was extracted from 200 μl of sera samples using high pure viral nucleic acid kit (Roche Germany) according to the manufacturer's instruction. The extracted DNA was stored in DNase free micro-tubes at −20°C. To detect CMV viremia, semi-nested PCR was carried out to amplify 293 or 296 bp fragments of gB gene (according to the strain). PCR master mix contained 0.1 μM of both sense gB1319 (TGGAACCTG-GAACGTTTGGGC) and external antisense gB1676 (TGACGCTGGTTTGGTGATG) primers, 1 U of Taq DNA polymerase, 2 mM MgCl2, 0.2 mM each dNTP, 2.5 μL of 10× PCR buffer, and 3 μl of the extracted DNA and the final volume was adjusted to 25 μl by H2O. PCR was carried out by 5 min at 95°C followed by 25 cycles of 95°C for 60 sec, 55°C for 90 sec, 72°C for 120 sec, and the final extension of 5 min at 72°C for the first round PCR. The second round of amplification was carried out by the same sense primer as the first round, but by other antisense primer, gB1604 (GAAACGCGGGCATTGCG). Two microliter from the first round PCR product was used as the template in the second round amplification. The semi-nested PCR reaction was carried out by 5 min at 95°C, which was followed by 30 cycles of 60 sec at 95°C, 60 sec at 65°C, 72°C 60 sec, and the final extension time of 5 min at 72°C, as described previously with some modification [Aquino and Figueiredo, 2000]. Amplification products were analyzed by electrophoresis in a 2% agarose gel containing 500 ng ethidium bromide per millilitre and visualized under ultraviolet light.

To determine the sensitivity of semi nested PCR, one positive plasma sample, quantified by qRT-PCR, underwent fourfold serial dilution and amplification by nested PCR. Our results demonstrated that the HCMV DNA was detected by nested PCR even though the viral load was as low as 500 copies/ml.

**Restriction Enzyme Digestion and Fragment Pattern Analysis**

Unpurified PCR products obtained from the second amplification were digested by HinfI and Rsal enzymes (Fermentas, Lithuania) in two separate reactions. Each reaction was carried out in a master mix containing 2 μl of 10× Tango buffer (Fermentas Lithuania), 10 μl PCR product, and 0.2 μl (10 U/μl) enzymes and adjusted to the final volume of 20 μl by adding double distilled water. The reaction tubes were incubated at 37°C for one hand the reaction was stopped by heating at 80°C for 20 min. The digested PCR products were electrophoresed on 3% agarose gels in 1X TBE buffer (134 mM Tris–HCl, pH 10; 68 mM boric acid; 2.5 mM EDTA) containing 500 ng ethidium bromide per millilitre. The RFLP pattern was then evaluated under ultraviolet light.

**Verification of RFLP Results by Sequencing**

Sequences obtained from five samples and examined by RFLP were determined and compared with the representative UL55 gene sequences retrieved from GenBank for gB1, gB2, gB3, and gB4 (M60929, M60931, M85228, and M60926, respectively) using neighbor joining method for phylogenetic analysis. UL55 sequences were clustered with relative genotypes and confirmed RFLP results.

**Statistical Analysis**

Data were analyzed by IBM SPSS software (SPSS Inc., Chicago, IL), version 20.0, to determine any statistical relations. Multivariate logistic regression was used to analyze the relationships between variables and statistically significant differences among
the groups of patients and $P$ values of $<0.05$ were considered statistically significant.

**RESULTS**

**Incidence of Viremia**

Two hundred and sixty-two male and 172 female recipients with the mean age of $42.5 \pm 15$, maximum 77 and minimum 4 years old, were enrolled in this study. Among 434 recipients, CMV UL55 (gB) sequences were detected in 68 (15.9%) patients. The median time to detect viremia was the 17th week after transplantation, ranging from 4 to 700 weeks. Viremia was detected in 35 out of 112 (31.2%) recipients in the first 3 months post-transplantation, 8 out of 26 (30.7%) in 4–6 months, 7 out of 40 (17.5%) between 7–12 months, 4 out of 39 (10.2%) in 13–24 months, and 14 out of 218 (6.4%) in the period more than 24 months post-transplantation. There was no statistically significant difference in the rate of CMV viremia among the male and female recipients ($P$-value $= 0.581$). The occurrence of viremia among the recipients within 6 months post-transplantation was significantly higher than that of other groups ($P$-value $= 0.000$) (Table I).

Out of 434 recipients, 75 (17.4%) had diabetes mellitus. CMV viremia was detected in 22 (29.3%) of diabetic and 45 (12.6%) of non-diabetic recipients. The rate of viremia was twice higher among diabetic than non-diabetic recipients ($P$-value $= 0.001$).

**Distribution of CMV gB Genotypes**

Genotyping was examined in 68 viremic recipients using RFLP technique. The pattern of the digested bands that distinguish gB genotypes 1–4 is shown in Figure 1. Genotypes gB1, gB2, gB3, gB4, and mixed genotype infection were detected in 18 (26.5%), 14 (20.6%), 12 (17.6%), and 4 (5.9%) of the recipients, respectively. Twenty (29.4%) patients presented more than one genotype. There was no association between CMV genotype and age, gender, diabetes, and post-transplantation period.

**DISCUSSION**

In the present study, we examined CMV viremia in a group of 434 kidney transplant recipients by PCR. Subsequently, the distribution of CMV genotypes was investigated by RFLP. To our best knowledge, this work was the first study investigating the distribution of CMV gB genotypes among Iranian renal transplant recipients.

We carried out the conventional nested PCR on CMV viral genome extracted from the plasma.
samples. There are several techniques for the identification of CMV infection, among which nucleic acid amplification is a widely available diagnostic tool in solid organ transplantation. PCR can detect viral sequences in various samples, including peripheral blood leukocytes [Manez et al., 1996], whole blood, and cell free body fluids such as serum [Cunningham et al., 1995] and plasma [Eriksson et al., 1996]. One concern regarding PCR performance on the extracted viral genome from leukocytes is the amplification of very low copies of latent cell-associated virus, which cannot be differentiated from the replicating virus. It has been hypothesized that active viral replication could result in the release of virus from leukocytes into serum [Sia et al., 2000]. Therefore, several studies have attempted to compare CMV PCR between the extracted DNA from leukocyte and serum samples of renal recipients. They have suggested that the detection of viral DNA from sera samples by CMV PCR is a sensitive and specific indicator of early infection [Van Dorp et al., 1992; Geddes et al., 2003].

In the present study, the incidence of CMV viremia in renal transplant recipients was 15.9%, which was similar to the rates reported by other studies using CMV-pp65 antigenemia technique [Razeghi et al., 2007; Einollahi, 2012] or quantitative PCR [Nafar et al., 2014]. The incidence of CMV infection among the renal transplant recipients not receiving antiviral prophylaxis has been reported to vary from 8% up to 32% [Weikert and Blumberg, 2008]. Donor seropositivity is the most important risk factor for post-transplant CMV infection. Seronegative recipients who receive kidney graft from seropositive donors are at the increased risk of CMV disease [Schnitzler et al., 1997; Brennan, 2001]. Although we were not able to present the serostatus of the patients in this report, our data (unpublished) indicated that almost all the recipients were IgG seropositive.

CMV viremia was developed in the one third of our recipients within the first 6 months post-transplantation, which was significantly higher than the rate of infection in other recipients. It has been shown that CMV viremia is more common in the first 6 months after renal transplantation and can increase up to 50% [Falagas and Snydman, 1995; Helanterä et al., 2010; Einollahi, 2012], which can be contributed to the strong immunosuppressive regime in the first 6 month period after transplantation [Einollahi, 2012].

A similar distribution rate of gB1, gB2, and gB3 was found in the renal transplant recipients, which was in agreement with other studies [Falagas and Snydman, 1995; Aquino and Figueiredo, 2000; Coaquette et al., 2004a]. Similar to other reports [Aquino and Figueiredo, 2000; Carraro and Granato, 2003b], genotype gB4 was rarely found (5.9%) in our recipients. Predominance of gB1 genotype was reported more frequently among the renal transplant recipients [Woo et al., 1997a] and congenitally infected newborns [Bale et al., 2000]. However, high prevalence of gB2 genotype has been found by other studies in renal transplant recipients [Carraro and Granato, 2003b], HIV-infected patients [Fidouh-Houhou et al., 2001], or CMV retinitis [Shepp et al., 1996]. The reasons for the observed differences in genotype distribution are not fully elucidated, but might be contributed to the geographical variation of CMV genotypes and shedding of a particular genotype according to the cause of immunosuppression [Zipeto et al., 1998; Carraro and Granato, 2003b].

In the present study, one third of the recipients were infected with more than one genotype. These data were in contrast to 96.3% and 90% of patients infected with only one gB genotype reported in previous studies on renal transplant recipients [Woo et al., 1997b; Carraro and Granato, 2003a]. Previous reports have implied that immunocompromised people including pregnant women [Boppana et al., 2001] can have dual [Baldanti et al., 1998] or multiple [Thomas et al., 2001] CMV infections. Among renal transplant patients, the rate of mixed CMV infections vary from low [Carraro and Granato, 2003b].

| Table I. Characteristics of Renal Transplant Recipients |
|----------------|----------------|----------------|----------------|----------------|
|                | CMV positive | CMV negative | Total recipients | OR (95%CI) |
| Diabetic       |               |               |                 |             |
| Yes            | 22 (29.3%)    | 53 (71.7%)    | 75              | 2.703       |
| No             | 46 (12.8%)    | 312 (87%)     | 358             |             |
| Post-transplant period (months) |               |               |                 |             |
| ≤3             | 35 (31%)      | 77 (68%)      | 112             | 1.013       |
| 4–6            | 8 (30.7%)     | 18 (69.3%)    | 26              |             |
| 7–12           | 7 (17.5%)     | 33 (82.5%)    | 40              |             |
| 13–24          | 4 (10.2%)     | 35 (89.8%)    | 39              |             |
| ≥24            | 14 (6.4%)     | 204 (93.6%)   | 218             |             |
| Recipient age  |               |               |                 |             |
| Female         |               |               |                 | 0.985       |
| Male           |               |               |                 | 1.321       |
| CI, confidence interval; OR, odds ratio.

In the present study, the rate of CMV viremia in the diabetic patients was significantly higher than non-diabetic patients. Similar results were reported by other group [Kosmadakis et al., 2013]. Cytomegalovirus has been reported to associate with type 1 diabetes [Lehr et al., 1985], but there are conflicting reports among transplant patients. Hjelmesaeth et al. [2004] suggested that kidney recipients who had symptomatic or asymptomatic CMV infection were at higher risk for developing new-onset diabetes post-transplantation. In contrast, analysis of Indian transplant patients did not find any significant association between CMV infection and new-onset diabetes post-transplantation [Sharma et al., 2003]. These differences may be partly due to using various criteria for the diagnosis of both CMV infection and post-transplantation diabetes.

In conclusion, the present data showed that the frequency of active CMV infection in Turkish renal transplant patients was high and the most frequent genotypes in these patients were gB1 and gB2 (47.1%). Moreover, CMV infection with more than one genotype occurred in high proportion (29%) of our patients.

REFERENCES


Razeghi E, Hadadi A, Mansor-Kiaei M, Molavi M, Khashayar P, Pourmand G. 2007. Clinical manifestation, laboratory findings, 2003a] to high [Coaquette et al., 2004b]. Prospective analysis of CMV gB genotypes carried out by Aquino and Figueiredo [2000] on 34 renal transplant recipients implied that 70.6% of the patients were infected with more than one gB genotype during the follow-up period. Debate about the polymorphism of gB gene and its correlation with clinical manifestations is still continuing. Although some studies have reported that gB1 and gB2 genotypes correlated with high mortality rate in hematopoietic stem cell recipients [Banaz et al., 2009], in renal transplant recipients, the data are conflicting and the results of such studies have been commonly unconvincing [Manuel et al., 2009].


