Original Article

Molecular Analysis of Ganciclovir-Resistant Cytomegalovirus in Renal Transplant Recipients with High Viral Load

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Abstract

Background: Gancyclovir-resistant (GanR) cytomegalovirus (CMV) remains an issue, especially in solid organ transplant (SOT) recipients. Some mutations in *UL54* and *UL97* confer this resistance. Long-lasting high-dose drug exposure, high viral load, together with lack of sufficient compliance with treatment may account for these mutations. The aim of this study was to detect *UL97* and *UL54* putative mutations conferring ganciclovir-resistance in renal organ transplant recipients with high CMV load.

Methods: In this cross-sectional study, 58 serum samples were collected from renal transplant recipients who had referred to three hospitals in Tehran from January 2014 to June 2015. Specific criteria such as CMV syndrome, presence of CMV in blood and organ dysfunction were considered. Then, they were tested for viral load in their early fourth month of intravenous ganciclovir treatment. Fifty cases revealing more than 200 copies/mL were analyzed for mutations. Two fragments of *UL54* and *Ul97* genes were amplified and sequenced bidirectionally. Sequence alignment and statistical analysis were performed by Mutation Surveyor software and *t*-test respectively.

Results: A significant difference was observed in viral load between seronegative and seropositive recipients (P = 0.036). The most frequent mutation was related to D605E in *UL97* gene with the rate of 25%. Regardless of viral load, neither putative mutation nor simultaneous mutation was detected in either *UL97* and *UL54* regions.

Conclusion: In spite of high viral load and persistence of symptoms, our population study did not reveal putative mutations. Hence, the direct relationship between the presence of high quantity of CMV and the occurrence of putative mutation cannot be considered. Non-putative gancyclovir resistant mutations and prolonged drug exposure may have a role in these manifestations.

Keywords: *Cytomegalovirus* (*CMV*), solid organ transplant (SOP), *UL97*, *UL54*

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Introduction

Human cytomegalovirus (HCMV) infection is a major issue in solid organ transplant (SOT) recipients, particularly those who receive lung, kidney, or kidney/pancreas transplants, most notably the seropositive-donor, seronegative-recipient (D+/R−) subset.[1–4] Although development of prophylaxis and preemptive procedures has significantly improved consequences in CMV infections, virologists have described an increasing incidence of antiviral resistance (AVR).[5] Mutations within *UL97* and *UL54* genes involved in ganciclovir (GCV) anabolism and encoding protein kinase and DNA polymerase respectively are the main causes of resistance.[6,7] Practically, CMV AVR was considered as rising or raised viral load in the presence of sufficient antiviral therapy administered for more than two weeks with confirmed resistance on genotypic and/or phenotypic testing.[5] The most likely reasons for advent of drug resistant HCMV are prolonged high-dose therapy, high viral load, as well as lack of sufficient compliance with treatment.[6,8]

GCV is a guanosin analog that is inactive as administered and requires the HCMV *UL97* gene product acting as a serine/threonine kinase to mediate its phosphorylation for antiviral activity. As a result, mutations which disturb the UL97 kinase function lead to emergence of ganciclovir-resistant cytomegaloviruses. The three common antiviral drugs, i.e. GCV, foscarnet (FOS) and cidofovir (CDV) target DNA polymerase (*UL*54); therefore, mutation in *UL54* may cause resistance to them.[7,9–11]

The drug of choice for treatment of systemic HCMV infections is GCV. This antiviral drug is commonly used in Iran for solid organ transplant recipients and has also remained as the first-line treatment for CMV infections in immunocompromised patients. It has been shown that the majority of ganciclovir-resistant CMV isolates carry mutations in the *UL97* phosphotransferase gene.[9,12,13] Putative mutations in *UL97* have been dominantly clustered at codons 460, 520, and 590–607 while the mutations in *UL54* are more diffusely spread throughout the gene, from codon 300 to 1000.[7,14,15]

Genome isolations of probable ganciclovir-resistant CMV obtained from Iranian patients were subjected to regional molecular analysis in the relevant viral UL54 DNA polymerase and UL97 kinase genes to assay the acquired mutational pattern in those motifs.

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Materials and Methods

This transversal analysis focused on kidney transplant recipients with high titer of CMV load following GCV antiviral therapy. Serum samples were collected from 58 patients, comprising 38% females and 62% males, who had referred to three hospitals in Tehran during a 15-month period from January 2014 to June 2015. Having received intravenous ganciclovir for more than 90 days post-transplant, these patients showed clinical feature of CMV disease. The following definitions of CMV disease were used: CMV syndrome, the presence of both CMV in blood, fever and the presence of malaise, leukopenia, atypical lymphocytosis, thrombocytopenia, elevated hepatic enzymes and serum creatinine, and evidence of organ dysfunction. DNA was extracted from 200 microliters of serum samples by QIAamp DNA Mini kit (Qiagen®), in accordance with the manufacturer's instructions. The quality of extracted DNA samples was checked with a spectrophotometer (NanoDrop 2000) and stored frozen at -20˚C until tested. After measuring viral load of samples by artus CMV RG PCR (Qiagen®), samples which exceeded safe viral load threshold of 200 copies/mL serum were retrospectively assessed for mutation detection.

Amplification of *UL97* and *UL54* regions was accomplished using the primers designed by Oligo software (version 6-DBA Oligo, Inc. USA). The sequence of these primers is shown in Table 1. Amplicons of *UL97* and *UL*54 harbored regions of VIB, VII, VIII, IX and IV/ExoII, delta-C/ExoIII respectively in which most of phenotypically validated drug resistance mutations are clustered. The primers and PCR condition were optimized using standard laboratory strain AD169. Amplification of DNA targets was performed in a thermocycler (BioRad) in the following profile: 3 min at 95°C followed by 30 cycles at 95°C for 1 min, 57°C for 45 sec and 72°C for 45 sec, with a final extension step at 72°C for 5 min. PCR products (658 bp and 583bp in length related to *UL97* and *UL54,* respectively) were loaded on 1% (w/v) agarose gel electrophoresis and stained with ethidium bromide and purified by the *AccuPrep* PCR Purification Kit (Bioneer, Korea) using the protocol recommended by the manufacturer. PCR fragments were then subjected to automatic sequencing bidirectionally using specific forward and reverse primers of each fragment. Alignment of chromatograms was carried out by Mutation Surveyor software version 5.0.1; all sequences were compared with reference strain of AD169.

Results

Serological assay indicated that 47 out of 58 kidney transplant recipients were seropositive and had previous exposure to CMV. In order to detect the DNA load in CMV patients, 58 specimens were tested by *artus* CMV RG PCR from kidney transplant recipients receiving intravenous ganciclovir for more than three months. In 50 specimens (86%), the cytomegalovirus DNA load was above the aforementioned threshold with the range of 10×10^3 to 1.1×10^6 copies/mL serum. A mean DNA load (± standard deviation [SD]) of $3.9 (\pm 3) \times 10^4$ copies/mL in seropositive group (39 patients) and a mean DNA load of $1.4 (\pm 3.1) \times 10^5$ copies/mL in seronegative group (11 patients) was observed. Statistical analysis (*t*-test) revealed significant differences in viral load between seronegative and seropositive recipients ($P = 0.036$). All eight recipients with viral loads under 200 copies/mL were seropositive.

In comparison with the reference genome sequence, outputs from DNA sequencing showed the presence of a total of 18 mutations in ten patients including seven seronegative and three seropositive. Sixteen mutations were related to *UL97* region, and two other cases occurred in *UL54* gene. Forty CMV-positive patients did not reveal any mutation in these regions. As shown in Table 2, the most frequent mutation is related to D605E with the rate of 25% in *UL97*. No case of simultaneous mutations in both *UL97* and *UL*54 regions was realized.

In addition to the above-mentioned changes, sequencing analysis and output results in the clinical specimens showed several silent mutations which are listed in Table 2. Silent nucleotide substitution at the position of 1794 (T®C) was common among all samples.

Discussion

Widespread use of antiviral therapy in most cases has led to drug resistance; this concern motivates investigators to monitor CMV patients for a long time. Our objective in this study was to genetically assay mutation in *UL97* and *UL54* genes in kidney transplant recipients who had persistent CMV viremia in spite of undergoing ganciclovir therapy. The patients had variable loads of CMV during more than three months of GCV therapy.

There are a few point mutations which are attributed to GCV resistance. Development of GCV resistance mainly occurs because of mutations in *UL97* gene, especially in its putative ATP binding (M460V/I, H520Q) and substrate recognition (C592G, A594V, L595S, and C603W) sites.[9,11,16,17] Lately, worldwide usage of GCV poses an unanswered question as to whether non-putative mutations are able to confer resistance or not. In the present study, we reported some non-putative mutations N461K, I464Y, N470T, C480G, C495W, and L516R in *UL97* and also D428N and F432Y in *UL54* gene. However, in *UL97* gene mutations which are very close to putative mutations cannot be assumed to have the same role in resistance.[7] Therefore, the significance of mutations like N461K, I464Y, and L516R requires recombinant phenotype assay and marker transfer experiments. It seems probable that suboptimal and non-continuous ganciclovir treatment along with high viral load may account for such mutations. Thanks to the rapidity and the chance of detecting resistant virus subpopulations, genotypic drug resistance testing of HCMV is becoming the method of choice. However, practical application of this approach relies on phenotypic characterization of both likely resistance-associated

Table 1. Oligonucleotide primers used in PCR reactions for *UL*97 and *UL*54 targets.

Gene	Primer sequence
*UL*97 (1292 to 1937)	F: 5′-GCTACCGACGTGCCTTTTGC3′
	R: 5′-ACGCGACACGAGGACATCTTG3′
*UL*54 (2005 to 2587)	F: 5'-AAAGATGACACGCCGCAACG-3'
	R: 5'-AAAGATGACACGCCGCAACG-3'

Table 2. Overview of renal transplant recipients with detected mutations and serological status.

Patient number	Gender	Gene	Sense mutation	Non-sense silent mutation	Viral load	Serum status	
						Recipient	Donor
1	M	*UL97*	D605E	1410 C→T 1794 T→C	28888	-	+
2	F	*UL97*	D605E	1410 C→T 1575 C→T 1794 T→C	62863	-	+
3	M	*UL97*	N461K, I464Y, D465R,V466M, N470T, C480G	1378 A→T 1413 C→T 1428 C→A 1794 T→C	34562	-	+
4	F	*UL97*	V466M	1368 C→T 1737 C→T 1657 C→T 1794 T→C	17229	+	+
5	F	*UL97*	N461K, C480G, D605E	1378 A→T 1410 C→T 1575 C→T 1587 G→A 1794 T→C	66203	-	+
6	M	*UL97*	S472N, C495W	1368 C→T 1657 C→T 1737 C→T 1794 T→C	10220	-	+
7	M	*UL97*	D605E	1410 C→T 1575 C→T 1794 T→C	31490	-	+
8	F	*UL97*	L516R	1869 C→G 1737 C→T 1657 C→T 1794 T→C	16680	+	+
9	M	*UL54*	D428N	1282 G→A	110000	+	+
10	F	*UL54*	F432Y	-	20501	-	+

M = male; F = female

mutations detected in *UL97* and *UL54*, and combinations of mutations found in patients' HCMV isolates.[18]

Although mutation in *UL97* gene vastly outnumbers *UL54* gene, CMV isolations have demonstrated that mutation only in *UL97* confers low-level resistance in comparison with simultaneous mutations in *UL97* and *UL54*.[19] Most of high level GCV resistance is observed in dual *UL97* and *UL57* mutations.[20] D428N and F432Y mutations in *UL54* are rare mutations whose exact role in drug resistance is open to more examination.

Approximately, 8% of kidney transplant recipients showed controversial mutation D605E in *UL97*. This kind of mutation has been also reported by Zhou *et al.* and Sun *et al.* with varying frequency in SOT. Researchers have suggested that the role of mutation D605E in GCV resistance is not noticeable.[21] Notwithstanding the host immunity may show a great impact on genetic variations, owing to high level of viral load in the present research, the cause of this mutation may be beyond host immunity. In addition, presence of mutations D605E has been reported by some investigators even before exposure to GCV; thus, it is regarded as a polymorphism and sequence changes.[7,22,23] V466G in *UL97* is an infrequent point mutation which confers low-grade GCV resistance.[7] This noncanonical mutation together with L405P has been recently reported in clinical specimens.[23] Contrary to current substitution, we observed V466M in two patients. Besides, some researchers have reported that V466M has no significant effect on GCV resistance and considered it as a change from a pretreatment sequence after therapy.[23,24] Nevertheless, whether this sequence change is associated with resistance or not demands further investigation.

The incidence of confirmed or probable ganciclovir resistance mutations was closely linked to high viral load (≥10,000) and D+/R- status.[12] All identified mutations belonged to renal transplant recipients who had high viral loads and a negative serological picture. These results indicate that these two factors have a significant influence on mutation rate. Neither D+/R+ status nor mild viral load condition revealed mutation.

Failure of treatment cannot always be ascribed to drug resistance; in addition, GCV resistance can be detected during clinical improvement.[25] So, failure to treat renal transplant recipients with GCV can be misleading. Taking into consideration that our assay encompassed most probable putative mutation sites in *UL97* and that the occurrence of *UL54* resistance mutation in the absence of a *UL97* mutation is rare,[7,19,24] the likely reasons for high viral load with no putative mutation involve unidentified gancyclovir resistant mutations and prolonged drug exposure. Furthermore, the viral loads in seronegative recipients were significantly greater than seropositive recipients.

In conclusion, antiviral drug resistance is generally suspected when there is rising or persistently high viral load and normal recommendation for discontinuance or beginning of antiviral

treatment is based on the viral load.[7,26,27] Hence, determination of viral load threshold in which renal transplant recipients should receive gancyclovir treatment contributes to prevention of long-term exposure. As a result, the likelihood of drug resistance may diminish.

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