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CLINICAL STUDY

Cytomegalovirus UL97 ganciclovir resistance mutations in kidney transplant recipients

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ABSTRACT

OBJECTIVES: Widespread and prolonged therapy with ganciclovir (GCV) may result in the emergence of GCV-resistant mutations in human cytomegalovirus (HCMV) genome. The aim of this study was to detect the UL97 mutations associated with GCV resistance in kidney transplant recipients.

METHODS: Forty-nine kidney recipients with positive HCMV DNAemia, who received GCV therapy were included in this study. A 707 bp fragment of UL97 gene spanning codons (436 to 655) was amplified by nested PCR and sequenced.

RESULTS: Thirteen (26.5 %) out of 49 recipients contained mutations associated with amino acid changes. Two UL97 mutations related to GCV resistance were detected in 2 recipients (4 %), including alanine to valine (A594V) and proline to leucine (P521L). The D605E mutation was identified in 8 out of 49 (16.3 %) recipients. Silent mutations G598G, G503G, L553L, L634L, D456D and G579G were commonly observed.

CONCLUSION: Our results indicate that mutations in the UL97 gene associated with GCV resistance may occur in 1 in 25 recipients treated with GCV. In addition, a higher mutation rate of D605E was detected in our recipients. This study provides the first evidence of the prevalence and pattern of GCV related mutations in Iranian Turkish recipients *(Tab. 2, Fig. 1, Ref. 28)*. Text in PDF *www.elis.sk*

KEY WORDS: human cytomegalovirus, UL97 mutations, ganciclovir resistance, kidney transplant.

Abbreviations: PCR – Polymerase chain reaction; HCMV – Human cytomegalovirus; GCV – ganciclovir; UV – Ultraviotet

Introduction

Human cytomegalovirus (HCMV) is known to be one of the major causes of opportunistic infections in immunocompromised patients. Most infections are the result of a latent virus activation. Emergence of drug-resistant viruses, as well as immunodeficiency, have increased morbidity and mortality in patients with HCMV (1, 2).

Several drugs are recommended for the treatment of cytomegalovirus infections, although ganciclovir (GCV) is considered the drug of choice for the treatment of systemic HCMV infection in kidney transplant recipients (3). Long-term treatment with GCV may lead to the development of resistant strains in recipients (4). Resistance to GCV may result from mutations in one or both of the UL97 and/or UL54 genes. More than 90 % of cases of GCV resistance are due to mutations in the UL97 gene (5). The phosphotransferase enzyme pUL97 encoded by the UL97 gene is essential to convert inactive GCV into an active triphosphate molecule that inhibits viral DNA polymerase (1). Most mutations in the UL97 gene occur in codons 460, 520 and 590 to 607(6, 7). These mutations lead to generation of a mutated protein which cannot activate GCV and causes an inadequate response to treatment (1).

In Iran, data on HCMV mutations associated with GCV resistance are limited and most studies have been conducted in the central region (Tehran province) (5, 8, 9). Aslani et al sequenced the 920 bp region of the UL97 gene from 112 GCV-treated renal transplant patients and reported 3 (2.6 %) patients with a single mutation (5). The aim of this study was to identify UL97 mutations in plasma samples of immunocompromised kidney transplant recipients from Urmia, the capital of West Azerbaijan Province, northwestern Iran.

Materials and methods

Patients

Blood samples were obtained from kidney transplant recipients who had received renal transplantation at Emam Hospital, Urmia.

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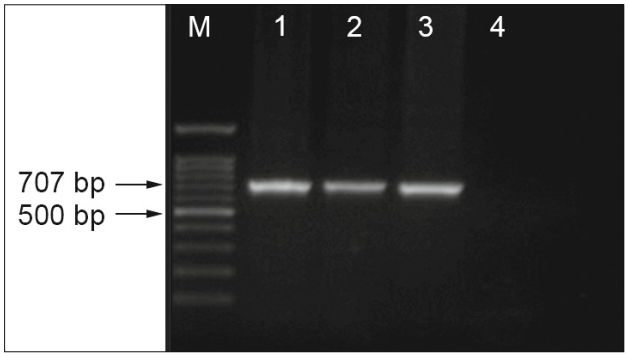

Fig. 1. Electrophoresis of the 707-bp product amplified from the UL97 gene by nested-PCR reaction on a 1.5 % agarose gel. Lane M: Molecular marker 100 bp DNA Ladder (Thermo Fisher Scientific, USA). Lane 1: positive control. Lane 2 & 3: positive UL97 samples. Lane 4: negative control.

Plasma was separated within 4 h of withdrawal using EDTA as an anti-coagulant and stored at –20 °C. Clinical data were obtained from the hospital medical records of transplant recipients. Samples were selected from recipients with positive CMV DNAemia with a history of GCV antiviral treatment. Informed written consent was obtained from patients and the study was approved by the Ethics Committee of Urmia University of Medical Sciences (approval number: IR.umsu.rec.1395.320).

Immunosuppressive and antiviral regimen

Kidney recipients received immunosuppressive therapy including cyclosporine 6mg/kg or tacrolimus 0.1–0.15 mg/kg and mycophenolate mofetil 2 g/day the day before surgery and intravenous methylprednisolone pulses 10–15 mg/kg 1 h before and daily up to 3 days after the operation. In highly sensitive recipients and when delayed graft function was suspected, thymoglobulin was given intravenously at the dose of 1 mg/kg daily for 7–10 days. Transplant recipients were treated with GCV for CMV infection prophylaxis or preemptive therapy (I.V, 5 mg/kg, and twice daily for 2 weeks). In high-risk recipients, valganciclovir (900 mg, twice daily) was extended for up to 3 months.

DNA extraction and PCR amplification of UL97 gene

HCMV DNA was extracted from plasma samples using the high pure viral nucleic acid kit (Roche, Germany), as described by the manufacturer's instructions. The viral DNA was stored at –20 °C until required. CMV DNAemia was detected as described previously (10). A 707 bp fragment (codons 436–655) of the UL97 gene, which encodes the C-terminal half of the enzyme and contains the most common GCV resistance mutations, was amplified by the nested PCR with specific primers (11). Briefly, 3 µl of extracted DNA was added to 22 µl of the reaction mixture containing PCR buffer, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, 5 pmoles for each of outer sense 1278 (GGCGTGCATCGACAGCTACCG) and antisense 2013 (GGCGTGCATCGACAGCTACCG) primers and 0.5 µl of the polymerase. Reactions were carried out under the following conditions: a denaturation step of 95 °C for 5 min then cycling of 94 °C for 1 min, 61 °C for 90 sec, and 72 °C for 1 min for 35 cycles. Finally, the reaction was heated to 72 °C for 5 min and cooled to 4 °C. Second round PCR was performed using inner sense 1292 (GCTACCGACGTGCCTTTTGCA) and antisense 1998 (AACAGACGCTCCACGTTCTTT) primers. Two microliters of first round product were added to the 23 µl reaction mix as for the first round. The cycling conditions of the second round PCR were the same as used for first round PCR. Second round PCR products containing the 707 bp fragments of the UL97 gene were subjected to electrophoresis on 1.5 % agarose gel, bands visualized under UV light as shown in Figure 1.

Tab. 1. UL97 mutations and polymorphisms in sequences isolated from kidney transplant recipients.

Transplant recipients	Changes	Age (y)/sex	Anti-viral treatment received	Symptoms	Post transplantion Time (days)	GCV Resistance	Region associated with GCV resistance
Recipient 1	A594V	63/M	GCV→VGCV	Leukopenia, ulcer in the colon	155	Marker confirmed*	Yes
Recipient 2	P521L	43/F	GCV	Fever, neutropenia	52	Marker confirmed*	Yes
Recipient 3	D605E	52/M	GCV→VGCV	Diarrhea	118	Reported**	Yes
Recipient 4	D605E	45/M	GCV→VGCV	Diarrhea	114	Reported *	Yes
Recipient 5	D605E	48/M	GCV	Diarrhea	34	Reported**	Yes
Recipient 6	D605E	61/F	GCV	None	45	Reported**	Yes
Recipient 7	D605E	60/M	GCV	None	63	Reported**	Yes
Recipient 8	D605E	28/M	GCV	None	474	Reported**	Yes
Recipient 9	D605E	60/F	GCV	Pneumonia	488	Reported**	Yes
Recipient 10	D605E	51/M	GCV	None	38	Reported**	Yes
Recipient 11	T438M	43/F	GCV	None	81	New	No
Recipient 12	I474V	55/M	GCV	None	1359	New	No
Recipient 13	N492S	18/M	GCV	None	74	New	No

A – Alanine; V – Valine; P – Proline; L – Lucine; D – Aspartic acid; E – Glutamic acid; T – Threonine; M – Methionine; I – Isoleucine; N – Asparagine; S – Serine; GCV – Ganciclovir; VGCV – Valganciclovir. *Mutations that have been shown to confer resistance by phenotypic testing and have been confirmed by gene marker transfer studies in vitro; **reported as GCV- sensitive polymorphism.

UL97 sequencing and analysis

The PCR amplified products were purified using the Accu-Prep PCR DNA Purification Kit (Bioneer, South Korea) according to the protocol recommended by manufacturer. Automated DNA sequencing was carried out using an ABI 377 sequencer (Applied Biosystems, Foster City, CA). DNA sequences were analyzed using BioEdit software (version 7.0.5.3) and compared with wild-type GCV-sensitive AD169 strain (accession number BK000394.5) as a reference. The UL97 sequences of the 49 clinical strains have been assigned GenBank accession numbers. MG978138 to MG978177 and MH185085 to MH185092.

Results

Demographic and clinical features of the recipients

Partial UL97 sequences were successfully amplified from 49 recipient blood samples, including 27 (55.1 %) males and 22 (44.9 %) females. The mean age was 46 ± 14 years, the maximum age was 65 years, and the minimum age was 8 years. The mean time to collect blood sample after transplantation was 18 ± 3 weeks. All these recipients had been exposed to GCV prior to sampling (15 days to 3 months). Some of the clinical characteristics of the recipients are listed in Table 1.

Amino acid alternations in UL97 gene sequences

Based on sequence analysis, 13 out of 49 recipients (26.5 %) had mutations associated with the amino acid changes (Tab. 1). Two mutations (4 %) were associated with GCV resistance. An amino acid change of alanine to valine (A594V mutation) was detected in one transplant recipient and a mutation of proline to leucine (P521L) was identified in a sample isolated from anoother transplant recipient. A substitution of aspartic acid to glutamic acid (D605E mutation) was detected in 8 (16.3 %) recipients.

In addition, novel mutations were detected in codons T438M (threonine to methionine), I474V (isoleucine to valine) and N492S (asparagine to serine).

Nucleotide alternations in UL97 gene sequences

Nucleotide changes were observed in 47 out of 49 (96 %) UL97 sequences obtained from recipients compared with strain AD169 (accession number BK000394.5). The most common silent mutations G598G, G503G, L553L, L634L, D456D, G579G and G557G were detected in 95.9 %, 91.8 %, 36.7 %, 34.7 %, 30.6 %, 30.6 % and 20.4 % of recipients, respectively (Tab. 2).

Tab. 2. Observed UL97 silent mutations.

Silent mutations	NO. observed	(%)
G598G	47	95.9
G503G	45	91.8
L553L	18	36.7
L634L	17	34.7
D456D	15	30.6
G579G	15	30.6
G557G	10	20.4
P525P	5	10.2
N470N	4	8
R494R	3	6.1
H454H	2	4
L634L	2	4
A497A	2	4
R516R	2	4
H537H	2	4
A590A	2	4
G623G	2	4
Other location, single occurrence	16	

G – Glycine; L – Leucine; D – Aspartate; P – Proline; N – R – Arginine; H – Histidine; A – Alanine; NO – number

Discussion

Prolonged treatment with GCV in transplant recipients is the most important cause of mutations in the UL97 gene and the development of resistance to GCV (9). Mutations at codons 460, 594, and 595 in the UL97 gene are the most common causes of GCV resistance (2, 12). There are limited studies regarding the genomic evaluation of the HCMV UL97 gene in Iranian kidney transplant recipients.

In present study, Sanger sequencing was used to identify mutations associated with GCV resistance in transplant recipients. UL97 sequences amplified from blood samples of 49 kidney transplant recipients with active viremia showed amino acid mutations in 13 recipients (26.5 %). GCV-related mutations were found in 2/49 (4 %) of the recipients.

The A594V mutation had a low prevalence (2 %) among our recipients. Consistent with our study, another Iranian study observed the A594V mutation in 1 (0.89 %) of 112 kidney recipients after 95 days of GCV treatment (5). The A594V mutation has been reported from Portugal, Washington, and Brazil, with a prevalence of 9 %, 8.34 %, and 1.2 %, respectively (13–15). The drug susceptibility analysis demonstrated that the A594V mutant could result in an 8.2-fold increase in the ganciclovir 50 % effective concentration (16).

The P521L mutation was present in 1 (2 %) of 49 recipients. This mutation as well as L405P and V466G mutations are outside the codon range in which they are normally found, but confer resistance to GCV (6, 7). In a study by Eckle et al (17), the P521L mutation was found in one (1.2 %) of the 79 children who received allogeneic stem cell transplantation and developed CMV encephalitis after transplantation. In another study, involving 561 patients with hematopoietic stem-cell transplantation, the P521L mutation was detected in one recipient (20). This mutation is associated with high-level of resistance to ganciclovir, cyclopropavir and maribavir (18, 19).

In this study, the D605E variant was identified in 8 (16.3 %) out of 49 kidney transplant recipients. This mutation has been reported by previous studies in Iran (5, 21). The role of the D605E mutation in resistance to GCV remains controversial. Zhou et al reported a high prevalence of the D605E mutation (78 %) in Chinese transplant recipients and suggested that this mutation should

be considered as a natural sequence variant. The prevalence of the D605E mutation was 91.8 %, 100 %, and 89.2 %, respectively, in recipients from Japan, Korea, and Taiwan and is therefore considered an important molecular marker of HCMV strains circulating in East Asian countries (2, 22–24). Some authors suggested that the D605E mutation could partially or fully compensate for the effects of the GCV resistance, conferred by M460V or A594P (25). In contrast to Asian countries, this variant was not frequently observed in the CMV strains circulating in Western countries. Studies in the United States, Argentina, and Germany have reported prevalence rates of 11.2 %, 2 %, and 0 %, respectively, in organ transplant recipients (13, 25–27).

In addition, 3 out of 49 recipients had a single amino acid mutation with uncertain impact on resistance: T438M, I474V and N492S. As far as we know, these mutations have not been previously reported. We were unable to draw any conclusions about the impact of these mutations on resistance to GCV without using phenotypic analysis.

In this study, a high rate of silent mutations in codons G598G and G503G was observed with a frequency of 95.9 % and 91.8 %, respectively. These nucleotide changes were detected in 100 % of HCMV strains isolated from Korea and in 98.4 % of HCMV strains isolated from Japan. In contrast, these changes were not frequently observed in western HCMV strains (13). Therefore, these polymorphisms can serve as genetic markers to detect Asian HCMV strains (13, 23, 28).

There are some limitations in our study. First, it was a retrospective study, and the associated bias was unavoidable. Second, mutations in the UL54 gene, which confer resistance to other antiviral drugs, were not investigated in this study. Therefore, the rate of drug resistance in CMV infection in our cohort was probably underestimated. Third, the Sanger sequencing method used to detect the mutations in this study failed to detect mutants that are present in less than 10 % to 20 % of the viral population. Finally, we were unable to perform phenotypic experiments to draw conclusions about the significance of mutations that have not been previously described in the literature.

In conclusion, our results identified the UL97 gene mutations in CMV infection in kidney transplant recipients from northwestern Iran. The prevalence of GCV resistance strains in kidney recipients with positive CMV DNAemia was 4 %. D605E substitution is moderately frequent in our recipients. This mutation is relatively prevalent in East Asian countries compared to Iran. Our study provides the first evidence about prevalence and pattern of GCV related mutations in Iranian Turkish kidney recipients and leads to more effective antiviral therapeutic approaches.

References

1. Silva DdFLd, Cardoso JF, Silva SPd, Arruda LMF, Medeiros RLFd, Moraes MM et al. HCMV UL97 phosphotransferase gene mutations may be associated with antiviral resistance in immunocompromised patients in Belém, PA, Northern Brazil. Revista da Sociedade Brasileira de Medicina Tropical 2018; 51: 141–145.

2. Yang SL, Lin TW, Lin HC, Wang H-, Chang PY, Wang PN et al. Molecular Epidemiology of Cytomegalovirus UL97 and UL54 variants in Taiwan. J Microbiol Immunol Infection 2021.

3. Lurain NS, Chou S. Antiviral drug resistance of human cytomegalovirus. Clinical Microbiol Rev 2010; 23 (4): 689–712.

4. Madi N, Al-Nakib W, Pacsa A. Does cytomegalovirus develop resistance following antiviral prophylaxis and treatment in renal transplant patients in Kuwait? Adv Virol 2011; 2011.

5. Aslani HR, Ziaie S, Salamzadeh J, Zaheri S, Samadian F, Mastoor-Tehrani S. Incidence of ganciclovir resistance in CMV-positive renal transplant recipients and its association with UL97 gene mutations. Iran J PharmRes 2017; 16 (2): 805.

6. Chou S. Recombinant phenotyping of cytomegalovirus UL97 kinase sequence variants for ganciclovir resistance. Antimicrob Agents Chemother 2010; 54 (6): 2371–2378.

7. Martin M, Goyette N, Ives J, Boivin G. Incidence and characterization of cytomegalovirus resistance mutations among pediatric solid organ transplant patients who received valganciclovir prophylaxis. J Clin Virol 2010; 47 (4): 321–324.

8. Hosseini SJ, Nemati E, Behzadian F, Einollahi B, Petrudy AR, Sohraby M et al., editors. Evaluation of ganciclovir resistance in cytomegalovirus infection of renal transplant recipients in Tehran. Transplantation proceedings; 2015: Elsevier.

9. Keyvani H, Saroukalaei ST, Mohseni AH. Assessment of the human cytomegalovirus UL97 gene for identification of resistance to ganciclovir in iranian immunosuppressed patients. Jundishapur J Microbiol 2016; 9 (5).

10. Khalafkhany D, Makhdoomi K, Taghizadeh Afshari A, Motazakker M. Prevalence and genotype distribution of cytomegalovirus UL55 sequence in renal transplant recipients in north west of Iran. J Med Virol 2016; 88 (9): 1622–1627.

11. Hu H, Jabs DA, Forman MS, Martin BK, Dunn J, Weinberg DV et al. Comparison of cytomegalovirus (CMV) UL97 gene sequences in the blood and vitreous of patients with acquired immunodeficiency syndrome and CMV retinitis. J Infect Dis 2002; 185 (7): 861–867.

12. Chou S, Erice A, Colin Jordan M, Vercellotti GM, Michels KR, Talarico CL et al. Analysis of the UL97 phosphotransferase coding sequence in clinical cytomegalovirus isolates and identification of mutations conferring ganciclovir resistance. J Infect Dis 1995; 171 (3): 576–583.

13. Castor J, Cook L, Corey L, Jerome KR. Rapid detection directly from patient serum samples of human cytomegalovirus UL97 mutations conferring ganciclovir resistance. J Clin Microbiol 2007; 45 (8): 2681–2683.

14. Campos AB, Ribeiro J, Vaz CP, Campilho F, Branca R, Campos Jr A et al. Genotypic resistance of cytomegalovirus to antivirals in hematopoietic stem cell transplant recipients from Portugal: A retrospective study. Antiviral Res 2017; 138: 86–92.

15. Silva DdFLd, Cardoso JF, Silva SPd, Arruda LMF, Medeiros RLFd, Moraes MM et al. HCMV UL97 phosphotransferase gene mutations may be associated with antiviral resistance in immunocompromised patients in Belém, PA, Northern Brazil. Revista da Sociedade Brasileira de Medicina Tropical 2018; 51 (2): 141–145.

16. Drouot E, Piret J, Boivin G. Novel method based on "en passant" mutagenesis coupled with a Gaussia luciferase reporter assay for studying the combined effects of human cytomegalovirus mutations. J Clin Microbiol 2013; 51 (10): 3216–3224.

17. Eckle T, Prix L, Jahn G, Klingebiel T, Handgretinger R, Selle B et al. Drug-resistant human cytomegalovirus infection in children after allogeneic stem cell transplantation may have different clinical outcomes. Blood 2000; 96 (9): 3286–3289.

18. Chou S. Approach to drug-resistant cytomegalovirus in transplant recipients. Curr Opinion Infect Dis 2015; 28 (4): 293.

19. Chou S, Ercolani RJ, Marousek G, Bowlin TL. Cytomegalovirus UL97 kinase catalytic domain mutations that confer multidrug resistance. Antimicrobi Agents Chemother 2013; 57 (7): 3375–3379.

20. Shmueli E, Or R, Shapira MY, Resnick IB, Caplan O, Bdolah-Abram T et al. High rate of cytomegalovirus drug resistance among patients receiving preemptive antiviral treatment after haploidentical stem cell transplantation. J Infect Dis 2014; 209 (4): 557–561.

21. Sohrabi M, Behzadian F, Hosseini J, Mohammad S, Lashini H. Molecular Analysis of Ganciclovir-Resistant Cytomegalovirus in Renal Transplant Recipients with High Viral Load. Arch Iran Med 2016; 19 (10).

22. Zhou L, Fan J, Zheng S, Ma W, editors. Prevalence of human cytomegalovirus UL97 D605E mutation in transplant recipients in China. Transplantation Proceedings; 2006: Elsevier.

23. Tanaka K, Hori T, Yoto Y, Hatakeyama N, Yamamoto M, Suzuki N et al. Human cytomegalovirus UL97 D605E polymorphism has a high prevalence in immunocompetent Japanese infants and children. Microbiol Immunol 2011; 55 (5): 328–330.

24. Lee G-C, Choi S-M, Lee CH, Lee D-G, Choi J-H, Yoo J-H. Detection of human cytomegalovirus UL97 D605E mutation in Korean stem cell transplantation recipients and donors. J Microbiol Biotechnol 2013; 23 (8): 1154–1158.

25. Puch SS, Ochoa C, Carballal G, Zala C, Cahn P, Brunet R et al. Cytomegalovirus UL97 mutations associated with ganciclovir resistance in immunocompromised patients from Argentina. J Clin Virol 2004; 30 (3): 271–275.

26. Zhou L, Qian J, Cai T. Study on cytomegalovirus gene mutations conferring resistance to ganciclovir in heart and lung transplant patients. Chinese J Infect Dis 1999 (01).

27. Schindele B, Apelt L, Hofmann J, Nitsche A, Michel D, Voigt S et al. Improved detection of mutated human cytomegalovirus UL97 by pyrosequencing. Antimicrob Agents Chemother 2010; 54 (12): 5234–5241.

28. Sung H, An D, Lee S-O, Choi S-H, Kim S-H, Chi H-S et al. Detection of a UL97 gene mutation conferring ganciclovir resistance in human cytomegalovirus: prevalence of the D605E polymorphism in Korean immunocompromised patients. Ann Clin Labor Sci 2012; 42 (4): 429–434.

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