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Detection of Human Cytomegalovirus UL97 D605E Mutation in Korean Stem Cell Transplantation Recipients and Donors

Gyu-Cheol Lee[1,2†], Su-Mi Choi[1†], Chan Hee Lee[3], Dong-Gun Lee[1*], Jung-Hyun Choi[1], and Jin-Hong Yoo[1]

[1]*Division of Infectious Diseases, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, Seoul 137-701, Republic of Korea*

[2]*Department of Microbiology, Water Analysis and Research Center, K-Water, Daejeon 306-711, Republic of Korea*

[3]*Department of Microbiology, College of Natural Sciences, Chungbuk National University, Cheongju 361-763, Republic of Korea*

Ganciclovir resistance of human cytomegalovirus is associated with mutations in the viral *UL97* gene and poses severe problems for immunocompromised patients. In this study, PCR-based restriction fragment length polymorphism and sequencing analyses detected the UL97 D605E mutation in all five clinical isolates from patients with ganciclovir-resistant human cytomegalovirus infection during prolonged ganciclovir therapy, whereas the M460V mutation was only present in 1 of 5 isolates. On the other hand, the detection rates of the D605E mutation in the stored available DNA samples from the donor and allogeneic stem cell transplantation recipients were 66.7% and 93.7%, respectively, suggesting that the presence of D605E mutation was not associated with the ganciclovir exposure. Although the D605E mutation may not be related to ganciclovir resistance, we suggest that this mutation could be an important molecular marker of human cytomegalovirus evolution in East Asian countries. Moreover, the restriction fragment length polymorphism method using the restriction enzyme *Hae*III, which is generally used to detect the UL97 A591V mutation, could also detect the D605E mutation and may therefore be a useful tool for future research on the investigation of *UL97* gene mutations.

Keywords: Ganciclovir, resistance, D605E, cytomegalovirus, stem cell transplantation

Introduction

Human cytomegalovirus (HCMV) can cause severe diseases in immunocompromised patients, especially those with acquired immune deficiency syndrome and transplantation recipients receiving immunosuppressive therapy [2]. Ganciclovir (GCV) is widely used for treating HCMV infection or diseases. However, since GCV resistance was first reported by Erice *et al.* [13] in 1989, the appearance of GCV-resistant HCMV has been an issue in the treatment of HCMV, particularly among immunocompromised patients. Numerous GCV-related mutations have been reported [4–6, 8–10, 12, 16, 18, 22]. The D605E mutation, for example, was first detected by Alain *et al.* [1]. Since then, there have been many reports about the mutation, but its exact

contribution to GCV resistance still remains unknown. Ijichi *et al.* [15] reported that the D605E mutation confers hypersensitivity to GCV by increasing GCV phosphorylation, whereas other studies have suggested that the mutation is not related to GCV resistance. Recently, a number of reports have surveyed the prevalence of D605E [3, 7, 20, 24], and Tanaka *et al.* [23] suggested that the mutation may represent a molecular marker of the HCMV strains circulating in East Asian countries.

In the present study, HCMV clinical isolates from Korean stem cell transplantation (SCT) recipients with suspected GCV-resistant HCMV infection or diseases were tested by restriction fragment length polymorphism (RFLP) analysis for GCV-related mutations. Furthermore, the detection rate of the D605E mutation in SCT recipients and

the donor group was also analyzed.

Materials and Methods

Patients and Blood Samples

GCV resistance was considered as antigenemia that persisted after 4 weeks of preemptive therapy with GCV or if the level of antigenemia continued to rise after 3 weeks of GCV therapy, according to the suggestion of the Centers for Disease Control and Prevention (CDC) [2]. From 2003 to 2004, HCMVs were isolated from blood samples of allogeneic SCT patients with suspected GCV-resistant HCMV infection during preemptive GCV therapy [17]. To study the detection rate of the D605E mutation, 247 available DNA samples were randomly selected from the stored blood bank from SCT recipients with HCMV antigenemia through October 2000 to April 2003, and 13 DNA samples were also selected from the stored blood bank from donors. The Institutional Review Board of Seoul St. Mary's Hospital approved the research protocol with a waiver of informed consent (KC12SISI0661).

Cells and Viruses

Human foreskin fibroblasts (HFF, CRL-2097) purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured at 37ºC in a 5% CO_2 incubator in Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville, Inc., USA), supplemented with 10% fetal bovine serum (US Bio-Technologies Inc., USA) and penicillin (100 units/ml)-streptomycin (100 µg/ml) solution (JBI, Korea). Clinical isolation and plaque purification of HCMV were performed as previously described [17].

RFLP Analysis of GCV Resistance-Related UL97 Mutations

Nested PCR-based RFLP was performed to analyze the GCV-resistant mutations in UL97 at positions 460 (M to V), 520 (H to Q), 591 (A to V), 592 (C to G), 594 (A to V), 595 (L to S or F), 603 (C to W), and 605 (D to E) [4, 11, 19]. For the first-round PCR, 100 pmol of primers (2 µl each), 5 µl of 10 mM dNTP, 5 µl of 10× PCR buffer, 5 µl of template DNA, and 0.5 µl of Taq polymerase (5 units/ml) were mixed with distilled water to a total volume of 50 µl. The PCR conditions comprised a hot start at 94ºC for 2 min; 40 cycles of 94.5ºC for 1 min, 55ºC for 1 min, and 72ºC for 1 min; and extension at 72ºC for 5 min. The second-round PCR (Nes 1–3, Table 1) comprised a hot start at 94ºC for 2 min; 40 cycles of 94ºC for 1 min, 58.5ºC for 1 min, 72ºC for 1 min; and extension at 72ºC for 5 min. The primer sets employed in the PCR are shown in Table 1. The PCR-amplified fragments were purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) and digested with eight different restriction endonucleases (New England Biolabs, Inc., USA), as indicated in Table 1. The products were subjected to electrophoresis on 4% agarose gels (MetaPhor Agarose, Cambrex Bio Science), stained with ethidium bromide, and photographed using a UV transilluminator. The presence of mutations was determined by distinctive restriction patterns (Table 1).

Nucleotide Sequence Accession Numbers

GenBank accession numbers for the five clinical HCMV isolates are as follows: SM301, AY729049; SM302, AY729050; SM303, AY659927; SM304, AY659928; and SM305, AY727868.

Results

Assessment of Genotypic GCV Resistance by RFLP Among Clinical Isolates

Five clinical HCMV isolates (SM301–305) were recovered from SCT patients with GCV-resistant HCMV infection. The PCR-based RFLP analysis was conducted on these HCMV DNA samples, and the resulting band pattern was compared with that of the HCMV wild-type strain AD169. As shown in Fig. 1, the *Nla*III cleavage at a single site

Table 1. Primer sequences and theoretical patterns of the nested PCR-based RFLP analysis.

PCR	Primer sequence (5′–3′)	Size (bp)	Restriction enzyme	UL97 mutation	RFLP (bp)	
					Wild type	Mutant
First	GTTGGCCGACGCTATCAAAT GGTCCTCCTCGCAGATTA	776				
Nes 1	GTTGGCCGACGCTATCAAAT GCATGGGTCGGAAAGCAA	266	*Nla*III	M460V	201, 65	Not cut
			*Alu*I	H520Q	Not cut	247, 19
Nes 2	CCTCATGCGGCTGTTGGACC CCATGCTCGCCCAGGAGA	189	*Hae*III	A591V	106, 83	Not cut
			*Fse*I	C592G	Not cut	104, 85
			*Hha*I	A594V	59, 38, 23, 18, 16, 12	59, 50, 23, 18, 16
			*Ava*II	C603W	172, 17	104, 68, 17
			*Hae*III	D605E	106, 83	83, 61, 45
Nes 3	CCTCATGCGGCTGTTGGACC GCAGTGCGTGAGCTTGCCGTTCTT	121	*Mse*I	L595F	67, 54	67, 30, 24
			*Taq*I	L595S	Not cut	96, 25
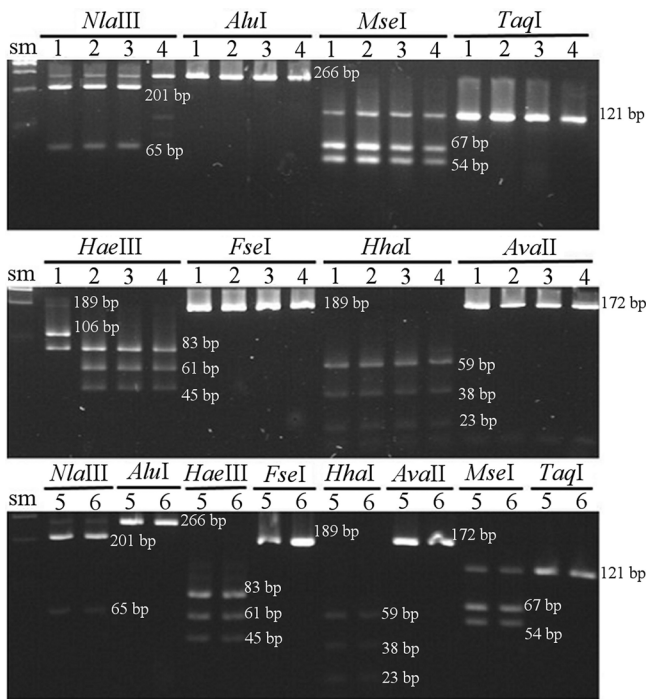
Fig. 1. RFLP analysis of the PCR-amplified *UL97* sequence.
For detection of GCV resistance-related mutations, DNA was obtained from the HCMV AD169 laboratory strain (lanes 1) or the clinical HCMV isolates (lane 2, SM304; lane 3, SM303; lane 4, SM305; lane 5, SM301; lane 6, SM302). The bands resolved on 4% agarose gels are indicated with their length sizes in bp. The results were consistent with the theoretical patterns of restriction enzyme digestions (see Table 1).

resulted in two bands of 201 and 65 bp for the wild-type AD169 strain and the SM301–304 isolates. In contrast, the PCR product from SM305 was not cleaved, and therefore only a single 266 bp band was observed. These results demonstrated that the HCMV SM305 isolate had the UL97 M460V mutation. Moreover, the UL97 PCR product from HCMV AD169 could be cleaved once by *Hae*III at the sequence encoding alanine (GCC) at position 591, resulting in the generation of two bands of 106 and 83 bp. Substitution of this A591 with valine (GTC) changed the *Hae*III restriction site from GGCC to GTCC, thereby preventing cleavage (Table 1). However, surprisingly, three unexpected bands (83, 61, and 45 bp) were observed for all clinical
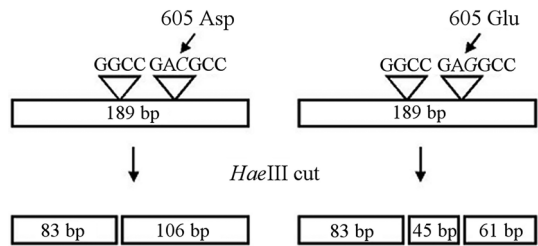
Fig. 2. Effect of the D605E mutation on *Hae*III RFLP.
Substitution of aspartate (Asp) at position 605 (GAC, left) with glutamate (Glu) (GAG, right) resultd in the generation of a new *Hae*III restriction site (GGCC, right).

isolates (Fig. 1). To investigate the presence of these bands after *Hae*III digestion, the UL97 PCR products were sequenced. The result showed that the sequence encoding aspartate (GAC) at position 605 was substituted with that encoding glutamate (GAG), thereby generating a new *Hae*III restriction site (Fig. 2). In summary, the M460V mutation was detected only in the clinical HCMV SM305 isolate, whereas the D605E mutation was detected in all five isolates. No other UL97 mutations were found by the RFLP analysis performed (Fig. 1), and no mutations in UL54 were observed in any of the five isolates (data not shown).

Detection Rate of the D605E Mutation Among Blood Samples

Among 247 DNA samples from SCT patients, UL97 PCR products could be obtained from 126 samples, 93.7% (118/126) of which displayed the D605E mutation (Table 2). Three of 13 DNA samples from the donor group generated the UL97 PCR products, and the D605E mutation was detected in 2 (66.7%) (Table 2). Moreover, from among the 129 UL97 PCR positive samples, 22 had been exposed to GCV (average, 13.7 days) before sample collection, and all of them had the D605E mutation. Interestingly, the mutation also existed in 91.6% of the GCV-naïve samples (98 of 107).

Discussion

Five clinical HCMV strains were isolated from SCT recipients with suspected GCV-resistant HCMV infection

Table 2. The detection rate of the D605E mutation among the donor and SCT recipient samples.

Group	Number of HCMV DNAemia	Number of PCR-positive	UL97 genotype		Detection rate of D605E (%)
			Wild type	D605E	
Donor	13	3	1	2	66.7
Recipient	247	126	8	118	93.7

in the previous study, based on the definition of GCV resistance suggested by the CDC [2]. Plaque reduction assays were performed and showed that the SM302 (IC_{50}, 9.5 μM) and SM305 (IC_{50}, 44.5 μM) isolates were phenotypically resistant to GCV [17]. The UL97 M460V mutation, which was detected in the clinical HCMV SM305 isolate, is known to be associated with GCV resistance [3]. Since M460 is located in close proximity to the proposed UL97 ATP-binding site involved in substrate recognition, mutation of this amino acid to valine may explain the GCV resistance of SM305 [14, 21].

On the other hand, the other four isolates (SM301–304) in the present study contained only the D605E mutation, and none of them were hypersensitive to GCV. Therefore, it is unlikely that the D605E mutation is associated with GCV hypersensitivity and is sufficient to confer GCV resistance.

A relatively lower detection rate of the D605E mutation was observed in donors than in SCT recipients (Table 2). Moreover, all 22 samples from patients treated with GCV had the D605E mutation, and therefore the possibility that D605E was a mutation resulting from GCV exposure still could not be ruled out. Alain *et al.* [1] and Sánchez Puch *et al.* [20] have reported that the D605E mutation may act as a compensatory mutation for the GCV resistance-related mutations. Interestingly, the results also showed that the D605E mutation was present in 98 (91.6%) of 107 GCV-naïve samples, demonstrating that the presence of D605E was not significantly associated with GCV exposure. However, the high detection rate of the D605E mutation in the GCV-naïve samples implies that although the mutation may not be caused by GCV exposure, it may have occurred naturally in the HCMV strains circulating in Korea. In fact, Zhou *et al.* [24] suggested that since the prevalence of the D605E mutation was 78%, the mutation may be regarded as a natural sequence variant. In contrast to its low prevalence among the HCMV strains circulating in Western countries, the D605E mutation was shown to occur more frequently (91.8%) in Japan and therefore has been suggested to represent a genetic marker for the HCMV strains circulating in East Asian countries [23].

*Hae*III RFLP analysis is generally used to detect the HCMV UL97 A591V mutation; we have shown here, for the first time, that it can also detect D605E (Figs. 1 and 2). Therefore, the PCR-based RFLP analysis using *Hae*III may aid future research on the prevalence of D605E. Furthermore, to our knowledge, this is also the first report on the detection rate of the D605E mutation in Korean SCT recipients and donors. Although the D605E mutation in HCMV UL97 may

not be related to GCV hypersensitivity or resistance, we suggest that it could be a molecular epidemiological characteristic of the main HCMV strain circulating in East Asian countries.

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