MAJOR ARTICLE

Phenotypic Evaluation of Previously Uncharacterized Cytomegalovirus DNA Polymerase Sequence Variants Detected in a Valganciclovir Treatment Trial

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Background. In a large randomized trial comparing oral valganciclovir and intravenous ganciclovir for treatment of cytomegalovirus disease in solid organ transplantation, confirmed genotypic drug resistance was uncommon (<5%), but definitive interpretation was limited by the detection of 110 uncharacterized UL54 viral DNA polymerase sequence variants.

Methods. Based on treatment history and genetic locus of the sequence changes, 39 of the sequence variants were prioritized for recombinant phenotyping by construction of cloned viral mutants and drug susceptibility testing in cell culture.

Results. Four amino acid substitutions were newly confirmed to alter ganciclovir susceptibility: A505V and I726T conferred a borderline decrease in ganciclovir and cidofovir susceptibility, while Q578L and G841S conferred slightly decreased ganciclovir and foscarnet susceptibility. A nonviable phenotype was found for 8 mutations distributed among amino terminal, exonuclease and catalytic domains. Retesting of stored study specimens could not confirm the original detection of >20 sequence variants, including the nonviable mutations and several resistance mutations.

Conclusions. Newly phenotyped UL54 sequence variants did not significantly change the reported incidence of drug resistance in the clinical trial. Unrecognized sequence variants in diagnostic genotyping reports should be confirmed by additional testing in order to improve clinical decision making.

Keywords. Antiviral drug resistance; cytomegalovirus; ganciclovir; valganciclovir.

The prevalent use of ganciclovir and its oral prodrug valganciclovir as prophylaxis and therapy for cytomegalovirus (CMV) infection has increased the awareness of drug resistance, the risk of which increases with prolonged drug exposure, impaired host defenses, or insufficient drug delivery [1]. In high-risk populations such as seronegative recipients of a CMV-seropositive donor organ, the incidence of ganciclovir resistance among treated individuals is estimated to be in the 5%–12% range. Drug resistance is suspected when rising plasma viral loads or progressive disease occur during prolonged antiviral treatment. Although ganciclovir resistance was originally documented by phenotypic testing of CMV culture isolates, this is a slow process, unsuitable in contemporary clinical practice where viral isolation is rarely performed. Instead, genotypic resistance testing is now standard, and is based on the detection of diagnostic mutations in the UL97 kinase gene involved in the initial phosphorylation of ganciclovir or in the UL54 DNA polymerase gene that encodes the antiviral drug target for ganciclovir.

The accuracy of genotypic resistance testing depends on a comprehensive and validated database linking

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specific mutations with levels of drug resistance. Extensive clinical experience has defined a set of UL97 mutations that are the most frequent initial laboratory markers of ganciclovir resistance (M460V/I, H520Q, C592G, A594V, L595S, and C603W), accounting for about 80% of documented cases [1]. In the remaining cases, less common UL97 mutations (clustered at codons 590–607) or UL54 mutation may be the first genetic marker of ganciclovir resistance. UL54 mutations typically add to pre-existing UL97 mutation to increase the level of ganciclovir resistance and confer cross-resistance to other anti-CMV drugs. An ongoing difficulty with interpretation of UL54 mutations is the relatively frequent occurrence of natural sequence polymorphisms unrelated to drug resistance. This difficulty is compounded in prophylaxis studies, where no baseline comparator sequence is available. UL97 and UL54 sequence variants remain incompletely documented despite several surveys of clinical CMV sequences [1–6].

A randomized nonblinded treatment trial comparing 3 weeks of oral valganciclovir and intravenous ganciclovir, followed by 4 weeks of valganciclovir, in solid organ transplant recipients (VICTOR study, NCT00431353) involved 321 subjects [7], of which 275 were included in a published resistance substudy [8]. Study subjects included those with a prior antiviral treatment history. Based on genotypic testing of prospectively collected samples, 13 subjects (4.7% of those tested) were considered to have confirmed or probable ganciclovir resistance mutations during the treatment period (0–49 days). Among the 13 cases, 10 had UL97 mutations only, 1 had both UL97 and UL54 mutations, and 2 were considered to have a probable mixture of resistance-associated and wild-type UL54 sequences without UL97 change [8]. Left unresolved were 110 UL54 amino acid sequence variants that could not be classified as known resistance mutations or natural sequence polymorphism. This study aimed to resolve the status of these sequence variants in relation to ganciclovir resistance. The extensive recombinant phenotyping needed for this purpose relied on recent technical advances in the construction and testing of cloned CMV strains.

MATERIALS AND METHODS

Study Specimens

The VICTOR trial [7] and a resistance substudy [8] have been published. The present study analyzes the genotypes and associated phenotypes of previously uncharacterized sequence variants detected in the same specimens and subjects.

Genotypic Resistance Testing

This was performed as previously reported [8]. Plasma specimens from study subjects at days 0, 21, and 49 with polymerase chain reaction (PCR)–positive CMV loads >200 copies/mL and stored at −80°C were routinely tested. For the UL54 gene, oligonucleotide primers were used to amplify sequencing templates spanning UL54 codons 184–1017 in 45 and 40 nested cycles of PCR using Taq polymerase [9]. Following dideoxy chain termination sequencing (Big Dye, Applied Biosystems), amino acid sequence differences from standard strain AD169 (Genbank X17403) were tabulated, with mixed variant and wild-type sequences reported if mixed peaks were observed in the sequencing chromatograms. Some frozen plasma specimens were retested in the current study to investigate the reproducibility of unusual UL54 genotypes, using the same primers and reagents for PCR amplification but different thermal cyclers with faster temperature ramping and fewer total cycles (30 and 40 nested cycles). The sequencing method was not changed.

Prioritization of Sequence Variants for Phenotyping

Despite recent technical advances, it was impractical to phenotype all of the >100 uncharacterized sequence variants detected in the clinical trial. Therefore, antiviral treatment history and genetic locus of the observed variants were used to establish priority for phenotyping. Variants observed in patients with no history of CMV antiviral therapy were presumed to be unrelated to drug resistance, whereas variants that newly emerged and persisted during antiviral therapy would be given high priority. Because a majority of validated UL54 drug-resistance mutations occur at conserved functional residues [1], variants at these loci were also prioritized. Conservation is defined as the same amino acid encoding at homologous residues of the DNA polymerases of CMV, herpes simplex virus 1, varicella zoster virus, and human herpesvirus 6, as determined by pairwise BLAST (NCBI) amino acid sequence alignments with CMV. Conservation was identified at 226 of 1242 CMV residues. The viral load response to treatment was also considered, with priority given for persistent viral load >600 copies/mL or <10-fold reduction at day 21.

Recombinant Phenotyping

Recombinant phenotyping was predominantly performed in Portland using a standardized reporter-based yield reduction assay on recombinant viral strains produced by mutagenesis of a baseline bacterial artificial chromosome (BAC) clone derived from standard CMV strain AD169 [10, 11], as previously used to phenotype the variants observed in another valganciclovir clinical trial [12]. Mutant PCR primers were used to create a plasmid transfer vector containing the variant of interest and a selectable kanamycin resistance marker in a UL54 sequence context. This was recombined in an induced bacterial host into a baseline BAC clone (BA33) in which wild-type UL54 sequence had been removed. The selection marker was then removed through the induction of Flp recombinase. The resulting CMV BAC clone was checked for a correct restriction pattern. After BAC transfection into human fibroblast cultures, the recovered live virus was sequenced throughout UL54 to

confirm the intended variant. Drug susceptibilities for ganciclovir, foscarnet, and cidofovir were determined by the concentration required to reduce activity of the secreted alkaline phosphatase reporter by 50% (EC50) in comparison with control strain 3265 [11]. BAC clones noted to be nonviable after repeated transfection attempts were recombined with a wild-type transfer vector to restore the baseline sequence and demonstrate clone viability upon transfection. Additional recombinant phenotypes were determined in Quebec by analogous mutagenesis of a separate BAC clone (pHB5) of CMV strain AD169 and testing the derived viral strain for drug susceptibility by the standard plaque reduction assay [13].

RESULTS

Overview of UL54 Sequence Variation

Among the 275 subjects in the resistance substudy [8], 273 had at least 1 UL54 genotype result. Amino acid sequence variation from the strain AD169 sequence was noted in 263 subjects (96%), which in 173 cases was limited to known sequence polymorphisms [1] not affecting drug susceptibility (Table 1). The 4 most frequent polymorphisms resulted from reference strain AD169 not representing the consensus wild type at those codons. One subject had known resistance mutations UL54 A987G and UL97 C603W at study entry [8], while another with no antiviral treatment history had a knockout mutation L845P [14] mixed with wild-type sequence. The remaining 88 subjects (32%) had at least 1 of the 110 uncharacterized UL54 sequence variants; 43 of these subjects had received no prior anti-CMV therapy at the time the variant was first observed.

Sequence Variants Observed at Study Entry in Previously Untreated Subjects

Among the 45 such variants (Table 2), 33 were detected in only 1 subject each, 12 were detected only as mixtures with wild-type sequence, and 4 involved conserved residues. Although lack of prior antiviral exposure creates a strong presumption that the variants are unrelated to drug resistance, 3 were selected for phenotyping because of their conserved codons and/or proximity to known resistance mutations (F718L, G822D, and M828V), and 3 others because of a detectable viral load on day 21 of therapy (N345S, S660N, and E903G).

Sequence Variants Observed Only in Previously Treated Subjects

Table 3 lists the 65 variants, of which 37 (57%) occurred only as mixtures with wild-type sequence. All were observed in only 1 subject per variant, except that 1 study subject contributed the second instances of both variants P468S and E882K. Most such variants detected at study entry were not subsequently detected, implying no preferential selection during antiviral therapy and lower priority for phenotyping. Those first detected at study days 21 or 49 represented a change from baseline and almost all were selected for phenotyping (n = 24), irrespective of whether they were present in later specimens. No variant met the high-priority criterion of documented emergence and persistence during study treatment. Variants at all 8 conserved residues were phenotyped (E235G, D247N, D262N, A505V, Q578L, F718S, G841S, K947E).

Table 1. Frequency Distribution of Known UL54 Polymorphisms

Sequence Variant	Subjects (N)[a]
A885T	253
N898D	219
N685S	177
S655L	151
S897L	86
A688V	32
G874R	32
F669L	27
P887S	22
V355A	20
L890F	15
S884insS	11
P628L	9
S676G, A693T	4
S695T	3
D515G, T691A, A692V, V759M, E899K	2
G347D, G678S, A692S, Q868R, A972V	1

[a] Number of subjects with each of the variants listed.

Table 2. Sequence Variants Observed Without Prior Antiviral Therapy

Sequence Variant[a]	Subjects (N)[b]
G629S	4
I341T	3
(F718L)[c]	3
A269V, P375L, L394F, (H446Y), A472V, K519R, A614V, Q639H, P656S	2
G201E, S212F, (R264G), A285S, (V327G), *Q323H,* A336T, V337M, **(N345S)**[d], K426R, V450G, H465Y, A473V, (V483A), P617S, P628A, V634A, Q651E, G653S, **(S660N)**, R800C, ***(G822D)***[c], **(M828V)**[c], R847H, N855D, H863R, T892I, **(E903G)**[c], (G920S), V927M, S1000L, *D1005N*, (R1006C)[d]	1

[a] Variants in parentheses were detected only as mixtures with wild type. Variants in italics are at conserved residues. Variants in bold were selected for phenotyping.

[b] Number of subjects noted to have each listed mutation. At least 1 subject per mutation had no history of prior anti-cytomegalovirus therapy.

[c] Not found on resequencing of any of the involved specimens.

[d] Mixture confirmed on resequencing of the involved specimen.

Table 3. Sequence Variants From Subjects With Prior Antiviral Treatment

Sequence Variant[a]	Subjects[b]	Specimens[b]	Study Day[c]	Resequencing Result[d]
(P468S), (E882K)	2	2	0	
P206S, P342L, **T437M,** G451S, **S612N,** (A636T), (A636V), (F638S), **V654G,** H686T. H886insA, T691S, P747L, K853E, G878E, A885M, (S893L), R900W, (R917H), V998I, L999I	1	1	0	
(A692G), ***(F718S),*** **(Q795P)**	1	1	0	Not found
A505V, G657D, G659D, (G687S), **P859A,** D879G, (S880L)	1	2	0	
D515E, **A543S**	1	2	0	Confirmed
S675G	1	4	0	
(Q229K), ***(E235G), (D247N), (D262N),*** **(T271A),** (D279N), **(V284E), (D289N)[e], (Y380C), (F396L), (L424V), (F460L), (R581H), (G667N), (I726T), (E793V),** ***(G841S), (K947E)***	1	1	21	Not found
(V373G), **(S660G), (V902G), (M959T),** E1010V	1	1	21	
(V476G), (V482G)	1	1	21	Inconclusive[f]
Q578L, **Q795R**	1	1	49	Not found
(S664G)	1	1	49	
K316E	1	2	Past end	

[a] Variants in parentheses were detected only as mixtures with wild type. Variants in italics are at conserved residues. Variants in bold were selected for phenotyping.

[b] Number of subjects or specimens per listed variant.

[c] Earliest study day of detection of variant. Past end = post treatment.

[d] Results of re-extraction and resequencing of the involved specimen. No resequencing where results not shown.

[e] Originally reported as D288N and phenotyped as such (Table 4).

[f] V476G and V482G were originally detected in the same specimen. Resequencing with optimally positioned primer showed wild type at both codons; 1 of 2 other primers showed a variant-wild type mix at both codons.

Recombinant Phenotyping

CMV BAC clones were constructed to produce individual viral mutants that were tested for drug susceptibility. This was successfully completed for 31 of the 39 sequence variants selected for phenotyping; for the remaining 8 mutant BAC clones, no live viruses could be generated. Genotypes and phenotypes of the recombinant viruses generated in Portland are shown in Table 4. Control strain 3265 and well-known UL54 resistant mutants (UL54 A809V and A987G) were used to calibrate the reporter-based yield reduction assays [11]. A published uncharacterized mutation I726V ([15] erratum) was newly phenotyped. Among 28 tested recombinant viruses, only 3 had any detectable effect on drug susceptibility, with A505V and I726T conferring borderline ganciclovir-cidofovir resistance similar to I726V, while G841S conferred low-grade ganciclovir and foscarnet resistance with a slow-growth phenotype. Recombinants generated in Quebec are shown in Table 5. Among the 3 recombinant viruses tested, Q578L conferred low-grade ganciclovir and foscarnet resistance.

Mutations Conferring a Nonviable Phenotype

Table 6 shows the 8 mutations that appeared to have a nonviable phenotype based on inability to recover live virus following transfection of BAC clones into fibroblasts. These knockout mutations were widely distributed among functional domains of the polymerase. The exonuclease residues involved are not well conserved among herpesviruses, while residues F718 and G822 are conserved polymerase residues [16]. Because mutagenesis of BAC clones by recombination may cause extraneous errors and deletions impairing viability, multiple corroborating experiments were performed. Mutations E235G and V482G were independently transferred into different BAC clones in separate laboratories. Mutation F718L was independently recombined into the same baseline BAC clone twice in Portland, once by itself and once in combination with the 4 most common polymorphisms listed in Table 1 (and present in the clinical F718L-containing sequences), and neither BAC clone was viable. All nonviable BAC clones in Portland were sequenced to rule out errors throughout the region of the transfer vector (4.8 kb), and viable revertant BACs were successfully made from the nonviable clones by a second recombination with the wild-type transfer vector to remove the UL54 mutation. This makes it unlikely that remote errors and deletions caused the BAC nonviability. Finally, viability of both the F718S and M828V BACs was directly rescued by cotransfecting into fibroblasts each BAC DNA with a restriction fragment of wild-type transfer vector representing UL54 codons 324–1107. The recovered live virus was wild type at codons 718 and 828. These experiments rule out a defective BAC DNA preparation or transfection technique as a cause of nonviability.

Table 4. Genotypes and Phenotypes of Recombinant Viruses (Portland)

	Cidofovir				Ganciclovir				Foscarnet			
UL54 Variant[a]	EC50[b]	SD[c]	N[d]	Ratio[e]	EC50[b]	SD[c]	N[d]	Ratio[e]	EC50[b]	SD[c]	N[d]	Ratio[e]
Control strains												
wt	0.21	0.06	134		1.06	0.3	124		39	10	132	
I726V	0.40	0.08	12	**1.9**	2.06	0.41	11	**1.9**	45	6	8	1.2
A809V									140	32	87	**3.6**
A987G	0.94	0.29	120	**4.5**	5.37	1.33	9	**5.1**				
Newly phenotyped variants (N = 28)												
Q229K	0.20	0.06	8	1.0	1.32	0.19	11	1.2	47	15	10	1.2
D247N	0.19	0.06	8	0.9	1.00	0.17	7	0.9	52	12	13	1.3
D262N	0.22	0.03	8	1.0	1.16	0.17	7	1.1	45	16	10	1.2
T271A	0.15	0.04	10	0.7	1.04	0.18	9	1.0	37	14	12	0.9
V284E	0.22	0.06	8	1.1	1.28	0.14	8	1.2	47	16	18	1.2
D288N	0.24	0.07	8	1.2	1.27	0.40	9	1.2	45	8	8	1.2
F396L	0.20	0.06	8	0.9	1.51	0.30	10	1.4	35	7	14	0.9
L424V	0.27	0.08	10	1.3	1.38	0.26	9	1.3	46	10	14	1.2
T437M	0.21	0.07	8	1.0	1.05	0.18	8	1.0	31	9	8	0.8
F460L	0.25	0.05	8	1.2	1.28	0.26	8	1.2	47	9	10	1.2
A505V	**0.39**	0.14	26	**1.9**	**1.97**	0.60	27	**1.9**	41	13	9	1.0
A543S	0.21	0.04	8	1.0	1.07	0.33	9	1.0	38	8	8	1.0
S612N	0.21	0.04	14	1.0	0.97	0.26	10	0.9	29	7	7	0.7
V654G	0.24	0.05	9	1.1	1.18	0.32	8	1.1	42	12	8	1.1
S660G	0.18	0.01	10	0.9	1.05	0.32	11	1.0	43	9	8	1.1
S660N	0.21	0.04	7	1.0	1.26	0.26	9	1.2	32	6	8	0.8
G667N	0.17	0.05	8	0.8	0.91	0.31	12	0.9	39	5	10	1.0
A692G	0.19	0.05	8	0.9	0.86	0.25	8	0.8	39	12	10	1.0
I726T	**0.36**	0.09	10	**1.7**	**2.14**	0.59	11	**2.0**	42	9	8	1.1
E793V	0.14	0.05	9	0.7	0.92	0.26	13	0.9	45	11	10	1.1
Q795P	0.19	0.05	13	0.9	0.91	0.27	9	0.9	41	10	9	1.1
Q795R	0.19	0.05	8	0.9	1.11	0.28	8	1.0	40	15	10	1.0
G841S	0.22	0.09	12	1.1	**2.30**	0.64	15	**2.2**	**83**	18	7	**2.1**
P859A	0.20	0.06	11	1.0	0.89	0.14	9	0.8	31	10	8	0.8
V902G	0.21	0.07	10	1.0	1.27	0.36	9	1.2	39	9	10	1.0
E903G	0.21	0.04	13	1.0	1.21	0.25	8	1.1	36	9	8	0.9
K947E	0.22	0.07	7	1.0	1.03	0.34	13	1.0	41	7	8	1.1
M959T	0.20	0.04	7	0.9	1.27	0.38	9	1.2	47	11	10	1.2

Bold: Results associated with decreased drug susceptibility.

Abbreviations: BAC, bacterial artificial chromosome; EC50, concentration required to reduce activity of the secreted alkaline phosphatase reporter by 50%; wt, wild type.

[a] Mutation introduced into BAC and recombinant virus.

[b] Mean drug concentration (μM) required to reduce secreted alkaline phosphatase growth by 50% at 6–7 days post infection.

[c] Standard deviation of the EC50 values.

[d] Number of assays (performed over at least 4 separate dates).

[e] Ratio of EC50 to baseline strain.

Correlation of Altered Phenotypes With Treatment Outcomes

UL54 mutation A505V and UL97 mutation M460V were detected at baseline and day 21 (subject 523 [8]). Treatment response was poor as reflected in nondeclining 4-log copy/mL plasma CMV loads on days 21, 28, and 35 that remained at approximately 30% of baseline. In contrast, no clinical impact was discernible for the 3 subjects with detection of mutations Q578L, I726T, and G841S, respectively (Table 3). All of these subjects had viral load declines of >1 log/mL at 17 days, >2 log/mL at 28 days at which time the load was undetectable in 2 cases, and 1000 copies/mL in 1 case (I726T). None of the 3 mutations were subsequently detected. Plasma viral load half-lives

Table 5. Genotypes and Phenotypes of Recombinant Viruses (Quebec)

	Cidofovir		Ganciclovir		Foscarnet	
UL54 Variant[a]	EC50[b]	Ratio[c]	EC50[b]	Ratio[c]	EC50[b]	Ratio[c]
Control strain						
wt	1.00		1.60		112	
Newly phenotyped variants (N = 3)						
N345S	0.67	0.7	1.94	1.2	99	0.9
V476G	0.80	0.8	1.50	0.9	50	0.4
Q578L	0.84	0.8	**3.00**	**1.9**	**332**	**3.0**

Bold: Results associated with decreased drug susceptibility.

Abbreviations: BAC, bacterial artificial chromosome; EC50, concentration required to reduce activity of the secreted alkaline phosphatase reporter by 50%; wt, wild type.

[a] Mutation introduced into BAC and recombinant virus.

[b] Drug concentration (μM) required to reduce plaque formation by 50%.

[c] Ratio of EC50 to baseline strain.

were 15 days for subject 523 and averaged 7.4 days for the other 3, as compared with 7.75 days for subjects with wild-type UL54 sequences previously reported [8].

Eight newly characterized knockout mutations (Table 6) were detected in 9 specimens from 9 subjects, with a median viral load of 1755; all as mixtures with wild-type sequence. None were detected more than once per subject. Mutations at codon F718 were remarkable for the 4 separate subjects involved, including 2 with F718L at baseline and no history of antiviral treatment. Mutations G822D and M828V were detected with variants N345S and E903G (Table 2) in the same pretreatment specimen in a subject with no prior antiviral history. Mutation V482G was detected with V476G (Table 3) at day 21 after a viral load decline from 108 000 to 8550 copies/mL. Mutation E235G was detected at day 21 in a specimen with 428 copies/mL. Mutation Y380C was detected in the same subject as G841S above (subject 27 [8]). Mutation R581H was detected with known resistance mutation P522A and variant G667N (Table 3), all as mixtures with wild-type sequences at day 21 (subject 605 [8]). The day 49 viral load was below the limit of quantitation.

Table 6. Nonviable UL54 Mutants

Mutation	Structure Domain	Conserved Residue[a]	Revertant BAC Viable	Laboratory
E235G	Amino terminal 1	Yes	Yes	P, Q
Y380C	Exonuclease	No	Yes	P
V482G	Exonuclease	(VIL)	Yes	P, Q
R581H	Amino terminal 2	(RRK)	Yes	P
F718L	Palm	Yes	Yes	P
F718L[b]	Palm	Yes	Yes	P
F718S	Palm	Yes	Yes	P
G822D	Finger	Yes	Yes	P
M828V	Palm	(LLL)	Yes	P

Abbreviations: BAC, bacterial artificial chromosome; P, Portland; Q, Quebec; I, isoleucine; K, lysine; L, leucine; R, arginine; V,valine.

[a] Amino acids in parentheses found in herpes simplex virus 1, varicella zoster virus, and human herpesvirus 6.

[b] Cloned with polymorphisms S655L, N685S, A885T, and N898D.

Resequencing Analysis

The isolated appearance of multiple simultaneous unrecognized and mixed sequence populations in various specimens raised issues of technical artifact in the originally reported genotypes. Review of original sequencing chromatograms supported the diagnosis of double peaks compatible with mixed sequence subpopulations, but did not address the possibility of artifacts arising from PCR amplification. Available stored frozen original plasma specimens were assessed by re-extraction and resequencing. Results are shown as part of Tables 2 and 3 but were not available at the time of prioritization for phenotyping. Although all baseline polymorphisms were confirmed on resequencing, none of the knockout mutations (including L845P) could be detected again in the original specimens, except that V482G was variably present in some resequencing chromatograms. Among phenotypically validated resistance mutations, several were not detected after retesting, including G841S and P522A originally reported (subjects 27 and 605 [8]), and the newly phenotyped I726T and Q578L. Overall, 33 uncharacterized variants were assessed by resequencing of original samples, resulting in 27 not redetected, 4 confirmed, and 2 inconclusive.

Revised Tally of UL54 Resistance Mutations and Frequencies

The mutations newly confirmed to affect drug susceptibility would have added 1 case each of induced resistance after ganciclovir or valganciclovir in the original statistics on genotypic resistance (Tables 1 and 2 in [8]). However, assuming the accuracy of noncorroborating resequencing data, the original statistics are altered by the removal of G841S and P522A, which reduces the cases of induced resistance by 2 in the ganciclovir arm, while the newly phenotyped A505V did not change the count of subjects because it occurred in the same subject with UL97 mutation M460V (subject 523). There is no change in the incidence of baseline resistance. Induced resistance in the valganciclovir arm remains at 5/140 (3.6%) and decreased in the ganciclovir arm to 1/135 (0.74%), with a new P value of 0.11. Compared with the original counts [8], there are now 11 instead of 13 subjects with confirmed or probable resistance mutations (4% of the substudy population of 275), of which 9 had only UL97 mutations and 2 had both UL97 and UL54 mutations.

DISCUSSION

This extensive genotype-phenotype correlation study resolved the status of many uncharacterized UL54 DNA polymerase sequence variants encountered during a major antiviral treatment trial. Several mutations modestly decreasing drug susceptibility were newly characterized, while many other variants were shown to confer no significant effect on drug susceptibility by recombinant phenotyping. A surprising incidence of knockout mutations documented the functional importance of specific polymerase residues. Inability to confirm the detection of many variants in the original clinical specimens suggests the need for careful investigation of unrecognized sequence variants, including the possibility of technical artifact. Quality control of CMV genotypic resistance testing is an ongoing concern, especially as next-generation sequencing technology is being introduced.

Amino acid sequence variation in UL54 is characterized by several high-frequency polymorphisms (Table 1), along with many unusual ones occurring at a frequency of 1% or less. The pretreatment baseline variants listed in Table 2 are new additions to the list, except that those found only as mixed populations were mostly not confirmed when available original samples were retested. This increasing database of polymorphisms should facilitate interpretation of genotypic resistance testing data.

Among the large number of variants found in those with a prior treatment history, the new resistance mutations were located at previously associated codons or domains, but the borderline or low levels of resistance conferred were not accurately predicted by existing data, thus reinforcing the need for individual characterization of mutations. Mutation A505V is located in the exonuclease domain where nearby mutations at codons 501, 503, and 513 confer higher levels of ganciclovir and cidofovir resistance [1]. The borderline resistance conferred by A505V may, however, increase the level of ganciclovir resistance in combination with the UL97 mutation M460V found in the same specimen [1, 17]. Mutation Q578L is located in an amino terminal polymerase domain containing 2 known resistance mutations D588N, which confers primarily foscarnet resistance [1], and Q578H, which was recently shown to confer 2.3- to 4.5-fold increases in EC50 for cidofovir, ganciclovir, and foscarnet [11]. Mutations in this domain and those in polymerase region III can confer clinically troublesome ganciclovir and foscarnet cross-resistance. Mutation I726T is close to critical conserved residues such as F718 and some well-known foscarnet resistance mutations [1], but the specific codon is nonconserved; both I726T and I726V conferred only borderline ganciclovir and cidofovir resistance. G841S is located close to other region III mutations with various multiresistant phenotypes [1], but this one confers a more severe growth defect than G841A [14].

Despite the new phenotypic confirmation of mutations that decrease ganciclovir susceptibility, the confirmed incidence of genotypic drug resistance in the clinical trial decreased slightly as a result of this study because of original sequencing data not confirmed on retesting. Although it is not proven that the retesting data are more accurate, circumstantial evidence supports this interpretation because the unconfirmed original genotypic findings were associated with viral load decreases compatible with successful therapy, and usually included multiple simultaneous unrecognized variants or knockout mutations not found in any other specimens from the same subjects.

The newly characterized UL54 knockout mutations illustrate the wide distribution of critical functional residues, including in an amino terminal domain not usually covered in genotypic testing (codon 235), and drawing attention to lesser-known polymerase domains than the exonuclease, finger, palm, and thumb structures. These functional knockout mutations add significantly to those previously documented in diagnostic genotyping reports, including UL97 mutations V466G and P521L [18–20], and UL54 mutations G698D [12] and L845P [14], the last of which was also nonreproducibly detected in this study. All reported occurrences were as isolated findings in single specimens per subject, as mixtures with wild-type sequence where information was provided, and no authentication by additional specimens, culture isolates, or independent resequencing. No documented treatment outcomes suggest that subpopulations of UL54 polymerase knockout mutants likely caused drug resistance. The remote possibility that knockout mutations may be viable in the context of specific UL54 sequence polymorphisms could not be validated for F718L, which was originally detected in 3 subjects.

The absence of many mixed variants upon retesting raises issues of technical artifact that have been given insufficient emphasis in the CMV drug resistance literature. The results reported here do not arise from contamination of specimens or PCR reagents, because the detected variants were typically unique. Instead, they probably resulted from the routine processing of specimens with a low or declining viral load (not usually ordered for diagnostic purposes), and/or technical factors in PCR amplification (enzyme, buffer, thermal cycling conditions), which influence the error rate and are poorly standardized. In this study, the original detection of many nonreproducible mixed sequence changes was probably related to the slower temperature ramping and more cycles of PCR than used later on. It is recommended that laboratories review their protocols to enhance the accuracy of amplification.

The current study supports the consensus of the existing literature [1]. The vast majority of ganciclovir resistance occurs initially by UL97 mutation, and resolving the status of UL54 mutations in this study strengthened this impression. Although in 2 instances UL54 mutation plausibly augmented the resistance conferred by a coexisting UL97 mutation, the detection of UL54 resistance mutations without any UL97 change was not reproducible in any of the 4 involved cases, and had no

discernible impact on the viral load response to treatment that was often observed with canonical UL97 resistance mutations. The emergence of ganciclovir resistance during a 7-week treatment period remains uncommon (4%) after considering the effects of numerous UL54 sequence changes. Interpretation of resistance genotyping reports should assess the levels of resistance conferred by specific mutations, and isolated findings of unrecognized mutations should be confirmed by retesting, preferably with independent specimens.

Notes

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