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Short Communication

Genotypic characterization of human cytomegalovirus UL97 phosphotransferase natural polymorphism in the era of ganciclovir and maribavir

David Boutolleau *, Sonia Burrel, Henri Agut

UPMC Univ Paris 06, ER1 DETIV, F-75013, Paris, France
Service de Virologie, Groupe Hospitalier Pitié-Salpêtrière, AP-HP, F-75013, Paris, France

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ABSTRACT

The molecular mechanisms of human cytomegalovirus (CMV) resistance to both ganciclovir and maribavir reported so far rely predominantly on the presence of mutations within UL97 phosphotransferase. The accurate interpretation of genotypic antiviral resistance assay results requires the clear distinction between resistance mutations and natural interstrain sequence variations. The objective of this work was to extend the catalog of CMV UL97 phosphotransferase natural polymorphisms. The full-length UL97 gene sequence analysis from 4 laboratory strains and 35 clinical samples from patients who had not received any previous anti-CMV treatment was performed. At the nucleotide level, the interstrain identity was >98%. At the amino acid level, ten natural polymorphisms never previously described were identified. Together with all previous results reported in the literature, a new map of UL97 phosphotransferase natural polymorphism could be settled in the era of ganciclovir and maribavir.

In the transplant setting, the emergence of human cytomegalovirus (CMV) resistance to antiviral drugs constitutes a raising therapeutic challenge. The virus-encoded UL97 phosphotransferase plays a major role in the antiviral treatment of CMV infection (Prichard, 2009). This viral enzyme performs the activation, by selective phosphorylation, of ganciclovir, a nucleoside analogue which constitutes together with its prodrug valganciclovir the first-line antiviral for prevention and treatment of CMV diseases in current clinical practice. Moreover, the benzimidazole L-riboside maribavir inhibits directly UL97 phosphotransferase enzymatic activity. This experimental anti-CMV drug may constitute an interesting alternative in case of CMV infection that is resistant and/or refractory to standard treatments. The molecular mechanisms of CMV resistance to ganciclovir and maribavir reported so far rely mainly on the presence of mutations within UL97 phosphotransferase (Chou, 2008). Ganciclovir resistance mutations are clustered at codons 460, 520, and 590–607, whereas maribavir resistance mutations are located upstream, at codons 353, 397, 409, and 411 (Chou et al., 2007; Chou and Marousek, 2008). To a lesser extent, mutations in UL54 DNA polymerase may account for ganciclovir resistance (Chou, 2008). Nowadays, genotypic assays, based on the identification of mutations within UL97 and UL54 genes, permit the determination of CMV antiviral resistance in a clinically relevant time frame. However, the accurate interpretation of genotypic assays requires distinguishing clearly resistance-associated mutations from interstrain natural polymorphisms. To date, UL54 DNA polymerase natural polymorphism has been extensively studied in numerous CMV strains (Chou et al., 1999; Fillet et al., 2004), whereas only few ganciclovir-sensitive clinical CMV strains have been investigated regarding UL97 phosphotransferase natural polymorphism (Chou et al., 1995a; Lurain et al., 2001). In fact, studies describing UL97 natural polymorphism mainly focused on codon range 460–607 where all known ganciclovir resistance mutations are located (Chou, 2008).

The aims of this work were to identify new natural polymorphisms within CMV UL97 phosphotransferase and to perform a review of the literature to provide a precise map of CMV UL97 natural polymorphism in order to improve the interpretation of CMV UL97 genotyping resistance testing.

This study included the analysis of 4 laboratory strains (AD169, Towne, Toledo, Davis) and 35 biological samples from unrelated patients experiencing CMV infection and who had not received any previous anti-CMV treatment. The biological samples collected were whole blood from transplant recipients ($n = 23$), urine from newborns with CMV congenital infection ($n = 8$), amniotic fluid from pregnant women ($n = 3$), and bronchoalveolar fluid from a HIV-infected patient ($n = 1$). Eighteen samples were previously used for the natural polymorphism study of CMV UL44 processivity factor (Boutolleau et al., 2009a). CMV DNA extraction was

* Corresponding author at: Service de Virologie, Bâtiment CERVI, Groupe Hospitalier Pitié-Salpêtrière, 83 boulevard de l'Hôpital, F-75013, Paris, France. Tel.: +33 1 42 17 72 89; fax: +33 1 42 17 74 11.
E-mail address: david.boutolleau@psl.aphp.fr (D. Boutolleau).

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performed using the MagNA Pure Compact Instrument (Roche, Meylan, France). A new method was developed for the sequencing of CMV UL97 phosphotransferase gene. The full-length gene (2124 bp) was amplified by nested PCR using the proofreading enzyme Expand High Fidelity (Roche). Thereafter, amplification products were sequenced using 4 overlapping primer pairs with the Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Courtaboeuf, France) and analyzed with the automated sequencer ABI 3730 Genetic Analyzer (Applied Biosystems). All primers for amplification and sequencing are indicated in Table 1. Nucleotide and amino acid sequences were compared with that of CMV reference strain AD169 (GenBank accession No. BK000394) using SeqScape v2.5 software. All UL97 phosphotransferase sequences determined in this study have been deposited in the GenBank database under accessions No. HQ158764 through HQ158798.

The sensitivity of UL97 nested PCR was assessed by testing serial dilutions of quantified genome from the laboratory strain AD169. After optimization, the detection threshold was 500 copies/mL.

At the nucleotide level, the interstrain identity of the full-length UL97 gene ranged from 98.8% to 100% among the 4 laboratory strains and the 35 clinical samples investigated (Table 2). In comparison with AD169 reference strain, a total of 102 nucleotide mutations were identified within the 2124 bp coding sequence. For each strain, the number of nucleotide mutations ranged from 0 to 25 (mean 17.6). The majority of these nucleotide mutations (84.3%) were silent. Thus, at the amino acid level, 16 different amino acid changes were identified (that is, 2.3% of the total codons of the protein). Each CMV strain harboured a mean number of 3 amino acid changes. Overall, the amino acid interstrain identity of the UL97 phosphotransferase was >99%. The frequency of these amino acid changes related to natural polymorphism ranged from 2.6% (1/39) for 9 of them to 92.3% (36/39) for 2 of them (Table 3). Ten changes were never previously reported: S7F, L14F, R112H, P132L, S133P, V171I, A238T, P247S, S249C and N467D. All those changes but one (N467D) were located outside the conserved domains of UL97 phosphotransferase. Of note, this latter amino acid change was reproducibly evidenced in another biological specimen collected from the same patient and using a different set of primers for PCRs and sequencing, as previously described (Boutolleau et al., 2009b) (data not shown). The new data provided by this work together with the data reported so far in the literature enabled to settle the map of CMV UL97 phosphotransferase natural polymorphism (Boivin et al., 2004; Chou, 2010; Chou et al., 1995a, 2002, 2005, 2007, 2010; Foulongne et al., 2004; Hu et al., 2002; Iwasenko et al., 2009; Lurain et al., 2001) (Fig. 1). This natural polymorphism is unevenly distributed alongside the 707-amino-acid long viral protein. Indeed, amino acid changes are mainly clustered in two distinct regions corresponding to codon ranges 1–249 (N-terminus) and 427–674 (C-terminus). Overall, 44 amino acid positions (corresponding to 47 different amino acid changes) are associated with natural polymorphism of CMV UL97 phosphotransferase, that is 6.2% of the total codons of the protein. Fifteen positions (corresponding to 17 different amino acid changes) are located within conserved domains: domains I ($n = 1$), VI ($n = 6$), VII ($n = 1$), IX ($n = 4$), and XI ($n = 3$). As indicated in Fig. 1 (in bold), 26 (59.1%) amino acid positions have been assessed as natural polymorphisms by marker transfer experiments (Chou, 2010; Chou et al., 2002, 2005, 2007; Iwasenko et al., 2009; Martin et al., 2006).

This study aimed to describe extensively the natural polymorphism of CMV UL97 phosphotransferase, the viral target for both antiviral drugs (val)ganciclovir and maribavir, especially within the first half of the protein, from the N-terminus to the conserved domain I (codon range 1–329). For this purpose, primer pairs were specifically designed to enable the sequencing of the full-length UL97 gene. The low detection threshold of this technique (i.e., 500 copies/mL) allowed the amplification and sequencing of the UL97 gene directly from blood samples obtained from patients experiencing a drug-resistant CMV infection (Boutolleau et al., 2009b).

Our results obtained from 4 laboratory and 35 clinical CMV strains demonstrated a very high level of sequence conservation of the UL97 phosphotransferase coding region, with a 99.2% average interstrain identity at the nucleotide level. At the amino acid level, 16 changes of natural polymorphism were identified, corresponding to an amino acid variation of 2.3% of the total codons of the protein. The weak variability of UL97 phosphotransferase is similar to the one previously reported for UL54 DNA polymerase, the other main viral target of anti-CMV drugs (Chou et al., 1999; Fillet et al., 2004). In this study, 6 out of the 16 amino acid changes identified were previously reported as UL97 phosphotransferase natural polymorphisms. The changes Q19E, N68D, S108N, L126Q, I244V were evidenced in phenotypically ganciclovir-sensitive CMV clinical strains (Chou et al., 1995a; Lurain et al., 2001). The M615V change, initially identified in a ganciclovir-resistant CMV clinical isolate (Foulongne et al., 2004), was recently demonstrated as a natural polymorphism by transfer marker experiments (Martin et al., 2010a). Furthermore, 10 amino acid changes were described for the first time in this study. All but one were located outside the conserved domains of the viral enzyme, within the N-terminus region of the protein (Fig. 1). The remaining N467D change was located in the conserved domain VI. However, other natural polymorphisms, such as A427V, A440V, A442G, Q449K/R, and V466M, have been previously described within this domain (Chou, 2010; Chou et al., 1995a,b, 2010; Lurain et al., 2001; Martin et al., 2006).

Until this study, 35 different amino acid positions related to natural polymorphism had been identified so far in CMV UL97 phosphotransferase, but only 10 (28.6%) were located in the first half of the protein (N-terminus). Our study led to the identification

Table 1
Primers used for amplification and sequencing of CMV full-length UL97 gene.

Function	Name	Sequence (5′ → 3′)
First-round PCR (outer primers)	UL97-F1	F: CGACGCCGTCTAACAGGTAT
	UL97-R1	R: CTCATCGTCGTCGTAGTCCA
Second-round PCR (inner primers)	UL97-F2	F: TCACGCCTCTGTTCAGATTTT
	UL97-R2	R: CGGTGGGTTTGTACCTTCTC
Sequence reaction	UL97-A	R: TCGTACTCGAACGACCACAT
	UL97-B	F: GAAACTTCGGCCATGTGGT
	UL97-C	R: CAGACCTCGCCGAAGGAG
	UL97-D	F: GACATGAGCGACGAGAGCTA
	UL97-E	R: AAAAGCCCAGCACGTTACC
	UL97-F	F: CTACGGCGTTATTGCATGTC
	+UL97-F2 and UL97-R2	

F, forward primer; R, reverse primer.

Table 2
UL97 phosphotransferase variation, both at nucleotide and amino acid levels, among 4 laboratory strains and 35 clinical strains of CMV in comparison with reference strain AD169 (GenBank accession No. BK000394).

Parameter	CMV UL97 phosphotransferase
Nucleotide identity (%)	98.8–100
Nucleotide mutations (No.)	102
Frequency per strain (mean)	0–25 (17.6)
Silent mutations (%)	86 (84.3)
Amino acid identity (%)	99.2–100
Amino acid changes (No.)	16
Frequency per strain (mean)	0–6 (3)
Variation of the total codons (%)	2.3

Table 3
Amino acid changes related to natural polymorphism of CMV UL97 phosphotransferase identified in this study in comparison with AD169 reference strain (GenBank accession No. BK000394).

Amino acid changes[a]	No. of strains	% of strains	References
S7F	1	**2.6**	
L14F	1	**2.6**	
Q19E	14	35.9	Chou et al. (1995a,b), Lurain et al. (2001)
N68D	36	92.3	Lurain et al. (2001), Chou et al. (2010)
S108N	14	35.9	Chou et al. (1995a,b), Lurain et al. (2001)
R112H	1	**2.6**	
L126Q	12	30.8	Lurain et al. (2001), Chou et al. (2010)
P132L	2	**5.1**	
S133P	1	**2.6**	
V171I	1	**2.6**	
A238T	1	**2.6**	
I244V	36	92.3	Lurain et al. (2001), Chou et al. (2010)
P247S	3	**7.7**	
S249C	1	**2.6**	
N467D	1	**2.6**	
M615V	1	2.6	Foulongne et al. (2004), Martin et al. (2010a,b)

[a] Amino acid changes never previously reported are in bold.

of 8 new additional positions in this part of the protein, which represents 40.9% of the total positions. Fig. 1 represents the precise map of CMV UL97 phosphotransferase natural polymorphism according to all these data. Domains I–IV, involved in kinase activity (He et al., 1997) are the highest conserved since only one natural polymorphism assessed by marker transfer, D329H, has been reported (Chou et al., 2002).

Among all the 47 amino acid changes related to natural polymorphism described so far, 28 (59.6%) have been assessed by marker transfer or recombinant phenotyping (Chou, 2010; Chou et al., 2002, 2005, 2007; Iwasenko et al., 2009; Martin et al., 2006). Further studies are warranted to demonstrate that the remaining amino acid changes do not confer resistance to ganciclovir or maribavir.

Some amino acid positions of UL97 phosphotransferase can be involved in both natural polymorphism and antiviral resistance. Indeed, as recently demonstrated using recombinant viruses with mutated UL97 genes, V466G change, but not V466M change, confers resistance to ganciclovir (Martin et al., 2010b). Similarly, K599T change was previously reported to induce ganciclovir resistance (Faizi Khan et al., 1998) whereas K599R change has been assessed as a natural polymorphism (Chou et al., 2010). This phenomenon of contrasting drug resistance phenotypes concerning the same location of mutation must be taken into account for the interpretation of CMV UL97 genotypic antiviral resistance testing.

Some natural polymorphisms may modulate the drug-resistance level provided by other mutations. Indeed, it has been evidenced by marker transfer that D605E change does not confer any ganciclovir resistance. Nevertheless, its potential effect on the degree of resistance conferred by other UL97 resistance changes, such as A594P or M460V remains to be ascertained (Ijichi et al., 2002; Sanchez Puch et al., 2004; Chou et al., 2005). Similarly, N510S change has been identified in phenotypically ganciclovir-sensitive CMV clinical isolates (Erice et al., 1998; Lurain et al., 2001), and it has been shown not to confer significant ganciclovir resistance (Chou, 2010). This N510S change has also been evidenced in a CMV clinical isolate harboring the 591–594 deletion associated with ganciclovir resistance, leading to the assumption that N510S change may be involved in the modulation of ganciclovir resistance induced by the 591–594 deletion (Chou et al., 1995b). However, the level of ganciclovir resistance of this CMV clinical isolate (EC_{50} [50% effective concentration] = 10.1 μM) was not higher than the one of a CMV isolate harboring the 591–594 deletion alone (EC_{50} = 10 μM) (Chou et al., 2002).

In conclusion, this work enabled to extend the catalog of CMV UL97 phosphotransferase natural polymorphisms. Studies are still needed to assess the role of some of them and to identify new ones. All these data are very useful for new search tools for the interpretation of genotypic drug resistance testing, as recently developed (Chevillotte et al., 2010).

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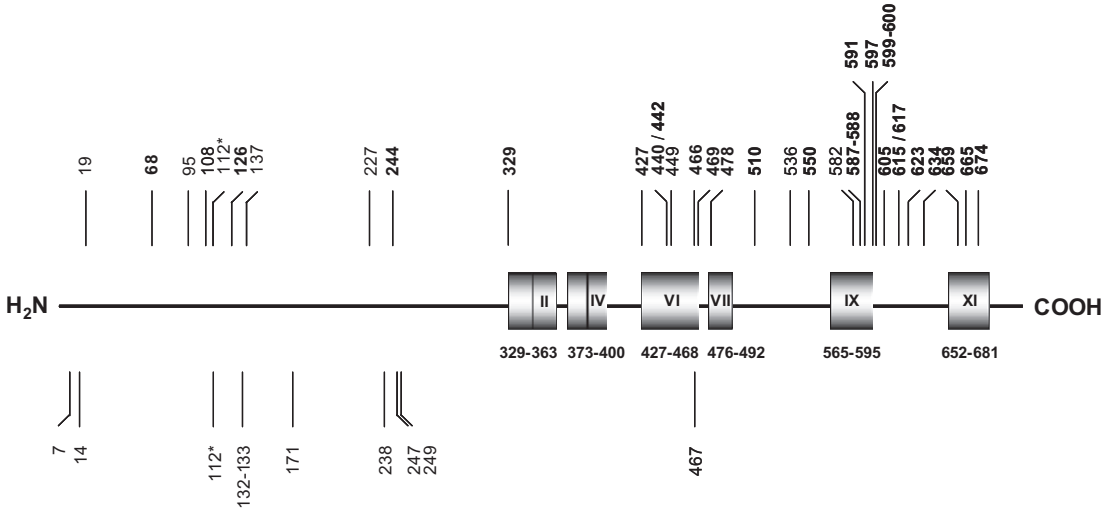

Fig. 1. Natural polymorphism map (to scale) of CMV UL97 phosphotransferase. Conserved regions are represented by the boxes. The positions (codon numbers) of these conserved regions are indicated under each box (according to Hanks et al. (1988)). All amino acid positions related to natural polymorphism reported so far in the literature are indicated above. Natural polymorphisms that have been assessed by marker transfer or recombinant phenotyping are indicated in bold. Amino acid positions related to natural polymorphism newly described in this study are indicated below. *Amino acid change R112C was previously reported by Lurain et al. (2001); amino acid change R112H was newly identified in this study.

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