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Short Communication

# Novel DNA polymerase mutations conferring cytomegalovirus resistance: Input of BAC-recombinant phenotyping and 3D model

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ABSTRACT

Long-term exposure to antiviral therapy in immunocompromised patients favors emergence of human cytomegalovirus (HCMV) resistance mutations. Two new UL54 DNA polymerase mutations (deletion of codon 524 and N408S substitution) identified in a kidney recipient and a bone marrow recipient respectively were characterized. Marker transfer experiment through recombination into a HCMV AD169 BAC demonstrated del524 and mutation N408S confer GCV and CDV resistance. These results suggest continued mutation of UL54 under selective antiviral pressure. Characterization of each new mutation is thus required to inform genotypic assays and to better understand the functional regions of UL54 for the development of novel antivirals.



Human cytomegalovirus (CMV) infections are common events in transplants recipients. Antiviral treatment or prophylaxis for CMV may result in the development of drug resistance (Boivin et al., 2012; Boivin et al., 2009). Resistance mutations are detected most often within the *UL97* gene, conferring resistance to GCV alone. Afterwards resistance mutations can emerge in the DNA polymerase gene *UL54*. Contrasting with UL97 mutations, which are confined to a relatively small genomic region, DNA polymerase mutations responsible for antiviral resistance are spread out across a wider region of functional domains encoded by codons 301 (DNA polymerase exonuclease I region) to 987 (domain V) (Lurain and Chou, 2010). Genotyping each CMV strain detected in patients not responding to antiviral treatment allows detection of newly emerging mutations. Though their impact on resistance has to be assessed. During systematic follow-up of two transplant patients, two new UL54 mutations were detected after routine genotyping as previously described (Alain et al., 2004; Hantz et al., 2010): a deletion of codon 524 (del524) in a renal transplant recipient (from (Couzi et al., 2011), and an amino acid substitution N408S in a haematopoietic stem cell (HSCT) recipient. To determine the real impact of each mutation, the phenotypic characterization was assessed *in vitro* with recombinant viruses obtained through HCMV bacterial artificial chromosome (BAC) mutagenesis.

Single UL54 mutations (del524 and N408S) were introduced into an EGFP-expressing HCMV BAC (Borst and Messerle, 2000) to generate two mutants - del524_BAC and N408S_BAC. The HCMV BAC contains an enhanced green fluorescent protein (EGFP) gene in the unique short region and was derived from parental strain pHB5, a BAC-cloned genome of the CMV laboratory strain AD169 (Borst et al., 1999). The recombinant CMV strains bearing the desired point mutation or deletion in UL54 were generated using the *en passant* mutagenesis, a two-step markerless Red recombination system for BAC mutagenesis in *Escherichia coli* strain GS1783

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(Tischer et al., 2010). The recombinant BACs were transfected into MRC-5 cells (Biomérieux, Lyon, France) by use of the liposomal reagent Transfast™ (Promega, Madison, USA) to reconstitute virus mutants according to manufacturer's instructions. Cell-free virus stocks of recombinant fluorescent strains (del524_RV and N408S_RV) and AD169-EGFP strain were prepared after propagation of the viruses on MRC-5 cells. The *UL54* gene of each stock virus was sequenced to confirm the presence of the desired mutations in the recombinant viruses. Phenotypic susceptibilities to ganciclovir, cidofovir and foscarnet were performed by focus reduction assay, as described previously (Ducancelle et al., 2004) and IC50 of the mutants were compared with that of the wild-type BAC-CMV. The growth properties of the mutant recombinant viruses over multiple cycles of replication were assessed by inoculating strains del524_RV, N408S_RV and AD169-EGFP (as wild-type control) as triplicates, in 48-well MRC-5 cultures at MOI of 0.1. On days 1, 2, 3, 4, 5 and 10 post inoculation, number of fluorescent cytopathic foci was quantified to establish viral growth curves for each recombinant. A theoretical structure of pUL54, calculated by homology modeling with the standalone version of MODELLER 9.9 (Eswar et al., 2006) (see supplementary data), was used to localize these new mutations into the whole protein and speculate about their putative mechanisms of action.

*Case patient 1* was the recipient of a first kidney transplant (CMV serostatus D+/R−) (Fig. 1A). CMV infection was detected by routine PCR analysis on day 31 post-transplantation. After more than 8 months of GCV therapy, the CMV viral load increased, associated with detection of UL97 mutation M460I and UL54 deletion of codon 524. Mutation M460I in UL97 kinase is known to induce GCV resistance alone. In contrast, the deletion of codon 524 in

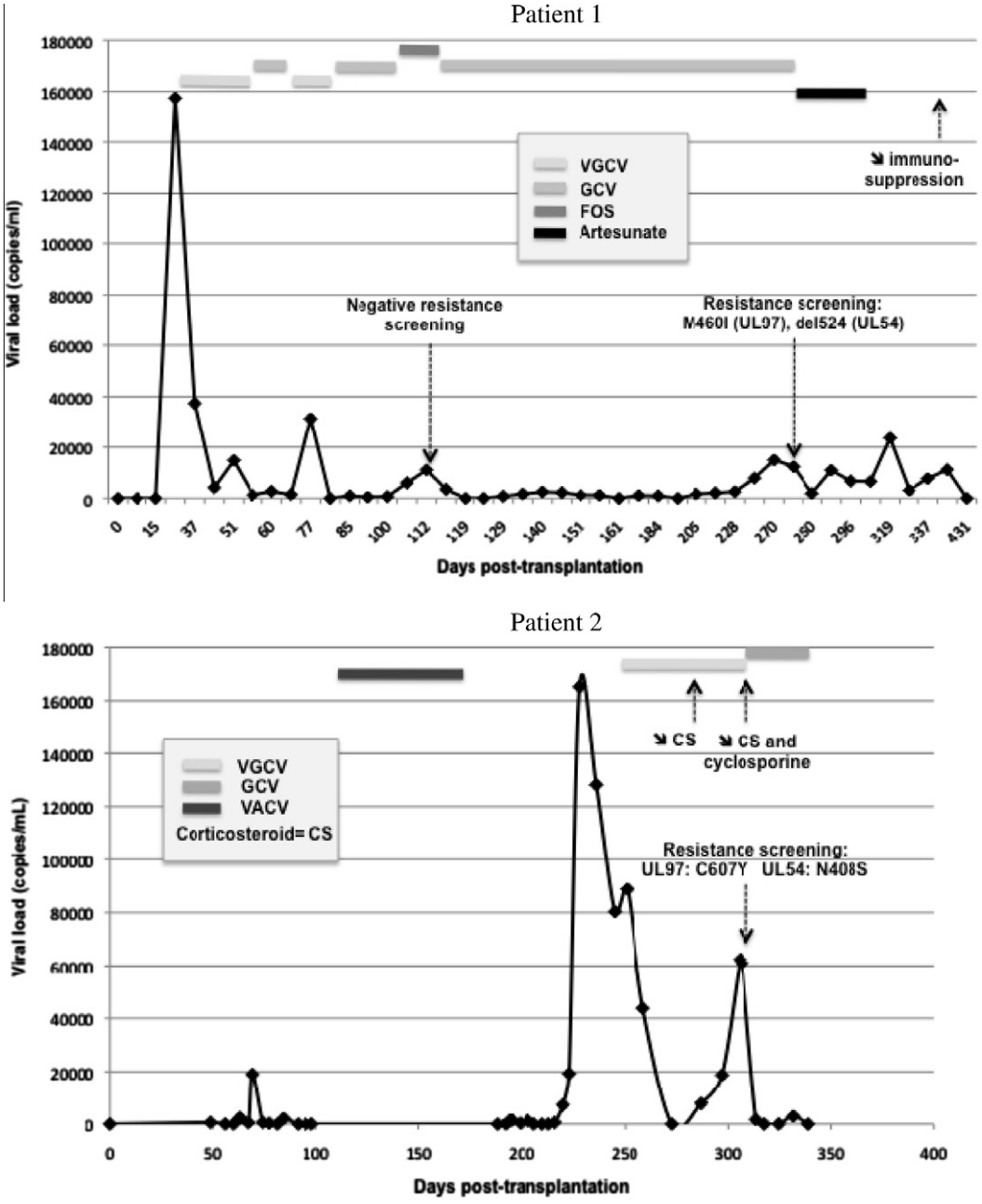


**Fig. 1.** (A) Time line of events and findings for patient 1. (B) Time line of events and findings for patient two (VACV: valaciclovir; VGCV: valganciclovir; GCV: ganciclovir; FOS: foscarnet; CS: corticosteroids).

UL54 polymerase was not described to date. At day 368 reduction of the immunosuppression was associated with a negativation of the viral load 2 months later that remained undetectable up to now. The UL54 del524_RV mutant shows low-level resistance to GCV (3.5-fold IC50 increase), high-level resistance to CDV (9.7-fold IC50 increase) and sensitivity to FOS (Table 1).

*Case patient 2* was a bone marrow recipient (CMV serostatus D−/R+) (Fig. 1B). Six months prophylaxis with valaciclovir (VACV 500 mg bid) was administrated post allograft. After an increase of immunosuppressive therapy for a digestive graft versus host disease, CMV replication was detected and VGCV therapy started. Despite treatment, CMV viral load still increased. Genotypic resistance assays highlighted the presence of mutation C607Y in UL97 and mutation N408S in UL54. Whereas the former is a well-known GCV-resistance mutation, the latter was not yet known despite aspartic acid or lysine substitutions at this position having already been associated with GCV and CDV resistance (Scott et al., 2007). VGCV treatment was changed to intravenous GCV treatment (300 mg bid) following a decrease in the immunosuppressive regimen, with reduced cyclosporine and corticosteroid dosage, which led to an undetectable viral load. The N408S_RV mutant shows low-level resistance to GCV (3.1-fold IC50 increase), high-level resistance to CDV (7.5-fold IC50 increase) and sensitivity to FOS.

In both cases analysis of replication fitness showed similar kinetics from day 1 to 10 between the mutant and wild type strains (Fig. 2).

The identification of these multi-drug resistance polymerase mutations emphasizes the increased risk of CMV resistance in transplant patients treated over a longer time with antivirals (Limaye et al., 2000). In regards to the kidney recipient, the CMV D+/R− serostatus and the high viral load increased the risk of this patient developing antiviral resistance, as previously reported (Couzi et al., 2011; Hantz et al., 2010; Limaye et al., 2002). Consistent with our findings for del524, the pressure from FOS therapy has not resulted in development of resistance mutations in UL54, as the phenotype of the mutated strain does not indicate resistance to FOS, and the duration of FOS therapy was short. Indeed, mutations of DNA polymerase domain ExoIII typically confer various levels of resistance to GCV and CDV but not to FOS (Cihlar et al., 1998; Lurain and Chou, 2010). The UL54 del524 is close to codon 522 where two substitutions - P522A (Chou et al., 2008) and P522S (Cihlar et al., 1998), resulted in moderate levels of GCV resistance and CDV resistance. The resistance phenotype of del524 strain is also consistent with the level of resistance induced by mutations of domain δC/ExoIII. A mutation C524R was described in a D+/R− kidney transplant recipient of the phase III trial IMPACT after 200 days of VGCV prophylaxis (Chou et al., 2010). This mutation (in addition to the published GCV susceptibility) also confers no CDV resistance (based on "personal communication" data from Sunwen Chou). Codon deletion is unusual for the DNA polymerase: only the two codons deletion 981–982 was already described in the region V with a significant effect on drug resistance. Three others deletions were also described in non-conserved regions, conferring no impact on resistance. The 3D modeling shows that the amino acid 524 is part of a helix. Removing one amino acid of a helix can change repartition of amino acids facing solvent or interior of the protein. Thus, the deletion could result in greater disruption of this part of UL54 than the previously observed substitution.

The N408S mutation represents the third mutation described at codon 408. Like other mutations of the ExoII domain, the mutation N408S confers GCV and CDV resistance. The CI50 ratios calculated were similar to those of mutation N408D and mutation N408K. It is interesting that substitution of a neutral polar residue (asparagine) for a smaller residue (serine) or an acidic residue (aspartic acid) or a basic residue (lysine) at this codon leads to similar resistance level to GCV and CDV *in vitro*. It is likely the prolonged VACV prophylaxis and VGCV therapy associated with high peak viral load resulted in the emergence of resistance mutations. This CMV infection was only resolved through a decrease of immunosuppressive regimen, as shown by viral load improvement.

**Table 1**
Antiviral susceptibilities of AD169-BAC derived mutants assessed by plaque reduction assay.

| Virus | Mutation | GCV | | CDV | | FOS | |
|---|---|---|---|---|---|---|---|
| | | IC50 (μM)* | Ratio** | IC50 (μM)* | Ratio** | IC50 (μM)* | Ratio** |
| AD169 BAC | Wild type | 4.4 ± 0.47 | | 0.4 ± 0.2 | | 89,6 ± 27.5 | |
| del524 BAC | Deletion of cystein 524 | 15.7 ± 3.67 | 3.5 | 2.5 ± 0.5 | 9.7 | 95 ± 32.1 | 1.06 |
| N408S BAC | N408S | 14.0 ± 5.25 | 3.1 | 2.1 ± 0.8 | 7.5 | 92.5 ± 32.1 | 1.03 |

* Mean values were calculated from seven replicate experiments.
** Fold increase in IC50 mean values compared to the average IC50 for wild-type sensitive strains.

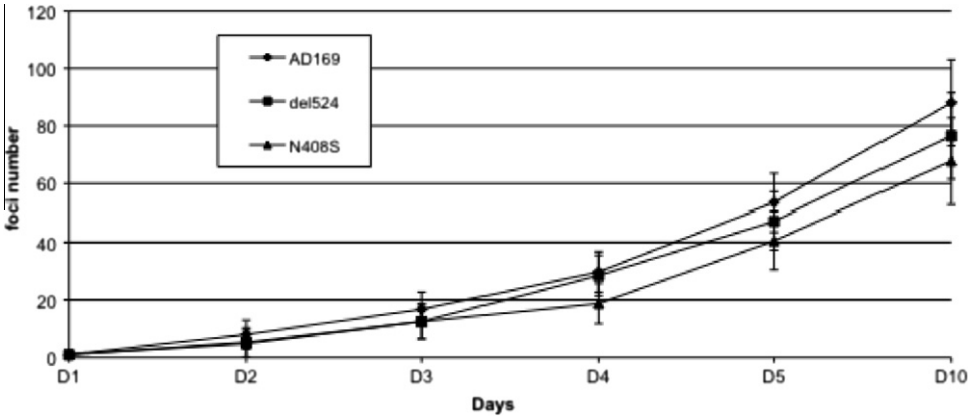


**Fig. 2.** Growth curves of UL54 del524_RV, N408S_RV and AD169-EGFP. Growth was assayed in MRC-5 cultures by counting number of fluorescent cytopathic foci at days 1–10. Each data point is the mean ± standard deviation of three replicates. Results shown are representative of three independent experiments.

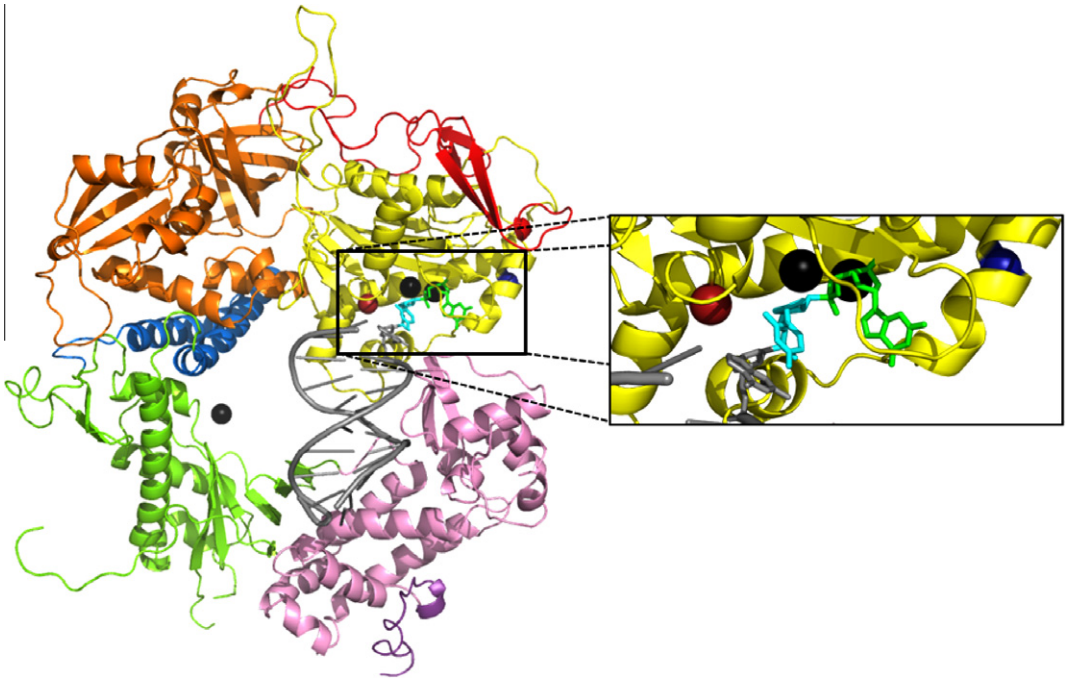

**Fig. 3.** Overall theoretical structure of pUL54 calculated with MODELLER represented in cartoon mode. The different domains according to homology with pUL52 from HSV1 (Liu et al., 2006) are colored as follow: residues 27–98 of pre-NH2-terminal in red, residues 99–295 and 555–600 of NH2-terminal in orange, residues 296–554 of 3′-5′ exonuclease in yellow, residues 678–765, 815–877 and 887–982 of palm in green, residues 766–824 of fingers in blue, residues 982–1112 and 1154–1222 of thumb in pink and residues 1223–1242 of the interaction domain with pUL44 in purple. In the inset, the DNA duplex is colored in gray excepted nucleotide i + 1 (green) and i−1 (blue) from scissile phosphate bound. Metal ions are colored in black, carbon alpha atoms of amino acids 408 and 524 in red and navy blue spheres, respectively.

Herein, we described two point mutations located in the 3′-5′ exonuclease domain (Fig. 3) both inducing higher resistance level for cidofovir than ganciclovir. The 3D homology model allows us to suppose that effects of these mutations are due to their close vicinity to catalytic ions i.e. less than 10 Å. Moreover, this difference of magnitude has already been described concerning mutants at codon F412 (C or V) and K513 (E or N). A common point between all those mutations is the reduction of the amino acid size but conservation of the general physicochemical properties (hydrophobic or dipolar). The primary function of the exonuclease domain of pUL54 lies in repairing mismatches during DNA synthesis. Thus, the protein has to fit its molecular morphology to better maintain a non-natural nucleotide for its subsequent excision.

Further characterization of emerging DNA polymerase and kinase UL97 mutations that confer resistance to antiviral agents, and those that result in antiviral sensitive phenotypes, is necessary to inform definitive detection of antiviral-resistant strains by genotypic assays. Use of BAC_CMV marker transfer and localization of new mutations on a 3D model together could allow better investigation of novel antiviral resistance mutations and understanding of the functional regions of CMV DNA polymerase.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2013.02.002.

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