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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.

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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

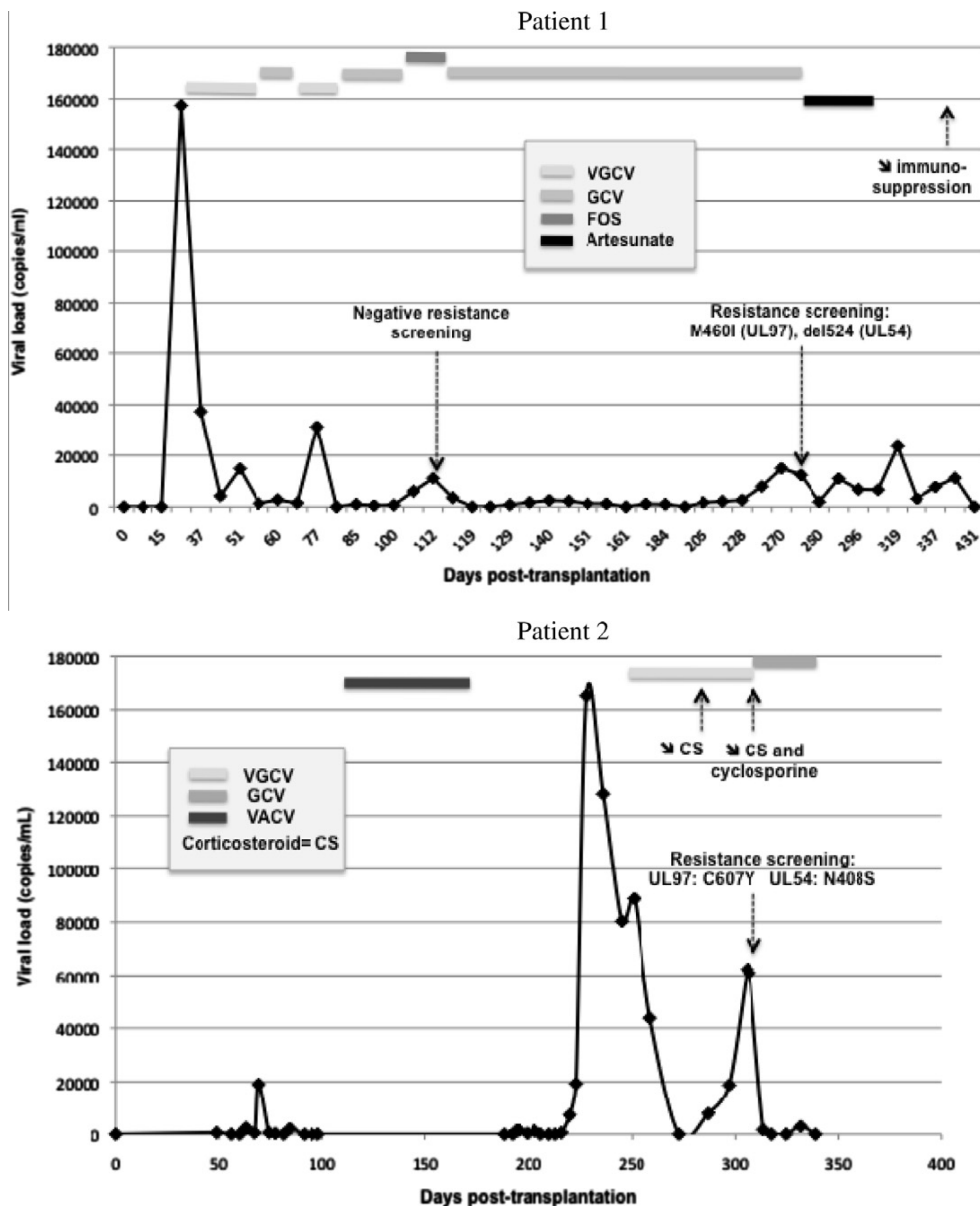

Fig. 1. (A) Time line of events and findings for patient 1. (B) Time line of events and findings for patient two (VACV: valaciclovir; VGCV: valganciclovir; GCV: ganciclovir; FOS: foscarnet; CS: corticosteroids).

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