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Identification of newly detected, drug-related HCMV UL97- and UL54-mutations using a modified plaque reduction assay

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ABSTRACT

Background: Drug-resistant cytomegalovirus causes major problems in immunocompromised patients and is due to mutations in the UL97-gene (phosphotransferase) and/or the UL54-gene (polymerase).
Objective: Three previously unknown UL97-mutations (E596D/Y and I610T), one UL54 single point mutation (D515E) and a UL54 triple mutation (D515E + L516M + I521T) were characterized for drug-resistance by marker transfer analysis using BAC-technology.
Study design: Mutations were introduced into the bacterial artificial chromosome TB40-BAC_{KL7}-UL32EGFP. In addition, mutations M460V (UL97) and I521T (UL54) served as drug-resistant control. Phenotypic resistance testing was performed by a modified plaque reduction assay using a mixture of infected fibroblasts and uninfected ARPE-19 cells which improved formation of clearly definable plaques considerably.
Results: Resistance testing showed ganciclovir (GCV)-resistance for UL97-mutations I610T and E596Y while mutation E596D was drug-sensitive. UL54-mutation D515E was resistant to GCV. The virus strain containing the UL54 triple mutation conferred cross-resistance to GCV and cidofovir (CDV). None of the mutations interfered with normal growth kinetics of the virus.
Conclusions: New mutations in the UL97- and UL54-gene of HCMV are still detected continuously. Furthermore, several mutations occurring in the same codon often show divergent phenotypes and the accumulation of multiple mutations in one virus strain may lead to increased or decreased drug-resistance. Therefore, characterization of newly detected mutations by marker transfer analysis is essential to confirm that genotypically detected mutations can produce phenotypic resistance. These results allow reliable interpretation of fast genotypic methods generally used in diagnostics.

1. Background

For the systemic treatment of HCMV-infected patients with compromised immune status, three drugs are currently used. Ganciclovir (GCV) is the first-line antiviral while cidofovir (CDV) and foscarnet (FOS) are mostly applied in the case of GCV therapy failure or severe side effects. Therapy failure is mainly due to drug-resistant HCMV-strains that arise spontaneously and are selected under the pressure of antiviral treatment [14,19]. In 94% of cases, GCV-resistance is due to mutations in the viral phosphotransferase-gene UL97 [3,4,15]. Severe immunosuppression and prolonged therapy often lead to the selection of additional polymerase (UL54) mutations, resulting in increased GCV-resistance or cross-resistance to CDV and/or FOS [29].

Genotypic methods such as amplification of the respective gene-region by PCR and sequencing are the method of choice in diagnostics as they are fast and easy to perform [20,26]. Nevertheless, distinction between resistance-associated mutations and polymorphisms is not possible. In order to get to know the phenotype of newly detected UL97- and UL54-mutations characterization by marker transfer analysis is essential.

Despite many efforts, phenotypic assays for the characterization of mutations with unknown phenotype still lack standardization [2,9,12,25]. Plaque reduction assays (PRA) that measure viral spread in fibroblasts in the presence of different drug-dilutions are considered the gold standard. But when highly passaged laboratory HCMV-strains are used where large amounts of progeny virus

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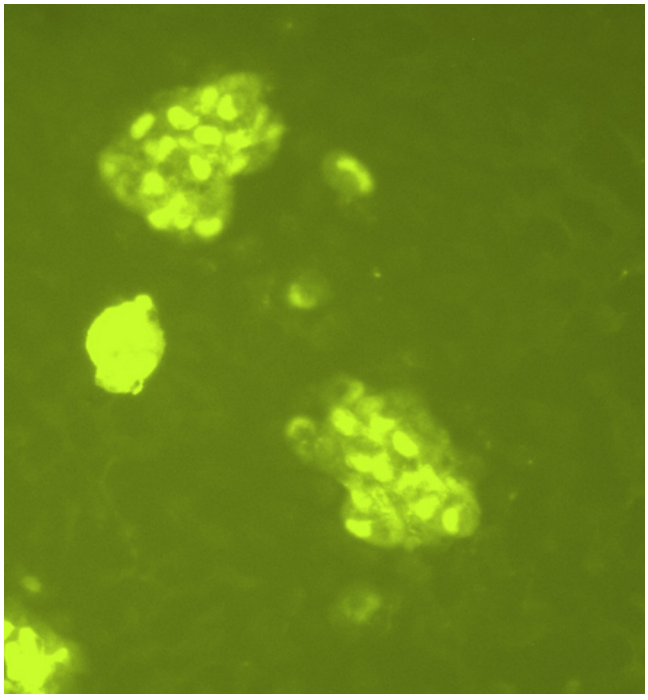

Fig. 1. Plaque formation in the modified plaque reduction assay using a mixture of infected HFF and uninfected Apre-19 cells. The formation of clearly distinguishable plaques as shown here facilitates evaluation of the assay considerably. One plaque was defined as a minimum of 5 infected cells per focus.

are released into the supernatant, the formation of single plaques can only be achieved by overlaying cells with methylcellulose or agarose-containing media. Here we describe a modified PRA derived from the original method by Prix et al. [25], using epithelial cells as target cells. Due to different entry routes of HCMV into different cell types the use of epithelial cells results in the formation of clearly distinguishable plaques that can be counted easily (Fig. 1). Therefore, the assay shows advantages in marker transfer experiments where point-mutations are introduced into CMV-BACs, derived from laboratory CMV-strains. Furthermore, no specific equipment is needed for the analysis and therefore the assay can be performed in any laboratory at low costs.

In this study we characterized some previously unknown UL97- and UL54-mutations associated with different outcomes to antiviral drug resistance. All mutations were detected in plasma samples of HCMV-infected, immunosuppressed patients of the transplant setting who were under antiviral treatment. The findings of this work contribute to a better understanding of antiviral drug resistance of HCMV and increase the number of mutations that can be determined by rapid genotypic diagnostics.

2. Study design

2.1. Patients

Patient 1 is a 43 year old patient receiving a liver transplant in April 2013. In surgical intensive care unit, a disseminated HCMV infection was detected 3 weeks later with rapid increase of viral load (VL) in plasma of $>1.5 \times 10^6$ copies/ml. The UL97 mutation I610T was detected in the plasma sample while in tracheal secretions only wild type sequence was detectable. The patient developed HCMV pneumonia and under FOS treatment virus replication could be controlled.

Patient 2 is a 5 months old girl with Tay-Sachs-syndrome receiving an allogeneic stem cell transplant (D+/R+). The patient received antiviral prophylaxis with GCV and PFA. Several intermittent donations of GCV/VGCV were applied during the course of infection. The UL97 mutation E596Y was detected in leukocytes and tracheal secretions following a tenfold increase in plasma VL under FOS-therapy. Mutation E596D was retrospectively found in plasma before occurrence of mutation E596Y.

Patient 3 is an 11 year old stem-cell transplant-recipient (D−/R+) developing fatal HCMV encephalitis with multi-drug resistant HCMV-infection [13]. The patient developed an HCMV infection during conditioning and received antiviral therapy with PFA and GCV during the course of infection [13]. The following point mutations were found in leucocytes and urine: A591V, C603W, M460I (UL97) and D515E, L516M, I521T and L802M (UL54). While the I521T-mutation is associated with GCV/CDV cross-resistance [10] and L802M is associated with GCV/FOS cross-resistance [5,11], mutation L516M was confirmed as drug-sensitive [16]. The UL97-mutation A591V is drug-sensitive [21] and mutations C603W and M460I confer GCV-resistance [29,5,33] The impact of mutation D515E on drug-resistance was still unknown and was therefore analysed both individually and in combination with mutations L516M and I521T.

2.2. Cells, BACs and bacteria

Human foreskin fibroblasts (HFF) were grown and maintained in minimal essential medium (MEM) (Gibco/Invitrogen, Burlington, Ontario, Canada), containing 5% fetal calf serum (FCS) (Gibco/Invitrogen, Burlington, Ontario, Canada) and 1% penicillin/streptomycin. HFF were used for transfection experiments, reconstitution and propagation of virus and for all phenotypic experiments.

Retinal pigmented epithelial cells (ARPE-19) were cultured in Dulbecco's minimal essential medium (DMEM-F12) (Gibco/Invitrogen, Burlington, Ontario, Canada) containing 5% FCS and 1% penicillin/streptomycin. ARPE-19 cells were used in co-culture with HFF for the plaque reduction assays.

For generation of HCMV with defined point-mutations all recombination events were carried out in *Echerichia coli* strain GS1783, harboring the TB40/E-derived bacterial artificial chromosome (BAC) TB40-BAC$_{KL7}$-UL32EGFP [18]. HCMV-TB40-BAC$_{KL7}$-UL32EGFP, reconstituted from TB40-BAC$_{KL7}$-UL32EGFP, served as reference strain.

2.3. Generation of recombinant viruses

Mutagenesis of BACs was performed by markerless "en-passant" mutagenesis [30,31]. Briefly, a PCR-product containing a kanamycin resistance gene and an I-Sce-I recognition site flanked by homologous HCMV-sequences containing the desired point-mutation was generated and introduced into TB40-BAC$_{KL7}$-UL32EGFP via homologous recombination. All foreign sequences were then removed by I-Sce-I cleavage of BAC-DNA followed by a second recombination step, leaving the point mutation as the only modification of the BAC genome. Successful mutagenesis of BACs was verified by sequencing. Primers used for mutagenesis-PCR and sequencing are listed in Table 1. Point-mutated BACs were transfected into HFF by chemical transfection (mammalian transfection kit, Stratagene) which resulted in the reconstitution of recombinant viruses HCMV-TB40-BAC$_{KL7}$-UL32EGFP/E596D, HCMV-TB40-BAC$_{KL7}$-UL32EGFP/E596Y, HCMV-TB40-BAC$_{KL7}$-UL32EGFP/I610T, HCMV-TB40-BAC$_{KL7}$-UL32EGFP/M460V (UL97) and HCMV-TB40-BAC$_{KL7}$-UL32EGFP/D515E, HCMV-TB40-BAC$_{KL7}$-UL32EGFP/I521T and HCMV-TB40-BAC$_{KL7}$-UL32EGFP/D515E,L516M,I521T (UL54).

After propagation of the virus in HFF, cell-free virus stocks were prepared and stored at −80 °C. Determination of virus titer was performed by 50% tissue culture infective dose (TCID$_{50}$)-assays