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Short Communication

Generation and characterization of a GCV resistant HCMV UL97-mutation and a drug sensitive UL54-mutation

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ABSTRACT

In transplant recipients, drug-resistant human cytomegalovirus (HCMV) infections remain a serious problem. Drug-resistance against ganciclovir (GCV), cidofovir (CDV) and foscarnet (PFA) is caused by mutations either in the phosphotransferase-gene (UL97) or in the viral polymerase (UL54). For characterization of newly emerging mutations marker transfer analysis is required. Two new HCMV-mutations, the UL54-mutation L516M and the UL97-mutation A613V, were characterized by this method.

HCMV antiviral drug-resistance remains a serious problem in immunocompromised patients and is due to mutations in the UL97- and/or UL54-gene (Baldanti et al., 2004). While mutations in UL97 can only lead to GCV-resistance, mutations in UL54 are associated with resistance to all currently available drugs. In routine diagnostics, resistance testing is mainly conducted by genotypic assays which allow a rapid detection of resistance-associated mutations. RFLP and LightCycler assays were developed for the UL97-gene but can only detect known mutations conferring resistance to GCV (Prix et al., 1999; Eckle et al., 2003; Göhring et al., 2006, 2008). For UL54, detection of drug resistance-associated mutations is done by sequencing as mutations are widely dispersed across the gene. For interpretation of new UL97- and UL54-mutations, marker transfer analysis has to be performed. This method allows a reliable differentiation between resistance-associated mutations and polymorphisms. Recent findings of drug-sensitive UL97-genotypes formerly associated with drug-resistance emphasize the impact of this method (Lurain and Chou, 2010).

Here we present the characterization of two new mutations, A613V (UL97) and L516M (UL54), by marker transfer. Both mutations were detected in clinical specimens of two stem-cell transplant recipients who suffered from leukemia. The viral isolate of the patient with the UL54-mutation L516M consisted of mixed virus populations with several UL97- and UL54-mutations. The impact of each mutation itself on drug-resistance was unknown. The clinical course of this patient was already published (Eckle et al., 2000). In the clinical specimen of the second patient, only the UL97-mutation A613V could be detected without any other amino acid changes in the UL97-gene compared to laboratory strain AD169.

A613V and L516M were individually transferred to a drug-sensitive virus strain using the markerless "en passant" mutagenesis technique (Tischer et al., 2010). Recombination events were carried out in *Escherichia coli* strain GS1783, harboring the TB40/E-derived BAC TB40-BAC_{KL7}-UL32EGFP (Laib Sampaio et al., 2013). Primers used for mutagenesis are listed in Table 1. Successful mutagenesis of BACs was verified by sequencing with primers 595F/R (UL97) and p1up/do (UL54) (Table 1). Chemical transfection (mammalian transfection kit, Stratagene) of human foreskin fibroblasts (HFF) resulted in the reconstitution of recombinant viruses HCMV-TB40-BAC_{KL7}-UL32EGFP-UL97/A613V and HCMV-TB40-BAC_{KL7}-UL32EGFP-UL54/L516M.

For phenotypic characterization of our recombinant virus strains by plaque reduction assay (PRA) three clinical HCMV-isolates were used as controls. Ro71132 contains the UL97-mutation A591V (drug-sensitive, Chou et al., 2007) and the UL54-mutations D515E, L516M, I521T and L802M. Multi-drug resistance of this strain is caused by mutations I521T (GCV, CDV) and L802M (GCV, PFA) (Eckle et al., 2000). pp6-M460V was plaque-purified from the clinical isolate pp6 (kindly provided by Dana Wolf, Jerusalem)

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Table 1
Sequences of UL97- and UL54-primers used for mutagenesis PCR and sequencing.

Region	Primer	Function	Sequence[a]
UL97	m A613V *forward*	en-passant mutagenesis	ctcacgcactgctccgacgcctgtctgctcattctggcgg**T**gcaaatgagctacggcgcctaggatgacgacgataagtaggg
UL97	m A613V *reverse*	en-passant mutagenesis	cgccatgctcgcccaggagaaggcgccgtagctcatttgc**A**ccgccagaatgagcagacagcaaccaattaaccaattctgattag
UL54	m L516M *forward*	en-passant mutagenesis	aacactatggccgagctttacctgcggcaacgcaaggatgac**A**gtcttacaaggacatcccaggatgacgacgataagtaggg
UL54	m L516M *reverse*	en-passant mutagenesis	cattagccacgaaacaacgcgggatgtccttgtaagaca**T**gtcatccttgcgttgccgcaggcaaccaattaaccaattctgattag
UL97	595 *forward*	Sequencing	cctcatgcggctgttggacc (Prix et al., 1999)
UL97	595 *reverse*	Sequencing	ccatgctcgcccaggagaca (Prix et al.,1999)
UL54	p1up	Sequencing	tctttacgatcggcacctgc
UL54	p1do	Sequencing	ccgtcgtacctttgctgtag

[a] The introduced mutation is highlighted enlarged and presented in bold font.

carrying only the M460V-mutation in UL97, which is responsible for GCV resistance. A clinical strain (H4397) isolated from urine of an untreated congenitally infected newborn was used as the reference drug-susceptible strain for this study. All clinical isolates used as controls were passaged frequently.

For the assay, 2×10^4 HFF/well were seeded in 96-well plates one day prior infection and kept at 37 °C. Cells were infected with cell-free virus at a multiplicity of infection (MOI) of 0.01; 0.005 and 0.001 for the BAC-derived viruses and a MOI of 0.2; 0.1; 0.005 and 0.002 for the clinical isolates. As infectivity of BAC-derived viruses and clinical strains varied significantly, different MOIs were used to obtain the same number of plaques in the control wells which, in turn, is essential to reliably compare the inhibitory concentration (EC_{50}) of all strains. Drugs were added in twofold serial dilutions, ranging from 0.5-50 μM GCV (Roche Pharma AG, Grenzach, Germany), 100–1000 μM PFA (AstraZeneca GmbH, Wedel, Germany) and 0.5–10 μM CDV. 12 hpi, cells were washed two times with phosphate buffered saline (PBS) and cultured in MEM + GCV (0.5–50 μM; Roche Pharma AG, Grenzach, Germany), MEM + PFA (100–1000 μM; AstraZeneca GmbH, Wedel, Germany) or MEM + CDV (0.5–10 μM; Gilead Sciences, Martinsried, Germany), respectively. 7 dpi cells were fixed and virus plaques were stained against the late viral protein pp65 (bioMérieux**,** Verniolle, France). One plaque was defined as a minimum of 5 infected cells per focus. For every virus strain, only the cell-culture that contained about 50 plaques/well in the control wells was counted. Evaluation of the EC_{50} was performed by Probit-Analysis. Drug-resistance was defined as a 2-fold decrease in drug-sensitivity in comparison to the sensitive reference strain.

Mutation A613V (UL97) was tested with regard to its sensitivity to GCV. While the parental strain showed a mean EC_{50} of 1.5 μM, mutation A613V increased the mean EC_{50} to 3.42 μM, representing a ratio of 2.3 (Table 2). As mutations in UL54 may result in resistance to all currently approved drugs, the PRA for L516M was performed with GCV, PFA and CDV. Using GCV, the mean EC_{50} was 1.92 μM (ratio 1.3). Using PFA, L516M showed a mean EC_{50} of 76.08 μM while the EC_{50} of the parental strain was 63.74 μM (ratio 1.2). In the CDV-assay, L516M had a mean EC_{50} of 0.532 while the EC_{50} of the parental strain was 0.478 resulting in a ratio of 1.11. Hence, L516M is sensitive to GCV, PFA and CDV (Table 2).

In order to see whether the low EC_{50}-values of all tested virus strains are due to our PRA or a characteristic of our BAC-derived viruses, the results were compared to the EC_{50} of the isolates H4397, Ro71132 and pp6- M460V (Table 2). H4397 had a mean EC_{50} of 3.3 μM GCV which is a 2.2-fold higher value in comparison to the BAC-derived, drug-sensitive HCMV-TB40-BAC_{KL7}-UL32EGFP. Both resistant reference strains showed an equally increased EC_{50} in comparison to the BAC-derived mutated HCMV strains. The same phenomenon could be observed using PFA.

For the growth experiments 1×10^5 HFF/well were seeded in 6-well plates one day prior infection. Cells were infected at a MOI of 0.5 and then cultivated for 7 days. Every 24 h, virus supernatants were harvested and replaced by fresh medium. Virus titers were determined by 50% tissue culture infectious dose ($TCID_{50}$)-assays (Mahy and Kangro, 1996). Growth experiments showed that the release of progeny virus started about 72 hpi for all BAC-derived virus strains and the virus titer increased without any significant interstrain differences. In contrast to that, pp6-M460V and Ro71132 showed a better replicative fitness with an earlier onset of virus release and higher titers (Fig. 1)

The characterization of the two new mutations, L516M (UL54) and A613V (UL97) was performed by PRA. Despite many efforts, standardization of this assay remains a problem (Prix et al., 1998; Schnepf et al., 2009). In our PRA, we infected HFF at different MOIs but only those cultures that showed about 50 plaques in the control-wells were chosen for evaluation. This method is based on

Table 2
Drug susceptibility testing of BAC-derived HCMV and clinical isolates by plaque reduction assay.

Origin	Virus strain	Mutation	GCV			PFA			CDV		
			μM[b]	Ratio[c]	S_D	μM[b]	Ratio[c]	S_D	μM[b]	Ratio[c]	S_D
BAC-derived	[a]*HCMV-Bac7-UL32-EGFP (drug sensitive)*	–	1.5	–	0.41	63.74	–	6.29	0.478	–	0.17
BAC-derived	HCMV-Bac7-UL32-EGFP/ A613V	A613V (UL97)	**3.42**	**2.3**	0.25	–	–	–	–	–	–
BAC-derived	HCMV-Bac7-UL32-EGFP/ L516M	L516M (UL54)	1.92	1.3	0.16	76.08	1.2	9.9	0.532	1.1	0.13
Clinical isolate	[a]*H4397 (drug sensitive)*	–	3.3	–	0.77	117.97	–	nd	–	–	–
Clinical isolate	[a]*pp6-ppM460V* (Göhring et al., 2006) *(GCV-resistant)*	M460V (UL97)	**51.4**	**15.5**	14.8	–	–	–	–	–	–
Clinical isolate	[a]*Ro71132* (Eckle et al., 2000) *(multidrug-resistant)*	A591V (UL97) D515E, L516M, I521T, L802M (UL54)	**>50**		nd	**484.81**	**4.1**	15.2	–	–	–

All values representing drug-resistance are in bold type.
[a] Reference strains are highlighted italic.
[b] Mean value of three individual assays.
[c] EC_{50} of mutant/EC_{50} of parental strain.

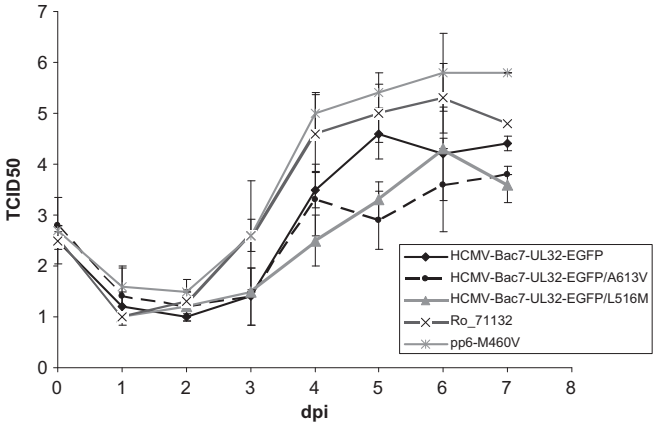

Fig. 1. One-step growth cycle of HCMV-TB40-BAC_{KL7}-UL32EGFP in comparison to the point-mutated strains HCMV-TB40-BAC_{KL7}-UL32EGFP-UL97/A613V and HCMV-TB40-BAC_{KL7}-UL32EGFP-UL54/L516M as well as the clinical isolates pp6/ M460V and Ro_71132.

the cell-associated PRA and allows comparing different virus strains despite their diverging infectivity (Prix et al., 1998).

All the BAC-derived virus strains showed lower EC_{50}-values than our clinical isolates. This difference could be based on the parental strain TB40/E which was used for BAC-generation. To our knowledge, the EC_{50} of TB40/E has never been evaluated but if this strain has a very low EC_{50}, all TB40/E-derived BACs naturally behave the same. This demonstrates that defining resistance based on drug-ratio is more reliable than using defined EC_{50}-values.

Nevertheless, the UL97-mutation A613V has a GCV-ratio of 2.3 in comparison to HCMV-TB40-BAC_{KL7}-UL32EGFP and was therefore classified as drug-resistant. It is hypothesized that mutations occurring in the codon range from 591 to 607 confer resistance by preventing the recognition of GCV as a substrate (Chou et al., 2002). With mutation A613V in the direct vicinity of this region, the same could be hypothesized for this mutation. This hypothesis is strengthened by the observation that HCMV-TB40-BAC_{KL7}-UL32EGFP-UL97/A613V shows almost the same growth behaviour as the parental strain. Therefore, the mutation seems not to interfere with the normal function of UL97 in replication.

The viral DNA-polymerase is encoded by UL54. Codon 516 is part of the conserved ExoIII/δ-region C that is, together with ExoI and ExoII, responsible for 3′5′-exonuclease activity (Shi et al., 2006). Several mutations in the ExoIII/δ-region C have a confirmed GCV/CDV-resistant phenotype, one of them being mutation L516R (Chou et al., 2003). Interestingly, mutation L516M does not confer resistance either to GCV or PFA. This illustrates that the phenotype of every newly emerging mutation has to be examined independently and gathering the chemosusceptibility of one mutation reflecting the phenotype of surrounding mutations is not reliable.

Although mutations in ExoIII do often affect the proofreading activity of pUL54, we could not observe any significant differences in growth behaviour of HCMV-TB40-BAC_{KL7}-UL32EGFP-UL54/L516M and the parental strain. The slightly different growth behaviour of our BAC-derived viruses and the clinical isolates is likely to be based on interstrain differences.

In conclusion, we showed that the UL97-mutation A613V leads to GCV-resistance. This finding increases the number of GCV-resistance associated mutations in the UL97-gene. The UL54-mutation L516M does not reduce drug sensitivity either to GCV, PFA or CDV. This information emphasizes the need of marker transfer analysis for each newly found mutation since it is possible that different mutations in the same codon have different drug resistant phenotypes (Lurain and Chou, 2010). As the replication activity of both virus strains was almost identical to that of the parental strain, the influence of these mutations on growth properties can be excluded.

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