Short communication

Rapid semiquantitative real-time PCR for the detection of human cytomegalovirus UL97 mutations conferring ganciclovir resistance

Katharina Göhring1, Elfriede Mikeler1, Gerhard Jahn1, Frank Rohde2 and Klaus Hamprecht1*

1Institute of Medical Virology and Epidemiology of Viral Diseases, University Hospital of Tübingen, 72076 Tübingen, Germany
2Hoffmann-La Roche AG, 79639 Grenzach-Wyhlen, Germany

*Corresponding author: E-mail: klaus.hamprecht@med.uni-tuebingen.de

Background: The development of infections with ganciclovir (GCV)-resistant human cytomegalovirus (HCMV) remains a serious problem in recipients of stem cell or organ transplants. Nearly all GCV-resistant clinical isolates have mutations in the viral UL97 gene. The rapid detection of GCV-resistant HCMV infections is necessary and the relative proportions of wild-type and mutant strains are predictive for the efficiency of antiviral therapy. To date, genotypical resistance screening has been limited to restriction fragment length polymorphism (RFLP) and sequencing analyses. Here, we present a comprehensive real-time PCR approach for the detection of most frequent mutations in the UL97 gene associated with GCV resistance.

Methods: The laboratory strains AD169 and Towne, different wild-type isolates and plasmids constructed by site-directed mutagenesis and overlap extension with specific point-mutations in the UL97 gene were analysed by LightCycler PCR and compared with UL97 RFLP and sequencing analyses.

Results: A new and comprehensive set of LightCycler PCRs was created using specific hybridization probes with melting-point analysis for the relevant codons 594, 595, 603 and 607. Different wild-type isolates and plasmids containing specific UL97 mutations conferring GCV resistance were investigated in the real-time PCR assay. Total processing time was 80 min per assay, whereas combinations of RFLP and sequencing needed at least 3–4 days. Proportions of co-existing wild-type and mutant strains in mixed viral populations can be obtained.

Conclusions: We established a rapid real-time PCR approach for the detection of most frequent HCMV UL97 mutations associated with GCV resistance. Moreover, the method allows semiquantitative differentiation of the proportions of co-existing wild-type and mutant strains. This approach represents a new alternative for laborious RFLP analysis.

Introduction

In human stem cell transplant and solid organ transplant recipients, the resistance of human cytomegalovirus (HCMV) to ganciclovir (GCV) is of growing importance [1,2]. This resistance is based on mutations in the HCMV phosphotransferase gene (UL97) and the HCMV polymerase gene (UL54) [1]. Over 90% of all mutations found in clinical GCV-resistant isolates have mutations in the UL97 gene between codons 400 and 665 [3,4]. The most frequent mutations occurring in the UL97 gene are mutations in codons 460, 594 and 595 [3,6]. Genotypical resistance screening is of key importance with respect to known UL97 mutations [5], because phenotypical resistance screening strongly depends on the availability of a clinical isolate. Even in immunosuppressed patients with GCV-resistant HCMV infection such an isolate often cannot be obtained, because even in the presence of a drug-resistant strain GCV is able to prevent the shedding of replication-competent virions into urine and saliva. It is known that the relative in vivo proportions of wild-type and mutant strains in mixed viral populations have a predictive effect on therapy efficacy [7,8]. To date, genotypical resistance screening has been performed by UL97 restriction fragment length polymorphism (RFLP) analysis and sequencing. These methods need at least 3–4 days to be performed. Additionally, relative proportions of non-dominating wild-type or mutant virus strains are often only detectable by RFLP analysis if viral...
DNA concentrations in the primary material are very low and, therefore, amplification is inefficient. In a previous study, we created a rapid LightCycler PCR assay for the detection of mutations in UL97 codons 460 and 520 [9], which can be performed in 1 day. RFLP assays for the detection of mutations in codons 460 [10], 520 [11], 594 [12], 595 [12], 603 [13] and 607 [7] have been developed previously. Here, we present a new set of LightCycler PCR assays that use specific hybridization probes with melting-point analysis for the characterization of the relevant UL97 codons 594, 595, 603 and 607. The relative frequency of the UL97 mutations A594V, L595S, C603W and C607Y was obtained for 39 out of 75 (52%) unrelated GCV-resistant isolates [5]. Together with the LightCycler assays for codons 460 and 520, we are now able to detect nearly 80% of all known mutations arising in the HCMV UL97 gene and conferring resistance to GCV.

Methods

HCMV strains
The laboratory strains AD169 and Towne and HCMV clinical isolates from human stem cell transplant recipients were used as reference strains.

Primers and hybridization probes
The assay for the simultaneous detection of mutations in the viral UL97 codons 594 and 595 was performed with one pair of hybridization probes labelled with LC Red dye 640 and fluorescein. The assay for the simultaneous detection of mutations in codons 603 and 607 was created using a dual-colour format with two pairs of hybridization probes each labelled with two different fluorescence dyes (LC Red dye 640 and LC Red dye 705). The detection of mutations was achieved by melting-curve analysis following PCR. The binding of the hybridization probe resulted in a specific melting point for the wild-type sequence. In mutant strains, however, the mismatch leads to an unstable binding of the probes decreasing the melting temperature. Primer design was performed using Oligo Primer Analysis Software Version 5.0 (NBI, Plymouth, UK). Hybridization probes and primers for assay 594/595 were designed by TIB MolBiol (Berlin, Germany) and purchased from this manufacturer. For assay of codons 603/607 primers and hybridization probes were designed with the LightCycler Probe design Software (Roche Diagnostics, Mannheim, Germany) and purchased from TIB MolBiol. Sequences of primers and specific hybridization probes are shown in Table 1.

Construction of recombinant plasmids containing specific UL97 mutations
The mutations C603W and C607Y were created by site-directed mutagenesis and overlap extension using primers m603F, m603R, m607F, m607R, 603/607F and 603/607R (Table 1). The resulting PCR products were cloned into the pGEMTEasy vector system (Promega, Mannheim, Germany) and after linearization the plasmids were used in the LightCycler PCR analysis described previously [9].

Real-time PCR
The composition of the reaction mixture was as described previously [9]. The program used for cycling was as described, although different annealing temperatures were employed: an annealing temperature of 57°C was used for mutation detection in codons 594/595 and 603/607 and for construction of the site-directed mutants A594V, L595S, L595S, C603W and C607Y.

Table 1. Sequences of HCMV UL97 primer and hybridization probes for the LightCycler assays 594/595 and 603/607 and for construction of the site-directed mutants A594V, L595S, L595S, C603W and C607Y

<table>
<thead>
<tr>
<th>Nucleotide position/primer</th>
<th>Primer description</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>nt142064–nt142083: 595 F</td>
<td>Forward primer (F)</td>
<td>gtcgcagaagtctctac</td>
</tr>
<tr>
<td>nt142440–nt142460: 595 R</td>
<td>Reverse primer (R)</td>
<td>gtcgcagacatcgtgta</td>
</tr>
<tr>
<td>nt142259–nt142277: 595 Sensor</td>
<td>Hybridization probe</td>
<td>cgccggctgagacgg-Fl*</td>
</tr>
<tr>
<td>nt142222–nt142239: 603/07 F</td>
<td>Forward primer (F)</td>
<td>gcggaggctgtctctt</td>
</tr>
<tr>
<td>nt142403–nt142420: 603/07 R</td>
<td>Reverse primer (R)</td>
<td>gcgcgcgttggagaacggtaag-Fl*</td>
</tr>
<tr>
<td>nt142259–nt142282: 603 Anchor</td>
<td>Hybridization probe</td>
<td>'LC640-agaagacgcgctagcagacg-P</td>
</tr>
<tr>
<td>nt142232–nt142329: 603 Sensor</td>
<td>Hybridization probe</td>
<td>gaggaggctgtctctt</td>
</tr>
<tr>
<td>nt142325–nt142348: 607 Anchor</td>
<td>Hybridization probe</td>
<td>gcgcgcgttggagaacggtaag-Fl*</td>
</tr>
<tr>
<td>nt142300–nt142323: 607 Sensor</td>
<td>Hybridization probe</td>
<td>'LC640-agaagacgcgctagcagacg-P</td>
</tr>
<tr>
<td>nt142281–nt142302: m603 F</td>
<td>Forward mismatch (g) primer (F)</td>
<td>ctcacgcactgcgtcctctc</td>
</tr>
<tr>
<td>nt142281–nt142302: m603 R</td>
<td>Reverse mismatch (c) primer (R)</td>
<td>gcggagtttggagaaggttaag-Fl*</td>
</tr>
<tr>
<td>nt142293–nt142314: m607 F</td>
<td>Forward mismatch (a) primer (F)</td>
<td>gacaggcgccgtggagaaggttaag-Fl*</td>
</tr>
<tr>
<td>nt142293–nt142314: m607 R</td>
<td>Reverse mismatch (t) primer (R)</td>
<td>Aatggagtattggagaaggttaag-Fl*</td>
</tr>
</tbody>
</table>

*Fluorescein. †Fluorescence dye LC Red dye 640. ‡Fluorescence dye LC Red dye 705. F, forward; R, reverse; P, phosphate; nt, nucleotide.
594/595 and a temperature of 53°C was used for the detection of mutations in codons 603 and 607 [9].

RFLP analysis
The RFLP analysis for the detection of mutations A594V, L595S and C603W was performed as described elsewhere [5,13]. The RFLP analysis for codon 607 [7] was modified by using primers 595F/R*[13], resulting in an amplicon of 189 bp. The mutation C607Y results in a loss of the PshAI cleavage site: the wild-type C607 was cut into two fragments of 57 bp and 132 bp, whereas the mutant strain C607Y was uncut.

Sequencing analysis
Sequencing analysis was performed by PCR with primers 595F/R*[13]. Sequencing products were cleaned with the Qiagen PCR purification Kit (Qiagen, Hilden, Germany).

Results
Sensitivity, specificity and reproducibility
The assay time for both LightCycler assays was 80 min and the sensitivity was 14 copies/assay using a commercially available HCMV AD169 quantitated

![Figure 1. Real-time PCR assay of HCMV wild-type and mutant strains](image)

(A) LightCycler assay for human cytomegalovirus (HCMV) UL97 codons 594 and 595 using melting-point analysis. (B) Simultaneous and semiquantitative detection of mixed viral populations in codons 594 and 595. The laboratory strains AD169 and Towne were used as wild-type and Iso-K (A594V) and Iso-6 (L595S) were employed as mutant reference strains. LightCycler assay for detection of the HCMV UL97 mutations (C) C603W and (D) C607Y using melting-point analysis. NTC, no template control; Tm, melting temperature.
DNA control (tebu-bio, Offenbach, Germany) with defined copy number/μl (Figure 1A, B). To check the sensitivity for the detection of wild-type and mutant strains in mixed viral populations, different ratios of wild-type and mutant reference strains were analysed in the real-time PCR assay. We were able to determine proportions of co-existing wild-type and mutant strains with a sensitivity of 10% of the non-dominating strain (Figure 1B). The specificity of the PCRs were tested using DNA from five different virus species (herpes simplex virus type 1 and 2, human herpes virus 6, Varicella zoster virus, Epstein-Barr virus and adenovirus) and no specific UL97 PCR product could be detected with these DNA specimens. Intrarun and interrun variability tests with triplicate repeats on 3 days were performed and the results showed a reproducibility of exactly 100% (data not shown).

Detection of UL97 mutations in codons 594 and 595
In our LightCycler assay for codon 594/595 the laboratory strains showed a specific melting point at 63.4°C. The clinical isolates with a mutation in codon 594 (A594V) reduced the melting point drastically to 55.1°C, while the GCV-resistant clinical strain Iso-6 and the plasmid with mutations in codon 595 decreased the specific melting point to 57.6°C (Figures 1A, B).

The RFLP analysis of the reference strains Iso-K (A594V) and Iso-6 (L595S) showed the mutation-specific restriction pattern in comparison with the wild-type A594 and L595 (Figure 2Ai, ii).

Sequencing analysis showed the wild-type sequence with GCG for codon 594 and TTG for codon 595. The reference strain for the mutation A594V Iso-K showed the sequence GTG, whereas the reference strain for the mutation L595S Iso-6 had the nucleotide sequence TCG (Figure 2Bi).

Detection of UL97 mutations in codons 603 and 607
In the LightCycler assay for the detection of mutations in codons 603 and 607, the laboratory strain Towne had a melting point of 66.2°C in channel 2 (for the detection of codon 603) and 66.2°C in channel 3 (for the detection of codon 607; Figures 1C, D). The plasmid and the reference strain Iso-3 containing the
mutation C603W had a specific melting point at 55.3°C (Figure 1C). The p607Y variant showed a melting point at 55.6°C (Figure 1D). In this semiquantitative assay we were also able to detect 10% of non-dominating wild-type or mutant strains out of mixed viral populations (data not shown).

The RFLP results showed the expected restriction pattern with an AvaII digest for codon 603 (wild-type fragments 173 bp, 16 bp; mutant fragments 104 bp, 57 bp and 16 bp) and a PstI digest for codon 607 (wild-type fragments 132 bp, 57 bp; mutant fragment 189 bp) for the wild-type and the recombinant mutant plasmid (Figure 2Aiii,iv). Sequencing analysis showed a TGC sequence for the wild-type C603 codon and a TGG sequence for the recombinant plasmid p603W. The codon sequence for C607 was TGT for the wild-type and TAT for the recombinant plasmid p607Y (Figure 2Bii).

**Discussion**

In conclusion, we have established a new comprehensive LightCycler real-time approach for the specific detection of the most frequent UL97 mutations in codons 460 [9], 520 [9], 594, 595, 603 and 607. In comparison with RFLP and sequencing analysis, our LightCycler assays allowed a very fast and sensitive detection of specific UL97 point-mutations associated with GCV resistance. Additionally, this procedure enabled the semiquantitative detection of different ratios of wild-type and mutant strains co-existing in vivo. Few reports have been published on the simultaneous detection by RFLP of co-existing wild-type and mutant strains in mixed viral populations [7–9,14]. With RFLP it is only possible to provide semiquantitative estimations of co-existing wild-type and mutant strains in vivo by densitometric analysis of the DNA fragments in an agarose gel. By contrast, the LightCycler assay with additional melting-point analysis can simultaneously detect different ratios of wild-type and mutant strains with a high sensitivity and offers a semiquantitative statement. With RFLP and direct sequencing it was not always possible to detect small amounts of mutant or wild-type strains within mixed viral populations [8,14], as sequencing analysis failed with small amounts of extracted DNA and, therefore, confirmation of RFLP results had to be omitted. By contrast, only 2 μl of extracted DNA is necessary for successful detection of UL97 mutations by real-time PCR.

Already known deletion mutants [3] or newly described UL97 deletions [15,16] cannot be specifically detected using our new real-time approach without sequencing, but alterations of the specific melting point will arise.

The presented set of UL97 LightCycler real-time PCR assays for codons 594, 595, 603 and 607, taken together with the similar established assays for codons 460 and 520 [9], will contribute to a better understanding of the in vivo dynamics of replication of HCMV UL97 wild-type and mutant strains and represents an important alternative to RFLP analysis.

**Disclosure statement**

The authors declare no conflict of interest.

**References**


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