Valganciclovir as pre-emptive therapy for cytomegalovirus infection post-allogenic stem cell transplantation: implications for the emergence of drug-resistant cytomegalovirus

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Objectives: Valganciclovir is a well established drug for the management of cytomegalovirus (CMV) infection in haematopoietic stem cell transplantation (HSCT). Data concerning its safety regarding the development of drug resistance are required. The aim of the present study was to retrospectively investigate CMV drug resistance in a group of HSCT patients experiencing relapses of CMV infection after a first-line pre-emptive antiviral therapy.

Methods: Thirteen adult HSCT patients out of 26 with asymptomatic CMV infection, experiencing relapsing infections 45–155 days after either intravenous (iv) ganciclovir (2 patients) or valganciclovir (11 patients), were studied. Genotypic assays for mutations in the viral phosphotransferase (UL97) and DNA-polymerase (UL54) genes were directly applied on patient specimens. Baseline CMV sequences were compared with those at the time of relapses to identify drug-resistant strains.

Results: UL97 mutations A594V and M460V known to confer drug resistance developed in one relapsing patient who received iv ganciclovir as first-line therapy, corresponding to a rate of 7.7% of relapses due to drug-resistant strains and an overall 3.8% rate of infections due to CMV drug-resistant strains. UL54 drug resistance mutations were absent. No evidence of drug resistance was found in patients on valganciclovir either as first-line therapy or as treatment for relapses.

Conclusions: The safety profile of valganciclovir as anti-CMV pre-emptive therapy was confirmed, as well as that monitoring CMV drug resistance with genotypic tests on sequential isolates over the time-course of therapy offers guidance to tailor antiviral treatment in a clinically relevant time frame.

Keywords: CMV, UL97, UL54, sequences, mutations

Introduction

Cytomegalovirus (CMV) is a major opportunistic infectious agent in haematopoietic stem cell transplantation (HSCT) and strategies such as prophylaxis and pre-emptive therapy have been adopted by HSCT centres to reduce CMV disease morbidity and mortality.¹–⁶ Major limitations to antiviral prophylaxis are either the exposure of a significant proportion of patients who will never develop CMV disease to prolonged courses of antiviral therapy and related side effects, enhancing the chance of developing drug resistance,⁷,⁸ or the late onset of CMV disease after prophylaxis discontinuation.⁹,¹⁰ An alternative approach to prophylaxis is the pre-emptive treatment of asymptomatic CMV-infected patients; this strategy is based on a strict virological monitoring of patients with viral parameters for the early detection of CMV infection in whole blood (WB) such as pp65 antigen test and/or the evaluation of CMV DNA by quantitative molecular techniques.²,³,¹¹–¹６ The pre-emptive treatment of CMV infection has proved to be beneficial to patient survival with a significant reduction in the incidence of CMV disease and is less costly than prophylaxis.¹⁴,¹⁵ Since CMV is an immunomodulated virus, patients can experience more than one
episode of infection according to the net state of immunosuppression and, as a consequence, they may be exposed to several courses of antiviral therapy or prolonged antiviral treatments.

Long or intermittent courses of antiviral drugs predispose to the development of CMV drug-resistant strains.17–21 A potential risk of developing drug resistance for long-term administration of intravenous (iv) ganciclovir, a nucleoside analogue inhibitor of CMV DNA polymerase (encoded by UL54) that is at present the first therapeutic choice for the treatment of CMV disease in immunosuppressed patients, has been described in HIV-1-infected patients (pre-HAART era) in whom ganciclovir-resistant isolates affected from 2% to 15% of patients after 3–18 months of therapy.22–25 In solid organ transplantation (SOT), the incidence of CMV infections due to ganciclovir-resistant strains is estimated at below 10%.18–21 Important studies in SOT patients showed that ganciclovir-resistant infections are associated with a poor outcome and a significant patient morbidity and mortality and graft failure.7,8,18,26

In HSCT patients, the emergence of CMV drug-resistant strains appears to be limited to case reports with the exception of children in whom antiviral resistance is a rapidly emerging problem, associated with long iv ganciclovir administration and a poor patient outcome.27–34 The recently introduced oral l-valyl ester prodrug of ganciclovir (valganciclovir) with a bioavailability of ~60% of the oral formulation of ganciclovir is currently used by many SOT and HSCT centres for CMV prophylaxis or infection treatment due to the combination of oral availability and excellent efficacy in viral suppression.4–6,35 Few published reports have addressed the issue of drug resistance related to valganciclovir in HSCT patients suggesting that valganciclovir resistance seems to very seldom occur so that treatment with valganciclovir appears to be safe.3,36,37 Since valganciclovir is actually a well established drug for the management of CMV infection in many HSCT centres, data about its safety in this setting concerning drug resistance are required.

Drug resistance to ganciclovir results from mutations in either the UL97 phosphotransferase gene (the kinase product responsible for ganciclovir phosphorylation and activation) or in the UL54 gene coding for the DNA polymerase or in both.18–21,38–41 UL97 mutations conferring ganciclovir resistance are more frequent than those in the UL54 region and limited to the coding region for the C-terminal half of the phosphotransferase gene, occurring within the putative ATP-binding (codons 460–520) and substrate recognition (codons 590–607) sites.21,39,40 UL54 mutations are associated with drug resistance not only to ganciclovir, but also to foscarnet and cidofovir and are widespread over a much larger stretch of nucleotides so that their identification is more complex.41 Standard phenotypic tests for CMV drug resistance based on the plaque reduction assay are presently too time-consuming to be clinically and therapeutically useful.42 Genotypic methods based on the detection of mutations by sequencing strains from viral culture are clinically limited by the slow growth of the virus and the long turnaround time, yielding results in 3–4 weeks.38,42 Therefore, the detection of CMV drug-resistant strains directly from patient specimens by genotypic assays is an issue of important value because it allows results to be available in a clinically relevant time frame, thus improving the management of patients with alternative choices of treatment. The limitation of CMV drug resistance to fixed sites enhances the feasibility of UL97 and, to a lesser extent, UL54 genotypic PCR-based tests directly applied to patient specimens during the virological monitoring of CMV infection in clinical settings.43–45 Major advantages of genotypic assays include a short turnaround time and the ability to detect mixtures of mutant and wild-type with as little as 10% to 20% of a mutant virus in a background of wild-type virus.18,21,40 There are also interesting observations that genotypic tests are more sensitive than phenotypic ones in detecting mixed populations.7,8,46 However, interpretation of results from genotypic tests (i.e. new mutations at other codons, discrimination between mutations associated with natural polymorphism from those related to drug resistance) requires confirmation with phenotypic testing and marker transfer studies.8,21,40

The aim of the present study was to retrospectively investigate for CMV drug resistance in a group of HSCT patients experiencing asymptomatic relapses of CMV infection after a first-line pre-emptive antiviral therapy with either iv ganciclovir or valganciclovir, as a group at high risk of developing drug resistance. Since valganciclovir was the first-line treatment of CMV infection in the majority of patients, attention was focused on detecting viral mutations in association with valganciclovir. Two genotypic assays for UL97 and UL54 mutations known to confer drug resistance (UL97 codons 439–641 and UL54 codons 380–848) were developed and directly performed on patient specimens by CMV DNA direct sequencing. Comparison of baseline sequences at the time of the first episode of CMV infection with those at the time of CMV relapses, allowed the identification of CMV drug-resistant strains.

Materials and methods

Clinical specimens

Between January 2005 and December 2007, blood samples (n = 35) from 13 adult patients with asymptomatic CMV infection after HSCT were retrospectively studied. Patients were selected from a group of 26 HSCT recipients with CMV infection receiving preemptive anti-CMV treatment for CMV DNA values >10000 copies/mL.14,15 The 13 study patients corresponded to those who experienced one or more relapses of CMV infection 45–155 days after a first episode of CMV infection and a first-line anti-CMV treatment. The remaining 13 patients were not evaluated because they promptly responded to the first-line antiviral treatment: CMV DNA became negative within 6 days of antiviral treatment with no subsequent recurrence of CMV infection.

CMV infection was defined according to published recommendations and the relapse of the infection by the recurrence of CMV DNA by PCR on WB from negative to positive confirmed in two different samples 1 week apart after a first-line anti-CMV therapy.27 Clinical drug resistance was suspected when blood CMV DNA failed to decline to undetectable levels despite 4 weeks of antiviral therapy.37

The first-line therapy for CMV infection was either iv ganciclovir (5 mg/kg twice daily for 14 days followed by 5 mg/kg once daily for 14 days, 2 patients) or valganciclovir (900 mg twice daily for 14 days followed by maintenance of 450 mg once daily for 14 days, 11 patients). Relapses of CMV infection were treated with foscarnet (induction dose: 180 mg/kg once daily for 14 days followed by a maintenance dose of 90 mg/kg once daily, for 14 days), valganciclovir or cidofovir (induction dose: 5 mg/kg once a week for
2 weeks, followed by 5 mg/kg every 2 weeks as maintenance). All doses were individualized for renal function. Success of treatment was defined by two negative PCR results for CMV DNA on two occasions.

Informed consent for bone marrow transplantation and all the procedures involved in it as well as those involved in infection monitoring and treatment was obtained from the study patients.

**CMV infection monitoring**

CMV infection was monitored weekly with the quantitative detection of CMV DNA on WB by real-time PCR starting 10 days after HSCT. A real-time PCR assay targeting the UL123 CMV genomic region (immediately early 1, exon 4) (Q-CMV Real-Time, Nanogen, Bittiglieri Alta, Italy) was applied to DNA extracted from WB, as published previously.13 The human β-globin gene was amplified as internal control of both DNA extraction and PCR.

The first blood sample that was positive for CMV DNA during the first episode of CMV infection and before antiviral treatment, as well as the first sample at the time of any CMV relapse, was studied for UL97 and UL54 mutations conferring drug resistance.

**Analysis of CMV UL97 and UL54 genes for drug resistance**

CMV DNA extraction from WB. DNA was directly extracted from 200 μL of WB using the QIAmp DNA Mini Kit (Qiagen, Milan, Italy) extraction system based on nucleic acid silica-gel affinity, following the manufacturer’s instructions.

Analysis of UL97 (phosphotransferase gene) mutations. A nested PCR was developed for amplifying the portion of UL97 from codon 439 to 641 in a single 626 bp PCR product including all of the known ganciclovir resistance mutation sites. The first PCR round was performed with 10 μL of DNA added to a 40 μL PCR mixture containing 1.5 mM MgCl2, 200 μM of each dNTP, 1 U of Taq DNA polymerase (Qiagen) and 20 pmol of primers F1278 (forward) and R2013 (reverse) as reported by Hu et al.25 Primer 3 (Steve Rozen and Helen J. Skaketsky, http://frodo.wi.mit.edu/primer3/input.htm) was applied to design the inner UL97-s1318 and UL97-as1924 primers (Figure 1a). The first PCR step consisted of an initial denaturation at 95°C for 5 min, followed by 40 cycles at 95°C for 40 s, 60°C for 30 s and 72°C for 40 s. Two microliters of the first-round PCR product was used in a second PCR round with similar conditions to the first PCR step, except that primers UL97-s1318 (5'-GCCAGGCTATATCAAATTCT-3') and UL97-as1924 (5'-ACATCTGGCCTCAACAGG-3') were used. Annealing temperature was set at 55°C and 25 cycles of reaction were performed. PCR products were visualized by gel electrophoresis with 2% agarose and ethidium bromide staining. Negative and zero DNA controls were set for every three samples and PCRs were performed according to the manufacturer’s instructions.

Analysis of UL54 (DNA-polymerase gene) mutation. A nested PCR for a segment containing the most common mutations was developed (codons 380–848, Figure 1b). Primer 3 (Steve Rozen and Helen J. Skaketsky http://frodo.wi.mit.edu/primer3/input.htm) was used to design UL54 primers. The first PCR round was performed with 6 μL of DNA added to a 24 μL PCR reaction mixture containing 1.5 mM MgCl2, 200 μM of each dNTP, 1 U of Taq DNA polymerase and 20 pmol of primers F-1050 (5'-CCTTCTGGCGGAGGTGTGATCT-3') and R-2558 (5'-CAGGGCTCTGAAAGGAGTTG-3'). An initial denaturation step at 95°C for 5 min was followed by 40 cycles at 95°C for 45 s, 50°C for 45 s and 72°C for 45 s. Two microliters of the first PCR product was used in a second PCR step consisting of three separate PCRs encompassing amino acids 380–848: one for the domain IV and the first part of δ-region C (UL54-1: codons 380–545), a second for the δ-region C (UL54-2: codons 544–720) and a third for domains IV to III (UL54-3; codons 670–848) (Figure 1b). Primer sequences were as follows: UL54-1, UL54-1141 (5'-GAGTTCTCCGGATGTACCA-3') and UL54-1857 (5'-ATCGGTGACAGACACAGCA-3'); UL54-2, UL54-1630 (5'-GTATTGGTGCGCGGATGCTGT-3') and UL54-2140 (5'-CCACGGGGTTCTTTGATGTA-3'); UL54-3, UL54-2039 (5'-GCGTTTCCACGACAATCAG-3') and UL54-2544 (5'-CTGTCGCTCTAGCATGTCT-3').

Sequencing of UL97 and UL54 PCR products. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen) and directly sequenced with the Big Dye Terminator Cycle Sequencing Kit (ABI, Foster City, CA, USA) on the ABI Prism 310 Genetic Analyzer. Sequences were analysed with the DNA Sequencing Analysis SoftwareTM version 3.7 and the BioEdit version 7.0.0 software package under ClustalW for alignments (bootstrap method: 1000 replicates). Sequences were compared with that of the laboratory strain AD169 (GenBank accession numbers BK000394 and X17403) as reference strain to determine the presence of known drug resistance mutations. Mutations were defined as changes in the UL97 and UL54 coding region known to confer resistance to anti-CMV drugs, while polymorphisms were defined as changes in the UL97 coding region known to not alter susceptibility to ganciclovir and other antivirals or for which the effect on drug susceptibility was unknown because marker transfer experiments had not been performed.38–41 To identify CMV UL97 new mutations, sequence alignment was performed with UL97 complete and incomplete sequences from laboratory and clinical strains reported by the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Seventy-seven UL97 sequences were examined [see Supplementary data, available at JAC Online (http://jac.oxfordjournals.org/)].

A commercially available panel of CMV DNA standards at concentrations of 500,000, 50,000, 5000, 500 and 0 genomes/mL (OptiQuant CMV DNA; Acrometrix Corp., Benicia, CA, USA) was used for assessing UL97 and UL54 nested-PCR sensitivity.

Statistical analysis

CMV viral load was reported as log10 copies/mL of CMV DNA. Viral load differences for categories were considered to be statistically significant for P values <0.05, according to Student’s t-test.

Results

Blood samples (n=35) from 13 patients with CMV relapsing infection were studied for UL97 and UL54 mutations conferring resistance to anti-CMV drugs. Thirty-one asymptomatic episodes of CMV infection were studied, corresponding to a mean of 2.4 episodes/patient. The number of samples investigated for both UL97 and UL54 was 2.7 per patient. Table 1 shows the characteristics of the study group, according to the treatment of CMV infection for both the first episode and further relapses. Eleven patients were treated with valganciclovir as first-line therapy, while iv ganciclovir was given to two patients. At the time of CMV relapse, infection treatment consisted of valganciclovir (7 episodes), foscarnet (10 episodes) or cidofovir (1 episode).

Two independent nested-PCR formats were designed to amplify UL97 and UL54 directly from WB of patients
undergoing CMV infection monitoring, allowing distinct final single PCR products including most of known CMV drug-resistant mutation sites. The sensitivity of two PCRs was assessed with a CMV DNA standard panel that showed how the two nested-PCRs efficiently amplified UL97 and UL54 with a 100% detection rate at 1250 copies/mL of CMV DNA (10 positive results out of 10 replicates). Sequences were available for 99% of the specimens.

At baseline, the wild-type strain of CMV was identified in all patients. At the time of CMV relapsing infection, four patients had evidence of UL97 mutations as either a single amino acid mutation (three out of four patients) or as a double mutation (one out of four patients), but only one of them harboured mutations known to be associated with ganciclovir resistance by phenotypic test and confirmed by marker transfer: A594V and M460V (Table 2, patient ID # 5).34,36,37 This patient underwent a short course of foscarnet followed by iv ganciclovir as first-line therapy for CMV infection; 63 days after the first episode, a relapse of the infection occurred with a wild-type CMV. The patient was treated with iv ganciclovir followed by valganciclovir. Two months later, the patient experienced a third episode of CMV infection due to a strain harbouring the drug resistance mutation A594V. Foscarnet was administered and CMV DNA declined to undetectable levels at the end of treatment. After 20 days, a new CMV infection occurred, with a strain harbouring a different UL97 drug resistance mutation.

Figure 1. PCRs developed for amplifying the UL97 gene (a) and the UL54 gene (b). (a) PCR design for amplifying the portion of the UL97 gene that includes all of the most common ganciclovir resistance mutation sites from codon 439 to 641. The first PCR round amplified a 755 bp region from codon 426 to 678, while the second PCR step amplified a single 626 bp product that was directly sequenced containing the most common UL97 mutations. (b) PCR design for amplifying the most common mutations from codon 380 to 848 that confer ganciclovir, cidofovir and foscarnet resistance. The first PCR round amplified a 1530 bp region from codon 350 to 859. The second PCR step consisted of three separate PCRs: the first one (UL54-1 PCR) amplified codons 380–545, the second (UL54-2 PCR) amplified codons 544–720 and the third (UL54-3 PCR) amplified codons 670–848.
M460V. A further course of foscarnet led to the final eradication of the infection.

Three patients harboured three different UL97 mutations at the time of CMV relapsing infection (Table 2, patient ID # 6, 9 and 13). One patient (patient ID # 9) developed the D605E mutation that has already been reported, but confirmed as not related to drug resistance by either phenotypic assay or marker transfer.27,38,39,42,48 No signs of clinical drug resistance were suspected in this patient and CMV DNA fell to undetectable levels after treatment with foscarnet. A new mutation (H602Y, patient ID #13), identified by comparing CMV UL97 sequences from our study with UL97 sequences from both laboratory and clinical isolates in GenBank [n = 77, see Supplementary data, available at JAC Online (http://jac.oxfordjournals.org/)], which has never been described as associated with CMV drug resistance, developed in a patient who received a regular course of valganciclovir as first-line treatment (CMV DNA became negative after 10 days of treatment). Forty-five days after the first infection, the virus relapsed and a second course of antiviral led the clearance of the virus. No clinical signs of drug resistance were present in this patient either in the first or second episode of infection, but further confirmation tests are required for a definite conclusion about the mutation H602Y. A recently reported UL97 mutation at position 526 (M526I) (S. I. Sanchez Puch and C. M. Vileda, GenBank accession number EU784737) was harboured by patient ID #6 who was treated with valganciclovir as first-line anti-CMV drug and relapsed 60 days after the first episode of infection. Our observation is the first one consistent with M526I as a new UL97 polymorphism not associated with clinical evidence of CMV drug resistance, since the patient cleared the virus after a second course of valganciclovir. However, a conclusive statement concerning the roles of both H602Y and M526I in CMV drug resistance can only be made after phenotypic testing by marker transfer.

Table 1. Characteristics of the study group, according to the treatment of CMV infection for both the first episode of CMV infection and CMV relapsing infections

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>D/R status</th>
<th>First-line therapy of CMV infection</th>
<th>No. of episodes of CMV infection</th>
<th>Therapy of CMV relapse(s)</th>
<th>Follow-up (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>3</td>
<td>foscarnet</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>D+/R−</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>D+/R+</td>
<td>VGCV</td>
<td>3</td>
<td>foscarnet</td>
<td>17</td>
</tr>
<tr>
<td>5</td>
<td>D+/R+</td>
<td>foscarnet—iv GCV</td>
<td>4</td>
<td>iv GCV—VGCV</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>2</td>
<td>VGCV</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>2</td>
<td>VGCV</td>
<td>4</td>
</tr>
<tr>
<td>8</td>
<td>D+/R+</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>D+/R+</td>
<td>VGCV</td>
<td>3</td>
<td>VGCV</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>D+/R+</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>D—/R+</td>
<td>iv GCV</td>
<td>2</td>
<td>VGCV</td>
<td>3</td>
</tr>
<tr>
<td>13</td>
<td>D—/R+</td>
<td>VGCV</td>
<td>2</td>
<td>foscarnet</td>
<td>7</td>
</tr>
</tbody>
</table>

D, donor; R, recipient; GCV, ganciclovir; iv, intravenous; VGCV, valganciclovir.

Table 2. UL97 mutations and resistance status according to published studies

<table>
<thead>
<tr>
<th>Amino acid mutation</th>
<th>Resistance status as reported by the literature</th>
<th>No. of observed patients</th>
<th>Patient ID (see Table 1 as reference)</th>
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<tr>
<td>M460V</td>
<td>marker confirmeda</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>A594V</td>
<td>marker confirmeda</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>D605E</td>
<td>reportedb</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>H602Y</td>
<td>new</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>M526I</td>
<td>newc</td>
<td>1</td>
<td>6</td>
</tr>
</tbody>
</table>

aMutations that have been shown to confer resistance by phenotypic testing and confirmed by gene marker transfer studies in vitro.34,36,37
bMarker transfer studies did not show any functional deficiency for the UL97-encoded phosphotransferase enzyme.27,38,39,42,48
cS. I. Sanchez Puch and C. M. Vileda, GenBank accession number EU784737.

Allice et al.
CMV drug resistance and valganciclovir pre-emptive therapy

Table 3. Comparison between baseline CMV strains and those at the time of CMV relapse in the four patients with UL97 mutations; CMV serostatus of donors and recipients before transplantation and the time between the first episode and the relapse of the infection is shown

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>D/R CMV serology</th>
<th>CMV UL97 strain at baseline</th>
<th>Number of episodes of CMV infection</th>
<th>CMV UL97 strain at relapse</th>
<th>Days after the first episode of CMV infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>D+/R+</td>
<td>wild-type</td>
<td>4</td>
<td>A594V</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>M460V</td>
<td>145&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>D−/R+</td>
<td>wild-type</td>
<td>2</td>
<td>M526I</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>D−/R+</td>
<td>wild-type</td>
<td>2</td>
<td>D605E</td>
<td>90</td>
</tr>
<tr>
<td>13</td>
<td>D−/R+</td>
<td>wild-type</td>
<td>2</td>
<td>H602Y</td>
<td>45</td>
</tr>
</tbody>
</table>

D, donor; R, recipient.

<sup>a</sup>See Table 1 as reference for patient characteristics.

<sup>b</sup>Days after the second episode of CMV infection. M594V was not present at that time. See text for details.

A good agreement between these and reference assays based on the study of drug susceptibility in viral isolates from culture has been established.<sup>23–25,38,45</sup> Moreover, interesting observations claim that genotypic tests are more sensitive than phenotypic ones in detecting mixed populations of wild-type and mutants.<sup>18,21,40</sup> Data from the literature suggest that the proportion of resistant virus detectable by direct DNA sequence would be above 10% to 20% of mutant virus in a mixture with the wild-type.<sup>18,21,40</sup> Even if the technique of direct sequencing may be more sensitive than phenotypic tests, it is not sensitive enough to detect a minority population of resistant viruses (<20%), which may be present and whose association with clinical failure is not understood, but it is generally accepted that for low levels of CMV DNA, CMV drug resistance is unlikely to be significant from a clinical standpoint.<sup>18,43,46</sup>

The CMV UL97 gene is by far the most frequent site of mutations conferring resistance to the drug of choice for CMV infection, which at present is ganciclovir.<sup>18–20</sup> Since UL97 is a highly conserved gene among clinical isolates and drug resistance mutations are located in fixed positions within codons 439 and 641, it is possible to develop a PCR-based genotypic assay for a single PCR product that includes known ganciclovir resistance mutation sites.<sup>42–44</sup> Mutations in the UL54 gene coding for the DNA polymerase can also occur during ganciclovir, foscarnet and cidofovir treatment.<sup>18–20,26</sup> Genotypic assays for the CMV UL54 region are more complex to design due to the fact that it is necessary to cover several codons that are widespread across the region, which is also larger than UL97.<sup>18,19</sup>

Two genotypic assays have been developed for UL97 and UL54 mutations conferring drug resistance with the purpose of being directly performed on blood specimens from patients undergoing infection monitoring after HSCT, to study the development of CMV drug resistance. Molecular assays were designed in order to make the procedure of identifying these strains simple, fast and with a reasonable turnaround time (~72 h) as requested when laboratory results need to be applied to clinical settings. To increase the sensitivity of the method and partially overcome the limitation of genotypic assays to discriminate between mixed viral populations of wild-type and mutants at 10% to 20%, a nested PCR was designed.<sup>18,21,40</sup> Both the assays showed excellent sensitivity as tested with a proficiency panel. The lower detection limit was 1250 copies/mL, but for levels below these values, ganciclovir resistance is unlikely to occur, as shown by published studies.<sup>18,19,42,43</sup>

CMV remains one of the leading opportunistic infectious agents in HSCT patients. In response to the widespread use of antiviral drugs as both prophylaxis and pre-emptive treatment and the awareness that long-term therapy and suboptimal drug concentration do increase the risk of drug resistance, the demand for CMV resistance assays has grown.<sup>37,43–45,49</sup> The availability of rapid assays for CMV mutations conferring drug resistance could allow the optimization of anti-CMV treatment and the choice of alternative drugs.

Genotypic PCR-based assays for the detection of CMV drug resistance are the most suitable tests for clinical purposes.
The study for CMV UL97 and UL54 resistance was retrospectively performed in a subset of HSCT patients experiencing relapses of CMV infection after a first-line treatment with ganciclovir or valganciclovir, as a group at high risk of developing drug resistance. Baseline viral sequences before antiviral therapy were started and sequences at the relapse of the infection were analysed and compared. CMV wild-type strain was identified in all the first episodes of CMV infection but UL97 mutations known to confer ganciclovir resistance were present in 3.8% (1 out of 26) of patients and in 7.7% (1 out of 13) of those with CMV relapsing infection, while resistance due to UL54 mutation was absent in all of them. Therefore, data from the present study confirm that the incidence of CMV drug resistance in HSCT patients is low in agreement with previously published work.5,28-30 Moreover, relapsing infections due to resistant strains were only a minority of all the infections (11%). In fact, CMV relapses, quite a frequent phenomenon in HSCT patients, are a reflection of an excessively immunocompromised status since immunological reconstitution plays an important role in the final eradication of the infection. Nevertheless, the issue of CMV drug resistance cannot be neglected, as the outcome in HSCT patients infected with drug-resistant strains can be poor.28-30

UL97 ganciclovir resistance mutations M460V and A594V were identified in two consecutive episodes of CMV infection in a patient who received iv ganciclovir as first-line therapy. No evidence of drug resistance was found in patients on valganciclovir either as first-line therapy or as treatment for relapses. Therefore, the present experience confirms the safety profile of valganciclovir, in agreement with previously published studies showing that resistance to valganciclovir occurs at a significantly lower level than with iv ganciclovir and only for more than 3 months of valganciclovir treatment.4,49 The switch to drugs other than iv ganciclovir such as valganciclovir, foscarnet and cidofovir can be considered to be a safer option as the emergence of CMV resistance is unlikely when these drugs are administered for short courses. Moreover, an investigational drug, maribavir, has recently been described as highly active against CMV isolates resistant to ganciclovir, cidofovir or foscarnet.50,51

Recently published data showing a remarkable incidence of CMV resistance in SOT patients while on valganciclovir prophylaxis can be related to a longer exposure to the drug as required by anti-CMV prophylaxis programmes (first 3 months after transplantation), than the much shorter exposure experienced by our study group (median time: 28 days), due to the different approach to CMV infection based on the pre-emptive therapy.5,20

Three UL97 mutations, M526I, H602Y and D605E, the last one already reported as not related to ganciclovir resistance by marker transfer experiments, were present in three patients undergoing valganciclovir as first-line treatment.38,42,43 None of these mutations was associated with clinical evidence of drug resistance since all patients successfully cleared CMV infection after a further course of antivirals. M526I lies outside the documented region for ganciclovir resistance (codons 460, 520 and 590–607), thus it is unlikely to confer drug resistance, but a conclusive statement can only be made after phenotypic testing by marker transfer. The other two (H602Y and D605E, the first a new one, the latter not confirmed by marker transfer studies that did not show any functional deficiency for the UL97-encoded phosphotransferase enzyme) lie within a region with a certain genetic instability.43 In fact, observations have been published suggesting a certain genetic variability at the site 590–607 of the UL97 gene.38,42,43 In this region, a considerable diversity of mutations and deletions have been linked to varying degrees of drug resistance, but not entirely validated by marker transfer, none of which was detected in the present study. However, the conclusive roles of M526I and H602Y require further confirmation analysis.

In conclusion, the safety profile of valganciclovir as preemptive therapy for CMV infection was confirmed, as well as that monitoring CMV drug resistance with genotypic testing on sequential isolates from the same patient over the time-course of therapy could offer guidance to tailor anti-CMV treatment in a clinically relevant time frame.

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Supplementary data
Supplementary data is available at JAC Online (http://jac.oxfordjournals.org).

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