Compartmentalization of Acyclovir-Resistant Varicella Zoster Virus: Implications for Sampling in Molecular Diagnostics

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Background. Acyclovir resistance of varicella zoster virus (VZV) may arise in stem cell transplant (SCT) recipients with VZV disease and is usually a result of mutations in VZV thymidine kinase (TK), which is the target protein of acyclovir. Early detection of such mutations is necessary to enable timely therapy adaptation, for example, to foscarnet. We aimed to investigate whether TK mutations arise over time, and what sample types might be the most useful for this method.

Methods. Spatially and temporally distinct samples from 3 SCT recipients with VZV disease unresponsive to acyclovir treatment were retrospectively investigated for the presence of TK mutations by polymerase chain reaction and sequence analysis.

Results. In all 3 patients, a mutation in the VZV TK coding region was found resulting in an amino acid substitution. TK mutations were not only temporally but also spatially compartmentalized. In particular, plasma samples frequently showed wild-type TK sequences, whereas cerebrospinal fluid or skin vesicle fluid acquired on the same day contained mutant sequences.

Conclusions. This study shows the importance of careful sampling for molecular diagnostics of acyclovir resistance in VZV disease. All affected body sites should be sampled and plasma samples may not be representative for the viral mutation status.

Varicella zoster virus (VZV) reactivation occurs in up to 50% of stem cell transplant (SCT) recipients [1]. The multitude of complications that may occur after reactivation can be divided into 4 groups: cutaneous, visceral, neurological, and ocular (reviewed in [2]). The standard treatment for VZV disease in these patients is the guanosine analogue acyclovir. However, resistance to acyclovir is increasingly reported, especially in the context of immunosuppression where prolonged viral replication occurs. Early recognition of resistance to acyclovir is necessary to enable an appropriately timed therapy switch (usually to foscarnet [3]). Phenotypical assessment of acyclovir resistance by plaque reduction assays was long considered to be the gold standard but has several disadvantages: these tests are time-consuming, they rely on the presence of a sufficient amount of infectious virions in the clinical sample, and the interpretation of the cytopathic effect may be subject to interobserver variability. Moreover, the presence of a heterogeneous population of resistant and wild-type (WT) viruses may preclude reliable analysis [4]. A suitable alternative to phenotypical resistance analysis is sequence analysis of the viral open reading frame (ORF) 36 encoding the viral thymidine kinase (TK) [5]. VZV TK is indispensable for the initial step in Acyclovir phosphorylation towards its active triphosphate form (reviewed in [6]). VZV TK sequence analysis correlates well with phenotypical resistance assays [4, 7, 8] and is feasible in a routine clinical diagnostic setting [8]. Compared with phenotypical assays, VZV TK sequence analysis has a much shorter time-to-result. This analysis
can be applied to samples that may contain any form of viral DNA—not necessarily infectious viral particles—and its read-out is not hampered by interobserver variability. At present, it is not known what sample type is preferred for VZV TK sequence analysis. In general, plasma samples are regularly obtained from patients with suspected VZV disease to confirm clinical observations and to monitor the effects of treatment [9, 10]. It would be convenient if these samples could also be used for the detection of TK mutations. In particular, the use of plasma instead of cerebrospinal fluid (CSF) could greatly reduce discomfort for patients who have neurological complications of VZV disease. Moreover, if plasma samples would reveal the presence of mutated VZV even before onset or progression of clinical symptoms, this would greatly facilitate timely therapy adaptation.

In this study, we have retrospectively sequenced the viral TK of VZV from temporally and spatially diverse clinical specimens from 3 SCT recipients who developed complicated VZV disease that was unresponsive to acyclovir therapy. Our aim was to determine (1) whether mutations in the VZV TK gene could be detected in these patients, (2) whether these mutations arose over time (probably induced by the presence of Acyclovir), and (3) whether the presence of these mutations could be monitored in plasma during or even before the onset of clinical complications.

MATERIALS AND METHODS

All clinical samples described were routinely collected (for surveillance purposes) from 3 SCT recipients at the Department of Medical Microbiology, Maastricht University Medical Center, the Netherlands (a 750-bed referral hospital), between March 2005 and December 2007. Patient characteristics are given in the results section; sample characteristics are given in Figure 1.

Nucleic Acid Extraction

Total nucleic acids from 200 μL of fluid samples (plasma, serum, CSF) were isolated using the MagNA Pure LC automated extraction system supplied with the Total Nucleic Acid Extraction Kit (Roche, Almere, The Netherlands), according to the manufacturer’s instructions. Skin vesicles were sampled by swabbing with a cotton bud that was subsequently swirled in 2 mL virus transport medium (Eagle’s Minimal Essential Medium (EMEM; Gibco/Invitrogen) supplied with 0.1% gelatin, 250 μg/mL vancomycin, 75 μg/mL gentamycin, 5 μg/mL amphotericin B, 2 mmol/L L-glutamine and 0.1 mmol/L nonessential amino acids (Gibco); 200 μL of this suspension was used for extraction as described above. Tissue samples (~25 mg/sample) were disrupted using a sterile surgical blade, and DNA was isolated using the QIAamp DNA Mini kit (Qiagen) according to the manufacturer’s instructions.

All nucleic acids were eluted in 120 μL. Prior to isolation, all samples and negative controls were spiked with a processing control consisting of 10^5 copies of a plasmid clone containing a fragment of mouse cytomegalovirus glycoprotein B (mCMV-gb). The Taqman assay for this processing control has been described elsewhere [11, 12].

VZV Polymerase Chain Reaction

The presence of VZV was routinely investigated by Taqman analysis using primers that amplify an 89-bp fragment of VZV ORF38 as described elsewhere [13, 14].

Sequence Analysis of the TK Gene

To enable sensitive amplification of the entire TK coding region and detection of any possible TK mutation present, one index sample with a high VZV load (as shown by low VZV Cycle Threshold (CT) values in the diagnostic Taqman assay; Figure 1) was selected from each patient. The entire ORF36 (TK) was amplified as described previously [8]; additional primers (Table 1) were designed to enable sequencing of this ORF amplicon. Subsequently, patient-specific primers flanking the respective mutations were designed to enable amplification and sequencing of shorter fragments in samples that had lower VZV loads and possibly fragmented DNA, such as plasma. Polymerase chain reaction (PCR) products were purified using the MSB Spin PCRapace kit (Invitrogen). Sequence analysis was performed using the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems). Sequences were compared with the prototype VZV sequence [5] using BLAST.

RESULTS

A graphical overview of the results is given in Figure 1.

Patient 1 (Figure 1A) was a 55-year old man who received a nonmyeloablative Matched Unrelated Donor (MUD) SCT because of Waldenström’s disease (WD). On day 136 after SCT, he was admitted to the hospital because of WD recurrence and various other complications including herpes zoster (HZ) on the skin of his head and his right ear. The HZ did not respond to oral valacyclovir, or to intravenous acyclovir. Because of suspected acyclovir resistance, foscarnet was started on day 165, which led to the resolution of HZ. Unfortunately, the patient’s condition deteriorated rapidly due to complications unrelated to VZV and he died on day 181.

The skin vesicle fluid from day 155 showed a nucleotide substitution (G → A) at position 142 of the TK ORF, resulting in an amino acid change (Glu → Lys) at codon 48.

PCR and sequencing with patient-specific primers flanking the mutation confirmed its presence in the sample of day 155. The mutation was not detected in vesicle fluid samples that were obtained after foscarnet had been started (days 168 and 169),
Figure 1. Time schedule of antiviral medication, clinical symptoms of varicella zoster virus (VZV) Disease, and results of VZV Taqman Assay and VZV thymidine kinase (TK) mutation analysis in various samples from (A) patient 1, (B) patient 2, and (C) patient 3. Symbols representing clinical samples: □ = cerebrospinal fluid; ○ = plasma; ◇ = skin vesicle fluid. Color of symbols: white = VZV negative; light gray = VZV positive with wild-type (WT) TK; dark gray = VZV positive with mix of WT and mutant TK; black = VZV positive with mutant TK. Numbers next to the symbols represent cycle threshold values of the VZV Taqman assay. HZ = herpes zoster.
preceding clearance of HZ. In addition, CSF obtained on days 155 and 169 as well as plasma samples obtained on days 148, 163, 167, and 176 did contain VZV, but all were WT.

Patient 2 (Figure 1B) was a 30-year old woman with Von Willebrand disease who received a NK-mismatched haploidentical SCT (from mother) because of recurrent acute myeloid leukemia (AML) several years after HLA-identical SCT. Forty-eight days after SCT, she developed graft-versus-host disease (GVHD) of the skin and digestive tract that was successfully treated with high dose steroids, cyclosporin and mycophenolate. On the 150th day after SCT, while still treated with cyclosporin and mycophenolate, she developed very painful HZ on the right side of the trunk. This was initially successfully treated with high dose valacyclovir (1000 mg t.i.d.), after which a maintenance dose was started (500 mg b.i.d.). On day 228 HZ reappeared at the same localization; the patient was readmitted on day 231, and intravenous acyclovir was started (5 mg/kg t.i.d.). On day 237, generalized HZ developed and the acyclovir dose was increased (10 mg/kg t.i.d.). On day 250, progressive visual impairment occurred because of VZV-induced neuritis optica and therapy was switched to foscarnet. Both the HZ and the neuritis optica did not improve upon foscarnet administration. In addition, an epileptic insult occurred on day 252, after which MRI was performed showing abnormalities consistent with encephalitis. The patient died on day 254 after having suffered severe rectal bleeding from recurrent GVHD and respiratory insufficiency that did not improve during mechanical ventilation. Autopsy confirmed the presence of generalized VZV disease with cerebral and pulmonary involvement.

The index sample of patient 2 was the CSF obtained on day 249; it showed a nucleotide substitution (T → C) at position 473, leading to an amino acid change (Leu → Pro) at codon 158. Using patient-specific mutation-flanking primers, this mutation was confirmed in the index sample, as well as in optic nerve, brain, and heart tissue samples that were obtained 7 days later at autopsy (not shown). In skin vesicle fluid samples, one of which was obtained 2 days after the index CSF, only the WT strain was detected. Moreover, the mutation was not detected in any of the plasma samples.

Patient 3 (Figure 1C) was a 23-year old Somalian man with recurrent AML. Because of this recurrence, and in the absence of an HLA-identical sibling or a matched-unrelated donor, the patient received a haploidentical SCT (from his sister). Two months after SCT, the patient developed urine retention and loss of muscular strength. CSF sampled on the 65th day was VZV positive, hence VZV-related transverse myelitis was diagnosed. This was successfully treated with intravenous acyclovir. VZV DNA became undetectable on day 100 and the myelitis did not progress. Acyclovir was then replaced by valacyclovir (500 mg t.i.d.). However, a CSF sample acquired on day 113 was again positive for VZV, and acyclovir was reinstalled. At the same time, the patient developed CMV retinitis for which he was treated with ganciclovir (until day 160), followed by valganciclovir (until day 200). Furthermore, the patient received cidofovir from day 208 to 220 to treat diarrhea caused by adenovirus reactivation. Donor lymphocyte infusions were given on day 153 (3 × 10E4 CD3 positive cells/kg), day 200 (1 × 10E4), day 214 (1 × 10E4), and day 238 (3 × 10E4) in an attempt to accelerate immune reconstitution. Indeed, gradual reconstitution of CD4 and CD8+ T-cells was observed in blood samples from day 238 onward (these were still undetectable on day 210).

VZV eventually became undetectable in plasma as well as CSF, but the patient died of bacterial pneumonia on day 261.

The index sample of patient 3 was a CSF obtained on day 162, during the second episode of VZV reactivation. This sample showed a nucleotide substitution (T → C) at position 275, leading to an amino acid change (Leu → Pro) at codon 92. Using patient-specific mutation-flanking primers, the mutation was confirmed in the index sample. Notably, the two CSF samples obtained on days 65 and 89 during the first episode of VZV myelitis, which had been successfully treated with acyclovir, were WT. Mixed sequences of WT and mutant virus in the CSF samples of days 113 and 116 indicate the emergence of the mutant strain. CSF samples from day 135 onward exclusively showed the mutant sequence, whereas plasma samples still yielded mixed sequences until day 154.

In all 3 patients, a polymorphism of nucleotide 863 was found, leading to an amino acid change (Ser → Leu) at codon 288. This is, however, probably not a clinically significant mutation because it is observed in all non-Dumas VZV strains [4, 7, 15, 16].

**DISCUSSION**

In this study, we have demonstrated the presence of mutations in the TK gene of VZV in 3 immunocompromised patients with VZV disease refractory to treatment with acyclovir.

The most striking result was the spatial compartmentalization of VZV TK mutations as seen in all 3 patients. Interestingly, in CMV-infected patients, mixed [17] and complete [18] compartmentalization of CMV carrying ganciclovir

**Table 1. Oligonucleotide Primers**

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence 5’- 3’</th>
<th>Purpose</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK-206F</td>
<td>aacctgccgtcttaagga</td>
<td>Sequencing All</td>
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</tr>
<tr>
<td>TK-712F</td>
<td>cagaaattcaaaatcgcagtg</td>
<td>Sequencing All</td>
<td></td>
</tr>
<tr>
<td>TK-29F</td>
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</tr>
<tr>
<td>TK-391F</td>
<td>caccaatgctctaatat</td>
<td>PCR/sequencing 2</td>
<td></td>
</tr>
<tr>
<td>TK-493R</td>
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<td></td>
</tr>
<tr>
<td>TK-298R</td>
<td>ttatgctagcgggacagga</td>
<td>PCR/sequencing 3a</td>
<td></td>
</tr>
</tbody>
</table>

* Primer TK-298R was combined with primer TK-206F for PCR/sequencing of samples from patient 3.
resistance-conferring UL97 mutations were described previously; it is not known whether this phenomenon exists in other human herpes viruses. At present, it is not clear what factors may have caused compartmentalization of mutant VZV.

In a previous study, de Miranda et al [19] demonstrated that acyclovir uptake into the brain may be lower than in other tissues or in plasma. Therefore, we hypothesize that in the case of patients 2 and 3, subtherapeutical levels of acyclovir in the brain combined with impaired immune surveillance may have resulted in the selection of TK mutated progeny in the course of prolonged viral replication. This is especially suggestive from the observations in patient 3, as a mutant strain arose when intravenous acyclovir was temporarily replaced with oral valacyclovir. In patient 1, who was naïve to acyclovir, selection of mutant VZV due to subtherapeutical drug levels is unlikely. We hypothesize this to be an example of acyclovir resistance conferring TK mutations in VZV that may arise spontaneously at low levels in acyclovir-naïve individuals, as shown previously for herpes simplex virus (HSV) [20, 21]. Despite their decreased virulence, these mutant strains may be more easily selected and/or more pathogenic in immunocompromised than in immunocompetent individuals.

Besides the difference in mutation status between skin and CNS, we observed that plasma did not accurately represent the mutation status of the virus. It is important to note that the precise origin of the viral DNA as detected by PCR in plasma is yet unknown. The isolation of infectious VZV from blood of patients is difficult [22]. Moreover, it has been suggested that VZV DNA in plasma is derived from lysed infected cells rather than from infectious virions [23], similar to Epstein-Barr virus and CMV DNA, which are both highly fragmented in plasma [24, 25]. Hence, in VZV patients with compartmentalization of acyclovir resistance, the plasma may contain a mixture of mutant and WT VZV DNA fragments in any possible proportion, which hampers accurate genotyping. In addition, for patients 2 and 3 the absence of mutant VZV in plasma can be explained by the poor permeability of the blood-brain barrier to herpes viruses [26], causing the mutant strain to largely remain in the brain and not enter the circulation. Concluding, although VZV load in plasma is a convenient parameter for standard therapy monitoring [9], this may not be the sample type of choice for molecular acyclovir resistance assessment.

In retrospect, the introduction of an antiviral other than acyclovir preceded viral clearance in 2 of the patients. In patient 1, clearance of the mutant strain occurred after foscarnet had been started, and this coincided with improvement of HZ lesions. In patient 3, VZV eventually disappeared during ganciclovir treatment of CMV retinitis. Ganciclovir has excellent antiviral properties to VZV both in vitro [27] and clinically [28]. Like acyclovir, ganciclovir needs an initial phosphorylation step by a viral kinase to become active. Interestingly, a kinase other than TK was shown to phosphorylate ganciclovir in VZV-infected cells [29], suggesting that ganciclovir may be used as a salvage therapy in acyclovir-resistant VZV disease. However, in patient 3, the rather large interval between start of ganciclovir treatment and clearance of VZV, together with the observed T cell reconstitution, suggests that the patient’s recovered immune system also contributed to VZV clearance.

Although biochemical characterization of TK proteins is beyond the scope of the present study, it is likely that the respective mutations observed were the cause of acyclovir resistance in the 3 patients. This can be deduced from clinical observations: In patients 2 and 3 an initial episode of VZV disease had successfully been treated with valacyclovir and acyclovir, respectively. During the subsequent episodes dominated by mutant virus, acyclovir treatment was not successful, indicating that the mutations indeed conferred resistance. In patient 1, improvement of HZ upon therapy switch to foscarnet strongly suggests that the mutation was related to acyclovir resistance. Interestingly, a mutation identical to the one found in patient 1 was described previously in a VZV strain obtained by random mutagenesis [30], which showed strongly reduced TK activity.

Returning to our aims of study, we have shown that (1) mutations in the VZV TK gene could be detected in clinical samples from 3 patients with acyclovir-unresponsive VZV disease, (2) mutations did arise over time and in 2 of the 3 cases were probably induced by selective acyclovir pressure, and (3) the presence of these mutations could not accurately be monitored in plasma.

In summary, our results indicate that molecular resistance monitoring of VZV should be performed frequently and in all body compartments where VZV disease is present or suspected. Although plasma VZV load measurement is generally considered useful [9, 10], we have shown that plasma samples may not be representative for the resistance status of the virus.

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Potential conflicts of interest. All authors: no conflicts.

References
