Analysis of Ganciclovir-Resistant Human Herpesvirus 6B Clinical Isolates Using Quenching Probe PCR Methodology

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Quenching probe PCR (QP-PCR) analysis was used to determine the frequency of ganciclovir (GCV) resistance among clinical isolates of human herpesvirus 6B (HHV-6B) obtained from patients with primary viral infection and viral reactivation. Forty-two HHV-6B clinical isolates were repeatedly recovered from 15 hematopoietic stem cell transplant (HSCT) recipients, and 20 isolates were recovered from 20 exanthem subitum (ES) patients. Of the 15 HSCT recipients, 9 received GCV during the observation period; however, none of the ES patients were treated with GCV. Two established laboratory strains, Z29 and HST, were used as standards in this study. Regions 1 and 2 of the U69 gene of all of the clinical isolates demonstrated the same melting temperature as regions 1 and 2 of the Z29 strain. For region 3, the melting temperatures of all clinical isolates fell between the melting temperature of the plasmid containing the A462D single nucleotide polymorphism (SNP) and the melting temperature of the Z29 strain, and the melting temperatures profiles of all clinical isolates were similar to the melting temperature profile of the Japanese HST strain. As expected, none of the 20 clinical isolates recovered from the ES patients and the 14 isolates recovered from the HSCT recipients who did not receive GCV treatment carried the six known SNPs associated with GCV resistance. Interestingly, these six SNPs were not detected in the 28 clinical isolates recovered from the 9 HSCT recipients who received GCV. Additional sequence analysis of the U69 gene from the 15 representative isolates from the 15 HSCT recipients identified other SNPs. These SNPs were identical to those identified in the HST strain. Therefore, the rate of emergence of GCV-resistant HHV-6B strains appears to be relatively low, even in HSCT recipients treated with GCV.

Primary human herpesvirus 6B (HHV-6B) infection causes exanthem subitum (ES) (1). Although this disease generally manifests as a benign self-limiting febrile illness (2), in rare cases it can cause severe complications, including encephalitis (3, 4) and fulminant hepatitis (5). HHV-6B reactivation has clinical manifestations in transplant recipients, such as skin rash and fever (6, 7) and bone marrow suppression and posttransplant acute limbic encephalitis (8–13). Antiviral drugs, such as ganciclovir (GCV) and foscarnet, have been used to treat transplant recipients with HHV-6B-associated encephalitis (8–13). To date there are limited data on the emergence of drug-resistant HHV-6B in these patients.

GCV was initially developed as an antiviral drug against human cytomegalovirus, which belongs to the same herpesvirus subfamily (the Betaherpesvirinae subfamily) as HHV-6A and HHV-6B. Resistance to GCV in cytomegalovirus has been mapped to the UL97 protein kinase that is responsible for the monophosphorylation of GCV (14–16). The antiviral effect of GCV against HHV-6B has been demonstrated using in vitro susceptibility assays (17–19). The in vivo efficacy of GCV remains inconclusive, despite its general use in patients with HHV-6 encephalitis posttransplantation (8–13). The U69 gene in HHV-6 is a homologue of the HCMV UL97 gene, and it phosphorylates GCV (20, 21). In vitro assays have revealed several hot spots in the HHV-6 U69 gene that were associated with GCV resistance (22, 23).

Although in vitro drug susceptibility assays are a reliable tool for determining the sensitivity of HHV-6B isolates to antiviral drugs, they are inappropriate for the rapid detection of drug-resistant viruses because of the long incubation period required for HHV-6B replication. Molecular methods for the detection of mutations in the U69 gene that are associated with GCV resistance would be useful for monitoring the emergence of GCV-resistant HHV-6B strains. The quenching probe PCR (QP-PCR) assay is a novel technique that can detect deviations as small as a single-base substitution in the sequence of a DNA fragment. Recently, a new and convenient molecular method for the screening of GCV-resistant HHV-6B strains using the QP-PCR technique was developed (24). This method could be appropriate for the rapid detection of GCV-resistant virus in transplant recipients being treated with GCV.

In the study described here, we utilized the QP-PCR method to elucidate the frequency of GCV-resistant HHV-6B strains in clinical isolates obtained from patients with primary viral infection and during viral reactivation. In contrast to a previous study of the emergence of GCV-resistant HHV-6B strains in transplant recipients based on viral DNA amplified by PCR (25), the present study...
TABLE 1 Characteristics of the 15 HSCT recipients with HHV-6B viremia

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Underlying disease</th>
<th>No. of HHV-6B clinical isolates analyzed</th>
<th>Length of GCV treatment (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F</td>
<td>62</td>
<td>MDS</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>48</td>
<td>AML</td>
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<td>1</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>9</td>
<td>ALD</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>48</td>
<td>AML</td>
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<td>32</td>
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<td>2</td>
<td>14</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>37</td>
<td>MDS</td>
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<td>—</td>
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<tr>
<td>11</td>
<td>F</td>
<td>17</td>
<td>MDS</td>
<td>2</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>M</td>
<td>13</td>
<td>AML</td>
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<td>13</td>
<td>M</td>
<td>10</td>
<td>NB</td>
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<td>—</td>
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<tr>
<td>14</td>
<td>F</td>
<td>36</td>
<td>AML</td>
<td>4</td>
<td>—</td>
</tr>
<tr>
<td>15</td>
<td>M</td>
<td>14</td>
<td>AA</td>
<td>2</td>
<td>—</td>
</tr>
</tbody>
</table>

a GCV, ganciclovir; F, female; M, male; MDS, myelodysplastic syndrome; AML, acute myeloid leukemia; AA, aplastic anemia; ALD, adrenoleukodystrophy; NB, neuroblastoma; —, no GCV treatment.

analyzed isolated viruses, which are reliable for the evaluation of active viral infections but not latency in transplant recipients.

MATERIALS AND METHODS

Clinical and laboratory isolates. Forty-two HHV-6B clinical isolates recovered from 15 hematopoietic stem cell transplant (HSCT) recipients (Table 1) and 20 isolates recovered from 20 ES patients were used in this study. Nine of the 15 HSCT recipients received GCV during the observation period. None of the ES patients were treated with GCV during the observation period. This study was approved by the Ethical Review Board at the Fujita Health University School of Medicine (no. 08-183). The patients or their guardians consented to participation in this study.

HHV-6B isolation and identification were performed as previously described (26). In brief, peripheral blood mononuclear cells were cocultured with cord blood mononuclear cells in culture medium. Viruses were identified primarily by morphological changes in cultured cells (i.e., characteristics of pleomorphic, balloon-like large cells), and then viral isolation was confirmed by immunofluorescence staining with an HHV-6B monoclonal antibody (OHV-3; provided by T. Okuno, Department of Microbiology, Hyogo College of Medicine, Hyogo, Japan). Cocultured cord blood mononuclear cells that were infected with each isolate were stored after 2 to 3 passages at −80°C.

The previously characterized HHV-6B strains HST and Z29 are established laboratory strains and were used as standards in the current study. HST was originally isolated from an ES patient in Japan, while Z29 was originally isolated from an AIDS patient in Zaire (27). The HST and Z29 strains were also propagated by cocultivation with cord blood mononuclear cells.

Control plasmids. DNAs containing the six known mutations in the U69 gene that are associated with GCV resistance (A to G, C to A, T to G, T to C, C to A, and G to A at positions 952, 1340, 1342, 1349, 1385, and 1388, respectively) were constructed using a PrimeStar mutagenesis basal kit (23). These mutations correspond to the amino acid substitutions M318V, A447D, C448G, L450S, A462D, and C463Y, respectively, in functional subdomains VIb and XII (Fig. 1). The DNAs were amplified by PCR and subcloned into pGEM-T vectors (Promega, Madison, WI). Plasmid DNAs were isolated using a Wizard Plus SV miniprep DNA purification system (Promega, Madison, WI).

DNA extraction. Viral DNAs were extracted from the stored clinical isolates and cord blood mononuclear cells infected with the HHV-6B laboratory strain (strains Z29 and HST) using a QIAamp DNA blood minikit (Qiagen, Chatsworth, CA). Extracted DNAs were eluted in 100 μl buffer and stored at −20°C until QP-PCR analysis.

QP-PCR for detection of GCV resistance-associated mutations in U69. The PCR amplification protocol used in this study to detect GCV resistance-associated mutations in the HHV-6B U69 gene was modified

![FIG 1 Map of the HHV-6B U69 (protein kinase) gene and protein. The U69 protein contains two functional domains. The six mutations (and the substitutions that they encode) that have been associated with GCV resistance are A952G (M318V), C1340A (A447D), T1342G (C448G), T1349C (L450S), C1385A (A462D), and G1388A (C463Y). (A) Positions of the synthesized primers and probes in the nucleotide sequence; (B) the corresponding positions of the six amino acid substitutions in the U69 protein.](http://aac.asm.org/content/300/2/2619/F1)
The primers and probes used for detection of GCV resistance mutations in U69 gene by QP-PCR analysis are shown in Table 2. As shown in Fig. 1, the six SNPs are located in three distinct regions (regions 1, 2, and 3) of the U69 gene, the 5' terminal region (the U69-S1 primer pair), the first middle region (the U69-S2 primer pair), and the second middle region (the U69-S3 primer pair). The PCR products amplified from the selected isolates were sequenced using a BigDye Terminator (v3.1) cycle sequencing kit and a Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA). The sequences of the HHV-6B U69 region were compared using the Clustal W computer program (DNA Data Bank of Japan).

**RESULTS**

Screening of the six SNPs associated with GCV resistance in clinical isolates. As the probes dissociate from unmatched PCR products at lower temperatures than perfectly matched PCR products, it is possible to detect mutations by melting curve analyses of the PCR products. Typical melting curves from the R1-QP-PCR, R2-QP-PCR, and R3-QP-PCR analyses are shown in Fig. 2. For regions 1 and 2, all clinical isolates demonstrated the same melting temperature as the Z29 strain (Fig. 2A and B). However, the melting temperatures of all clinical isolates were located between the melting temperatures of the A462D SNP and the Z29 strain (Fig. 2C). Then, the melting curves from the R3-QP-PCR for clinical isolates and DNA extracted from the laboratory strain, HST, were compared. As shown in Fig. 2C, the melting temperatures of all clinical isolates were the same as the melting temperature of HST. Sequence analysis of region 3 identified the C1383T SNP in HST and all of the clinical isolates (data not shown). Finally, as we expected, none of the 20 clinical isolates recovered from the ES patients and the 14 isolates recovered from the HSCT recipients who did not receive GCV contained any of the six GCV resistance-associated SNPs. Moreover, the six SNPs were not detected in any of the 28 clinical isolates recovered from the 9 HSCT recipients treated with GCV (Table 1).

In order to confirm our findings, sequence analysis of the entire U69 gene from 15 representative isolates consisting of the latest isolate recovered from each of the 15 HSCT recipients was carried out. The SNPs T63C, C132T, T240C, C348G, C450A, A438G, T693C, T1287C, and C1383T were discovered in all 15 isolates and were identical to the SNPs detected in the HST strain. Some additional unique silent mutations (C154T, G372T, C486T, A438G, T693C, T1287C, and C1383T were discovered in all 15 isolates). The PCR mixture (20 μl) contained 5 μl of extracted DNA, 5 μl of primers, 0.2 μM probe, and 10 μl of a master mix (TaqMan universal PCR master mix [Applied Biosystems, Foster City, CA]). The primers were made at a ratio of 1:5 (0.1 μM MR1F to 0.5 μM MR1R or 0.1 μM MR2R to 0.5 μM MR2F). The PCR was performed under the following conditions: 95°C for 5 min; 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 10 s; 72°C for 10 min; and 4°C for storage. Melting curve thermal cycle conditions were as follows: 95°C (a 1-s holding time), followed by a 60-s annealing step at 30°C with a slow ramp (0.3°C/s) up to 70°C with continuous detection through the ramp.

**Direct sequencing.** To confirm the results of the QP-PCR analysis, sequencing analysis of the entire U69 gene was performed using PCR. Table 3 shows the four primer pairs that were designed to amplify the four distinct regions of the U69 gene, the 5' terminal region (the U69-S1 primer pair), the first middle region (the U69-S2 primer pair), the second middle region (the U69-S3 primer pair), and the 3' terminal region (the U69-S4 primer pair). The PCR products amplified from the selected isolates were sequenced using a BigDye Terminator (v3.1) cycle sequencing kit and a Prism 3100 Avant sequencer (Applied Biosystems, Foster City, CA). The sequences of the HHV-6B U69 region were compared using the Clustal W computer program (DNA Data Bank of Japan).

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Region</th>
<th>Primer sequence (orientation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U69-S1</td>
<td>5' terminal region</td>
<td>5'-GAGAATTTGGAGCTGATGAAA-3' (sense)</td>
</tr>
<tr>
<td>U69-S2</td>
<td>First middle region</td>
<td>5'-TGAAATCCTGAGCTAGAAGAAA-3' (sense)</td>
</tr>
<tr>
<td>U69-S3</td>
<td>Second middle region</td>
<td>5'-AAATCGGAGGCGGACTATT-3' (sense)</td>
</tr>
<tr>
<td>U69-S4</td>
<td>3' terminal region</td>
<td>5'-TTGGCGCAATGCATTGATA-3' (sense)</td>
</tr>
</tbody>
</table>
critically ill patients (cases 4, 5, and 9). In most cases, the HHV-6B DNA load peaked once during the observation period, but either repeated elevation of the viral load or repeated isolation of the virus was demonstrated in several patients with severe immunosuppression (cases 1, 2, 3, 4, 5, 7, and 9). Persistent active viral infection (viremia) due to long periods of GCV administration was demonstrated in cases 2 and 4, which placed these patients at the highest risk for the emergence of GCV-resistant HHV-6B.

DISCUSSION

In order to elucidate the reliability of QP-PCR methods for the detection of GCV resistance-associated SNPs in HHV-6B clinical isolates, we initially examined clinical isolates recovered from ES patients not treated with GCV as representative samples without GCV resistance-associated SNPs. Regions 1 and 2 of all clinical isolates demonstrated the same melting temperatures as regions 1 and 2 of the Z29 strain; however, the melting temperatures of region 3 differed (Fig. 2C). Although both the Z29 and HST strains belong to HHV-6B, the Z29 strain was isolated from an AIDS patient in Zaire (27) and the HST strain was isolated from a Japanese ES patient (1). The melting temperature of the HST strain, which was same as that of the clinical isolates recovered from ES patients (Fig. 2C), differed from the melting temperature of the Z29 strain. Furthermore, sequence analysis of region 3 identified several sequence differences between the Z29 and HST strains (data not shown), which were consistent with the previously reported sequence data (GenBank accession numbers AB021506 and AF157706). These results indicated that the R3-QP-PCR is useful for the detection of GCV resistance-associated SNPs not only in the Z29 strain but also in the HST strain. Therefore, our QP-PCR method performed with probes specific for regions 1, 2, and 3 was effective for the discrimination of GCV resistance-associated SNPs from the wild-type sequences.

Manichanh et al. demonstrated that one of the five peripheral blood mononuclear cell specimens obtained from AIDS patients who were treated with GCV contained the A952G mutation in region 1 (25). To the best of our knowledge, although the sample size was too small, our study is the first one to analyze the frequency of GCV-resistant HHV-6B isolates in patients with primary viral infections and HSCT recipients with viral reactivation. As we expected, no GCV resistance-associated SNPs were detected in the isolates recovered from ES patients who were not treated with GCV. Additionally, no GCV resistance-associated SNPs were detected in any of the clinical isolates obtained from HSCT recipients. As demonstrated by in vitro experiments, persistent viral replication under GCV treatment is required for the emergence of GCV-resistant HHV-6B strains (22, 23). In contrast to cytomegalovirus reactivation, HHV-6B reactivation (viremia) regresses without antiviral treatment in most HSCT recipients. Although HHV-6B was repeatedly isolated from several HSCT recipients, these patients (cases 1, 3, 5, 7, and 9) did not receive concurrent prolonged GCV administration. Thus, the self-limiting clinical course of HHV-6B reactivation in HSCT recipients and the low frequency of active viral infection under GCV treatment may result in the low rate of emergence of GCV-resistant HHV-6B strains. As cases with prolonged HHV-6B viremia were rare in the recipients after HSCT, the number of study subjects was limited in this study. Therefore, a large number of patients with prolonged viremia during GCV treatment, which may be very rare, should be analyzed to elucidate the precise incidence of the emergence of GCV-resistant HHV-6 in HSCT recipients.

We failed to detect GCV resistance-associated SNPs in the isolates recovered from the two patients (cases 2 and 4) with persistent GCV administration (Fig. 3). Mutations in the U38 gene, which is another candidate gene involved in GCV metabolism, occurred in only one GCV-resistant clinical isolate obtained from an HSCT recipient (28). Therefore, further studies are needed to determine whether the isolates recovered from the two patients in the current study may also have mutations in the U38 gene which...
FIG 3 Clinical courses and kinetics of viral DNA loads in nine HSCT recipients treated with GCV. HHV-6 was isolated from peripheral blood mononuclear cells collected from the patients weekly. +, an HHV-6 isolate was obtained; *, sequencing analysis was performed; dotted lines, cytomegalovirus (CMV) DNA loads; solid lines, HHV-6B DNA loads.
may mediate their GCV resistance. We are currently developing a QP-PCR method for the detection of mutations in the HHV-6B U38 gene that mediate GCV resistance.

The use of clinical isolates in this study may have resulted in the selection of the virus during the virus isolation step; however, this is unlikely because no differences in the growth curves between the wild-type and GCV-resistant strains were demonstrated on the basis of in vitro assays (25). Previous studies suggested that mixed cytomegalovirus populations composed of wild-type and GCV-resistant strains occurred in immunosuppressed patients following GCV treatment (29, 30). Thus, further pyrosequencing analyses may be needed to determine the precise incidence of the emergence of GCV-resistant HHV-6B strains in immunocompromised patients following GCV administration.

Previous studies demonstrated HHV-6B reactivation between 2 and 4 weeks after transplantation (6, 7, 31), and similarly, in the emergence of GCV-resistant HHV-6B strains in immunocompromised patients following GCV administration.

The current study demonstrates that QP-PCR analysis can be used to monitor GCV-resistant HHV-6B strains in high-risk patients, such as transplant recipients. In contrast to the rate of emergence of GCV-resistant cytomegalovirus, our findings suggest that the rate of emergence of GCV-resistant HHV-6B strains appears to be relatively low even in HSCT recipients who have received GCV treatment.

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