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Detection and identification of U69 gene mutations encoded by ganciclovir-resistant human herpesvirus 6 using denaturing high-performance liquid chromatography

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A denaturing high-performance liquid chromatography (dHPLC) assay was developed to detect antiviral drug-resistance mutations of human herpesvirus 6 (HHV-6). Recombinant baculoviruses were created that contained wild-type and mutant forms of the HHV-6 U69 gene, which determines sensitivity to the antiviral drug ganciclovir (GCV). The mutations causing GCV resistance in HHV-6 U69 were single-base mutations adapted from known GCV-resistant DNA sequences of HCMV, and their ability to confer GCV resistance on recombinant baculoviruses was confirmed. Six characterized mutant sequences, including one reported previously that encodes a GCV-sensitive kinase-activity mutant, were used. DNA was extracted, and the levels of homoduplex and heteroduplex DNA in the PCR products from mixed wild-type and mutant viral DNAs were determined using dHPLC. The optimized assay could distinguish the different mutants, and could detect mutants representing only 10% of the DNAs. The new assay with dHPLC readout permitted the rapid (4 h), objective, and reproducible detection of HHV-6 drug-resistance mutations.

1. Introduction

Human herpesvirus 6 (HHV-6) was first isolated in 1986 from the peripheral blood of patients with lymphoproliferative disorders (Salahuddin et al., 1986). Comparison with other human herpesviruses by molecular and immunological analyses revealed that it has distinct characteristics (Lusso et al., 1988). HHV-6 replicates predominantly in $CD4^+$ lymphocytes (Takahashi et al., 1989) and may establish latent infection in cells of the monocyte/macrophage lineage (Kondo et al., 1991). Infection with this virus results in exanthem subitum (ES), a common illness in infants (Yamanishi et al., 1988), but has not been linked clearly to any disease in adults except in patients with immunodeficiency.

Because HHV-6 infects frequently immunocompromised individuals, especially transplant recipients, AIDS patients, and children with congenital immunodeficiency disorders, it is important to assay the susceptibility of HHV-6 clinical isolates to antiviral drugs. HHV-6 is amenable to therapy with the acyclovir derivative ganciclovir (GCV), which is a nucleoside analogue that is mono-phosphorylated by an encoded virus protein kinase (U69 in HHV-6) before being further phosphorylated by host cellular kinases to its active, triphosphate form. GCV triphosphate is a competitor of dGTP and inhibits the viral DNA polymerase (Erice, 1999). GCV-resistant HCMV mutants have been both isolated from patients and generated in the laboratory (Chou et al., 2005; Lurain et al., 2001; Smith et al., 1997). Little work has been done to elucidate the resistance of HHV-6 to GCV, to date, although GCV-resistant HHV-6 has been reported (Bolle et al., 2002; Isegawa et al., 2009; Manichanh et al., 2001; Safronetz et al., 2003), GCV-phosphorylation by U69 gene product has been shown (Bolle et al., 2002) and U69 mutations responsible for GCV resistance have been assayed using the baculovirus expression system (Safronetz et al., 2003).

Denaturing high-performance liquid chromatography (dHPLC) separates PCR products by size and sequence. It detects sequence divergences in DNA fragments, including single-base substitutions and short deletions and insertions. This method is sufficiently sensitive for the reliable detection of nearly 100% of DNA sequence variations at an optimized partially denaturing temperature (Xiao and Oefner, 2001). Previously, the susceptibility of HHV-6 to antiviral drugs was determined by real-time PCR (Isegawa et al., 2007). In this study, a new approach for detecting HHV-6 U69 mutations

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using dHPLC was developed and evaluated. With this approach, clinical samples can be processed in a 96-well format and loaded continuously onto the column for rapid analysis.

2. Materials and methods

2.1. Cells and viruses

Umbilical cord blood mononuclear cells (CBMCs) were separated on a Ficoll-Conray gradient, transferred to RPMI 1640 medium containing 10% fetal calf serum (FCS), and stimulated with 5 μg/ml phytohemagglutinin for 2 or 3 days. HHV-6 strain HST, which was isolated from a patient with ES and belongs to the HHV-6B group, was propagated in fresh human peripheral mononuclear cells (Yamanishi et al., 1988). When more than 80% of the cells showed cytopathic effects, the culture was frozen and thawed twice, spun at 1500 × *g* for 10 min, and the supernatant was stored at −80 °C as a cell-free virus stock. The viruses and DNA samples used for the U69 DNA sequence analysis were reported previously (Sjahril et al., 2009).

Recombinant baculovirus containing the full-length wild-type U69 ORF (strain HST) was constructed as described before (Isegawa et al., 2008). The M318V U69 mutant baculovirus constructed previously (Isegawa et al., 2009) was used. *Spodoptera frugiperda* 21 (Sf-21) cells (Invitrogen, Tokyo, Japan) were maintained and propagated in Sf-900 medium (Invitrogen) supplemented with 5% FCS, at 27 °C.

2.2. DNA sequencing and sequence analysis

To amplify the HHV-6 genomic DNA, the viral genome was isolated from virus-infected CBMCs with a QIAamp DNA blood mini kit (Qiagen K.K., Tokyo, Japan), and 100 ng of total DNA was subjected to 30 cycles of PCR with EX-*Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan), as described before (Sjahril et al., 2009). PCR for the DNA sequence analysis was carried out with the primer pair: U69-Met-NcoI and U69-Ter-NotI, which was reported previously (Isegawa et al., 2008). The corresponding sequences were confirmed by direct sequencing of the PCR products amplified from the DNA, using an ABI PRISM 3100 gene analyzer (Applied Biosystems Japan Ltd., Tokyo, Japan).

2.3. Construction of mutant recombinant baculoviruses

To construct recombinant baculoviruses expressing mutant U69 genes, the following mutations were introduced into the pAcH6 plasmid containing the wild-type U69: C–A, T–G, T–C, C–A, and G–A at positions 1340, 1342, 1349, 1385, and 1388, respectively, using the PrimeSTAR® Mutagenesis Basal Kit (Takara Bio). As shown in Fig. 1A, these mutations corresponded to A447D, C448G, L450S, A462D, and C463Y in functional subdomain XI. Sf-21 insect cells were transfected with pAcH6 plasmids containing the mutant U69 genes together with the linearized AcNPV baculovirus DNA (BaculoGold™, BD PharMingen, San Diego, CA), and the recombinant baculoviruses were expanded into high-titer virus stocks, following the manufacturer's instructions. The insertion of the U69 gene into the baculovirus genome was confirmed by PCR analysis.

2.4. Real-time PCR assay for recombinant baculovirus antiviral susceptibility testing

Cells (5×10^5) in each well of a 12-well culture plate were infected with recombinant baculoviruses at an MOI of 0.3 (100 μl) at room temperature. One hour later, the medium was changed to

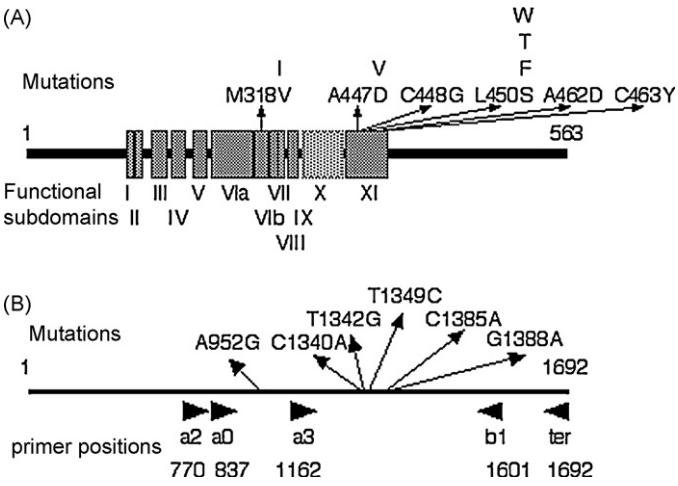

Fig. 1. (A) Map of the HHV-6 protein kinase (U69) functional subdomains, and predicted GCV-resistance mutations estimated from the UL97 mutation map of GCV-resistant HCMV. Gray boxes: subdomains of the HHV-6 U69 protein kinase. (B) Position map of synthesized primers and predicted GCV-resistance mutations in the HHV-6 U69 nucleotide sequence. Arrows: mutation positions. Arrowheads: primers; numbers show primer starting positions.

1 ml of Sf-900 medium with serial twofold dilutions of GCV (Wako, Osaka, Japan) ranging from 78 to 1250 μM. The plates were incubated at 27 °C for 3 days, then 1 ml of the culture was collected, the cells were separated from the baculovirus-containing supernatant by centrifugation (1100 × *g*, 1 min, 4 °C), and the DNA was extracted from the supernatant using the QIAamp DNA blood mini kit (Qiagen). The DNA samples were examined by real-time PCR or stored at −70 °C until assayed. Reference GCV-susceptible (HST) and GCV-resistant (M318V) recombinant baculoviruses were included as controls in each PCR assay, as shown in a previous report (Isegawa et al., 2009).

To quantify the recombinant baculovirus DNA, the DNA samples were subjected to quantitative, real-time PCR for HHV-6 U69 DNA as described previously (Isegawa et al., 2007).

2.5. PCRs

Several primer sets were designed and tested (Fig. 1B). Forward primers were dHPLC-a2 (770–790), CAAATTCCGTTTGTATGGATC; dHPLC-a0 (837–855), CGAAGATTGGGATGTCAGG; and dHPLC-a3 (1162–1181), TTGGTCAATGTATGCGAGGC. The reverse primers were dHPLC-b1 (1601–1585), CCATACTCGGACGACTG; and dHPLC-ter (1692–1674), TCACATCTGAAAGAGAGAT. The sizes of the PCR products obtained by the primer pairs dHPLC-a2 and -ter, dHPLC-a0 and -b1, and dHPLC-a3 and -b1 were 923 bp, 765 bp, and 440 bp, respectively. The suitability and assay conditions for the dHPLC analysis of the PCR products were predicted by the WAVE simulation software (Transgenomic, Inc., San Jose, CA).

2.6. Detection of mutations by dHPLC

The detection of mutations in the amplified PCR products was performed by dHPLC with the WAVE Nucleic Acid Fragment Analysis System (Transgenomic, Inc.). All buffers were supplied by Transgenomic. Buffer A contained 0.1 M triethylammonium acetate (TEAA) and 0.025% (vol/vol) acetonitrile, and buffer B was 0.1 M TEAA and 25% (vol/vol) acetonitrile.

In the WAVE system, the PCR products are separated on a chromatographic column (DNASep column) packed with C_{18} alkylated polystyrene-divinylbenzene polymeric beads. A positively charged ion-pairing reagent (TEAA) allows the negatively charged