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PCR with quenching probes enables the rapid detection and identification of ganciclovir-resistance-causing U69 gene mutations in human herpesvirus 6

Yuji Isegawa [a,*], Chisa Matsumoto [a], Kazuko Nishinaka [a], Kazushi Nakano [a], Tatsuya Tanaka [b], Nakaba Sugimoto [a], Atsushi Ohshima [c]

[a] Department of Infectious Disease Control, G-5, Graduate School of Medicine, Osaka University, 2-2 Yamada-Oka Suita, Osaka 565-0871, Japan
[b] Center for Medical Research and Education, C-10, Graduate School of Medicine, Osaka University Suita, Osaka 565-0871, Japan
[c] Gene Engineering Laboratory, Genomics Program, Nagahamabio Institute of Bio-Science and Technology, Nagahama, Shiga 526-0829, Japan

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ABSTRACT

A single-nucleotide polymorphism detection assay using PCR with quenching probes (QP-PCR) was developed for the rapid detection of antiviral drug-resistance mutations of human herpesvirus 6 (HHV-6). The mutations examined were in the HHV-6 U69 gene, and were single-base mutations in sequences known to be associated with ganciclovir (GCV) resistance in HCMV. We previously confirmed that they conferred GCV resistance to recombinant baculoviruses (Nakano et al., J. Virol. Methods **161**:223–230, 2009). Six characterized mutations, including a previously reported one that encodes a GCV-sensitive kinase-activity mutant (Isegawa et al., J. Clin. Virol. **44**:15–19, 2009), were used. The six mutations were separated into three groups based on their location in the U69 protein, and detected by the hybridization of three probes. We developed and validated a set of assays for these mutations using PCR followed by differential melting of a fluorescently labeled oligo probe, on a Roche Light Cycler platform. Nucleobase quenching was used to detect the hybridized probe. The optimized assay could distinguish the different mutants, and easily detected mutants representing 30% of the DNA in a mixed sample. This QP-PCR assay permitted the rapid (1.5 h), objective, and reproducible detection of drug-resistant mutations of HHV-6.

1. Introduction

Human herpesvirus 6 (HHV-6) was first isolated in 1986, from the peripheral blood of patients with lymphoproliferative disorders [1]. Molecular and immunological comparisons showed that HHV-6 was distinct from other known human herpesviruses [2]. A number of different properties have led to the identification of two HHV-6 variants [3–7], which are called HHV-6A (variant A) and HHV-6B (variant B). HHV-6 is known to replicate predominantly in $CD4^+$ lymphocytes [8] and can establish latent infections in monocyte/macrophage-lineage cells [9]. HHV-6 infection results in exanthem subitum (ES), which is common in infants [10], but has not been clearly linked to any adult disease except in immunodeficient patients.

Individuals who are especially vulnerable to HHV-6 infection include transplant recipients, AIDS patients, and children with congenital immunodeficiency disease. Therefore, it is important to assess HHV-6 clinical isolates for their susceptibility to antiviral drugs. HHV-6 infection is treated with ganciclovir (GCV), an acyclovir derivative. GCV is a nucleoside analogue that is monophosphorylated by a virally encoded protein kinase (U69 in HHV-6), and then is further phosphorylated by host kinases to its active, triphosphate form. GCV triphosphate competes with dGTP to inhibit the viral DNA polymerase [11]. GCV-resistant mutants of human cytomegalovirus (HCMV) have been both isolated from patients and generated in the laboratory [12–14]. To assess the contributions of the U69 gene mutations (M318V, A447D, C448G, L450S, A462D, and C463Y) to GCV resistance that were estimated from the UL97 mutants of GCV-resistant HCMV, we previously performed a baculovirus reduction assay, and showed that the U69 M318V, A447D, C448G, L450S, A462D, and C463Y substitutions are all associated with HHV-6 GCV resistance [15]. However, minimal work has been done to identify the mutants responsible for HHV-6's resistance to GCV [16–19].

A PCR with quenching probes (QP-PCR) assay was originally developed as a simple method for quantifying specific nucleic acid sequences without using calibration curves [20]. The method combines competitive PCR and fluorescence-nucleobase quenching. Nucleobase quenching is the product of an interaction between a fluorophore that is tethered to a cytosine residue on the probe and the complementary guanosine residue on the target strand. In addition, homogeneous PCR nucleobase quenching assays for

* Corresponding author. Tel.: +81 6 6879 3301; fax: +81 6 6879 3309.
E-mail address: isegawa@bact.med.osaka-u.ac.jp (Y. Isegawa).

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detecting mutations were recently developed [21]. The QP-PCR assay can detect deviations as small as a single-base substitution in the sequence of a DNA fragment. This assay, once optimized for the denaturing temperature and probe length, is sensitive enough to detect nearly 100% of the sequence variations in a DNA fragment. Macé et al. and we previously reported a procedure for determining HHV-6 antiviral drug susceptibility by real-time PCR [22,23] and another procedure for detecting HHV-6 U69 mutations using dHPLC [15]. Here we developed and evaluated a new approach for detecting specific HHV-6 U69 mutations using QP-PCR. This approach enables the processing and rapid analysis of clinical samples in a 96-well or a 384-well format.

2. Materials and methods

2.1. Cells and viruses

Umbilical cord blood mononuclear cells (CBMCs) were prepared by separation on a Ficoll–Conray gradient, followed by transfer to RPMI 1640 medium containing 10% fetal calf serum (FCS), and stimulation with 5 μg/ml phytohemagglutinin for 2 or 3 days. HHV-6 strain HST [10], which was isolated from a patient with ES, and M2 isolate [17] from a 4-year-old boy with acute lymphocytic leukemia who received an allogeneic SCT of positively selected CD34+ cells, were used. Both the HST strain and M2 isolate belong to the HHV-6B group. The virus was propagated in fresh CBMCs [10]. When more than 80% of the cells showed cytopathic effects, the culture was frozen and thawed twice, and then spun at 1500 × g for 10 min. The supernatant was stored at −80 °C as cell-free virus stock. DNA samples of HST strain and M2 isolate used for the QP-PCR assay were isolated with a QIAamp DNA blood mini kit (Qiagen K.K., Tokyo, Japan) from virus-infected MT-4 cells that had been cultured in RPMI 1640 medium containing 10% FCS, in a 5% CO_2 incubator at 37 °C.

2.2. Construction of mutant genes

The mutants used in this experiment were constructed as described previously [15]. They corresponded to M318V, A447D, C448G, L450S, A462D, and C463Y, and were located in the functional subdomains VIb and XI of the U69 protein kinase of HHV-6 (Fig. 1A).

2.3. PCR amplification

Two primer sets were designed and tested (Fig. 1B). The forward primers were U69-MR1F (827–852), TCTATAAGTTCGAAGATTGGGATGTC; and U69-MR2F (1282–1305), GATGCCGCGAAGCTCAGATATAC. The reverse primers were U69-MR1R (1020–999), GGACAAGCTGTAATCCGCCAAC; and U69-MR2R (1455–1431), TATATCAACCACGTCTCGGTAAAAC. The sizes of the PCR products obtained by the primer pairs U69-MR1F and -MR1R, and U69-MR2F and -MR2R were 194 bp and 174 bp, respectively. The suitability and assay conditions for PCR were predicted by shuttle PCR which had 2 reaction stages (denaturation and annealing/extension) and three-step PCR which had 3 reaction stages (denaturation, annealing, and extension) in a volume of 20 μl with the plasmid DNA (10^8 copies), 0.2 μM each of a pair of primers, 200 μM dNTPs, and 0.5 unit of EX-*Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan). The conditions for the three-step PCR were 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 55 °C for 20 s, and 72 °C for 10 s, and 72 °C for 10 min; or 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s, and 72 °C for 10 min. The conditions for the shuttle PCR were 95 °C for 5 min, 40 cycles of 95 °C for 10 s and 60 °C for 60 s, and 72 °C for 10 min.

2.4. Nucleobase quenching assays

The overall strategy for genotyping using a probe and melting curve analysis is to first generate an amplicon using asymmetric PCR, so there will be an excess of the DNA strand that hybridizes to the probe. This increases the signal by avoiding competition between probe hybridization and renaturation of the PCR product [24]. The probes are designed so that the fluorophores which link to cytosine residues is located close to deoxyguanylate residues on the opposite strand when the probe is base-paired to the amplicon. This causes a decrease in the fluorescent signal due to the quenching affect of the guanine base [25]. After probe hybridization, the temperature is raised, and the fluorescence is monitored continuously to identify the temperature at which the probe dissociates from the complementary strand. When the probe/amplicon hybrid dissociates (melts), the fluorescent signal increases, due to elimination of the quenching affect of the guanine bases. The use of the nucleobase quenching for genotyping was introduced by Crockett and Wittwer [25].

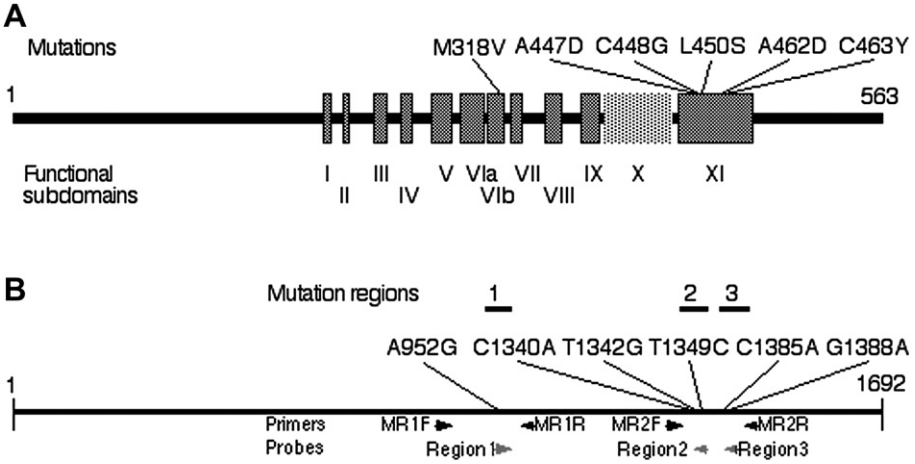

Fig. 1. (A) HHV-6 U69 protein kinase functional subdomains, and GCV-resistance mutations predicted from the GCV-resistant HCMV mutants in UL97. Gray boxes: HHV-6 U69 protein kinase subdomains. (B) Positions of synthesized primers and predicted GCV-resistance mutations in the HHV-6 U69 nucleotide sequence. Dark gray bars: Mutation Regions. Black arrowheads: primers and directions (5′–3′). Gray arrowheads: probes and directions.