Detection of Two Drug-Resistance Mutants of the Cytomegalovirus by High-Resolution Melting Analysis

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Background: Human cytomegalovirus (CMV) is an opportunistic pathogen that can be treated with ganciclovir. Mutations in the *UL97* gene of CMV render the virus ganciclovir resistance. These include H520Q and C603W mutations, against which we developed a novel genotyping assay for their identification. **Methods:** PCR reactions were performed to amplify fragments of the *UL97* gene containing H520Q or C603W mutations. High resolution melting analysis (HRMA) coupled with unlabeled DNA probes was employed to identify the shift in melting temperature of the probe–template complex, which reflexes the presence of point mutations. **Results:** Melting point

analysis performed on the dimeric DNA of PCR products of *UL97* gene could not identify mutations in the gene. When coupled to unlabeled probes, point mutations in *UL97* can be identified by analyzing the melting curve of probe–template complex. When WT and mutant *UL97* DNAs were mixed together to mimic heterogeneous viral population in clinical samples, the genotyping assay is sensitive enough to detect H520Q and C603W mutants that constitute 10% of total DNA input. **Conclusion:** Probe-based HRMA is effective in detecting H520Q and C603W mutations in the *UL97* gene of CMV. *J. Clin. Lab. Anal.* 30:319–325, 2016. © 2015 Wiley Periodicals, Inc.

Key words: cytomegalovirus; *UL97*; H520Q; C603W; high resolution melting analysis

INTRODUCTION

Cytomegalovirus (CMV) is a prevalent viral pathogen in humans. Majority of acute CMV infections is asymptomatic. Treatment of CMV infection involves the use

of antiviral drugs, including foscarnet, cidofovir, ganciclovir, and its prodrug valganciclovir. Ganciclovir is widely used for treating CMV-related diseases in patients with AIDS or organ transplants (1, 2). Prolonged treatment with ganciclovir may result in the emergence of drug-resistant CMV strains (3). Pharmacologically, ganciclovir acts as a nucleoside analogue that competes with

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deoxyguanosine triphosphate for DNA polymerase and inhibits DNA elongation once incorporated, thus serves as a DNA replication inhibitor (4–7). Inhibition on DNA synthesis requires phosphorylation of ganciclovir by the UL97 kinase encoded in CMV genome (8, 9). For this reason, the concentration of ganciclovir in its active form (tri-phosphorylated) is much higher in CMV-infected cells compared to normal cells. Long-term treatment of human patients against CMV infection selects for virus that harbor mutations in the *UL97* gene that blocks the phosphorylation and activation of ganciclovir (3, 10, 11). These include mutations in codons 460, 520, and 590–607 (3, 7, 10–12). Among them, five most frequently detected mutations (M460V/I, H520Q, C592G, A594V, L595S, and C603W) represent about 80% of all *UL97* mutation events, and confer a five- to tenfold increase in the IC₅₀ of ganciclovir, rendering the drug ineffective against CMV in clinical settings (13, 14).

Traditional methods for the detection of ganciclovir-resistant CMV rely on culturing CMV isolates at the presence of ganciclovir (15–18). While directly testing the efficacy of the antiviral drug, these assays have drawbacks, including the difficulty in obtaining viral isolates from patients and long duration of the test. Low speed in the replication of CMV causes the lengthy period (usually weeks) required for the diagnosis (19).

In contrast, direct genotypic analysis on the known mutations in CMV genes, which cause drug-resistance, is capable of obtaining reliable results within hours (20). Several genotypic assays have been developed for the diagnostic screening of ganciclovir-resistant CMV isolates, including line probe assay (LiPA; (21)), pyrosequencing (22, 23), real-time PCR (24, 25), and restriction fragment length polymorphism (RFLP; (10, 11)). Recent advances in techniques for genotypic analysis include instrumentation providing highly controlled temperature transitions and data acquisition, and the fluorescent DNA binding dyes with improved saturation properties (26). These advances allow a more accurate measurement of sequence variations based on melting analysis.

High resolution melting analysis (HRMA) with unlabeled probe is able to clearly distinguish single-nucleotide changes and is regarded as a powerful tool for genotyping (27, 28). This method combines unlabeled oligonucleotide probes with saturation dyes in an asymmetric PCR, and detects point mutations in target genes by measuring the melting point shift of the probe–PCR product complex. Here, we show a tailored protocol for the detection of H520Q and C603W mutations in the *UL97* gene of CMV, which is sensitive enough to detect mutant viral DNAs in a heterogeneous background containing mostly wild-type (WT) *UL97* genes.

MATERIALS AND METHODS

Cloning of the *UL97* Gene

CMV-containing sera samples were obtained from Peking University People's Hospital and PCR reactions were performed to amplify *UL97* fragments. Amplified fragment of *UL97* was cloned into a pMD18-T vector (Takara, Japan). H520Q and C603W mutations in the *UL97* fragments were generated by PCR-directed mutagenesis. All constructs were subjected to sequencing confirmation.

HRMA With Unlabeled Probes

High resolution melting point analysis (HRMA) with unlabeled probe was performed as previously described (29). Briefly, an asymmetric PCR reaction was performed with an S1000 Thermal Cycler (Bio-Rad, USA). The PCR reaction contains 0.5 U hot-start rTaq DNA polymerase (Takara, Japan), 1 × PCR buffer (Takara, Japan), 200 μM dNTPs (including dATP, dGTP, dTTP and dCTP), 0.05 μM forward primer, 0.5 μM excess reverse primer, 0.5 μM C3-blocked probe, and 10 ng plasmid DNA in a total reaction volume of 20 μl. PCR cycling was performed at 94°C for 120 sec, followed by 50 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 20 sec, and a final extension at 72°C for 300 sec. For each fragment, 10 μl PCR product was supplemented with 1 μl LCGreen Plus dye (Idaho Technology, USA) in LightCycler capillary (Idaho Technology, USA), then transferred to HR-1 system for melting point analysis. Samples were denatured at 95°C for 30 sec and rapidly cooled to 40°C for 30 sec. Melting point analysis was performed by raising reaction temperature from 55°C to 90°C with a 0.2°C/sec ramp rate.

RESULTS

Construction of WT *UL97* and Its H520Q and C603W Mutants

WT *UL97* fragment was amplified from serum samples containing CMV and cloned to a pMD18-T vector. Site-directed mutagenesis was used to introduce H520Q and C603W mutations.

Detection of H520Q and C603W Mutations by HRMA With Unlabeled Probe

The sequence of the probe used in HRMA is the same as WT *UL97*, and has one base-pair mismatch with the H520Q mutant (Fig. 1a). Likewise, the primers and probe for the detection of C603W mutation are shown in

(a)

CAGCCTCAGCGAGCCCTATCCGGATTACAACGAGCGCTGTGTGGCCGTCTTTCAGGAGACGGGC-
 ACGGCGCGCCGCATCCCCAACTGCTCGCACCGTCTGCGCGAATGTTAC**CAC**CCTGCTTCCGAC-
 CCATGCCGCTGCAGAAGCTGCTCATCTGCGACCCGCACGCGCGTTTCCCCGTAGCCGGCCTACG-
 GCGTTATTGCATGTCGGAGTTGTCGGCGCTGGGTAACGTGCTGGGCTTT

Probe
 H520Q: **CAG**

(b)

GTACGGAGGCGTTGCTCTTTAAGCACGCCGGCGCGGCCTGCCGCGCGTTGGAGAACGGCAA-
 GCTCACGCAC**TGG**CTCCGACGCCTGTCTGCTCATTCTGGCGGCGCAAATGAGCTACGGCGCCT-
 GTCTCCTGGGCGAGCATGGCGCCGCGCTGGTGTGCGACACGCTACGCTTTGTGGAGGCCAAG-
 ATGTCCTCGTGTGCGGTACGCGCCTTTCGCCGCTTCTACCACGAATGCTCGCAGACCATGCTG-
 CACGAATACGTAGAAAGAACGTGGAGC

Probe
 C603W: **TGG**

Fig. 1. Location of the probes for HRMA. (a) Sequence of the H520Q probe is shown as indicated in the amplified H520Q mutation region of *UL97*. (b) Sequence of the C603W probe are shown as indicated in the amplified C603W mutation region of *UL97*.

Figure 1b. As a result of the mismatch between the probe and mutant *UL97* DNA, WT *UL97*-probe complex was expected to have a higher melting temperature during HRMA than the mutant *UL97*-probe complex. Indeed, melting point analysis showed that the probe-template complex has a melting temperature ranging from 60°C to 75°C, and the melting point of the PCR product is between 89°C and 93°C (Fig. 2a). HRMA analysis on PCR products of WT and mutant *UL97* showed no distinguishable melting curve, suggesting that direct melting point analysis on double strand DNA of the PCR fragments is ineffective in identifying CMV mutant strains in clinical samples. In contrast, melting curve comparison between probe-WT and probe-mutant *UL97* complexes showed clearly distinct patterns (Fig. 2b). For H520Q mutation, probe-mutant *UL97* complex showed a melting peak at 65.5°C, whereas the probe-WT complex melted at 70.5°C (Fig. 2b). Lowered melting point of the probe-H520Q mutant complex is the result of one base pair mismatch between them (Fig. 2a) and provides the basis of the genotyping assay. We also employed melting point analysis using mixed PCR products that contain different ratios of mutant/WT fragments, to mimic clin-

ical cases where mixed WT and mutant strains of CMV infect human patients. Mutated *UL97* DNA comprising as low as 10% of total *UL97* DNA contents was clearly identified in this assay (Fig. 2b), suggesting that HRMA with unlabeled DNA probe is sensitive in detecting the presence of ganciclovir-resistant CMV strains in clinical samples prior to treatment-mediated enrichment of the mutant viral strain in patients. The normalized melting curves showed a much clearer discrimination for different genotypes, which could also serve as a standard curve for semiquantitative examination of the genotypes (Fig. 2c). We also tested the sensitivity of the HRMA assay in identifying the C603W mutation in *UL97* gene, and observed similar results (Fig. 3). Taken together, HRMA coupled with unlabeled DNA probes is a sensitive and fast method for detecting drug-resistance mutants in CMV *UL97* gene and may prove valuable in pretreatment diagnosis of human patients with CMV infection.

DISCUSSION

As a high-throughput and cost-effective method, HRMA has been used for genotyping identification

Fig. 2. HRMA for H520Q. (a) Melting peaks of the complex between unlabeled probes and PCR fragment flanking the codon 520. WT and H520Q genotypes are indicated in the probe region. (b) Normalized melting peaks of unlabeled probe region for genotyping of H520Q. The melting peaks are shown after normalization. WT *UL97*-probe complex showed higher melting temperature since there was no mismatch between the unlabeled probe and the template. (c) Normalized difference curves obtained by subtracting each peak from the 100% H520Q peak.

of mutation and SNP (26). HRMA is combined with unlabeled probe for the melting analysis on probe-template complex, which provides greatly enhanced sensitivity in detecting single nucleotide changes. During the melting process, a C3-blocked probe (~30 bp) is used to anchor to a target site (27,28). A single base pair mismatch between the probe and the template could incur a detectable shift in the melting temperature. The applications of genotyping with unlabeled probes include the detection of disease-related mutations in cystic fibrosis (30), single nucleotide polymorphisms in factor V Leiden (31), peroxisome proliferator-activated receptor (PPAR; (32)), and herpes simplex virus (33). Unlabeled probe HRMA is inexpensive, high-throughput, closed-tube genotyping that reduces the need for target gene sequencing, provided that the mutation sites are known. Traditional melting curve analysis on full-length PCR products is unable to distinguish the mutants from WT genes, while accuracy and sensitivity of HRMA is dramatically improved by using unlabeled probes as shown in our study. All the genotypes were discriminated clearly after normalization of the melting curves, which could serve as standard curves for semiquantitative examination of the genotype when clinical samples are employed.

Human CMV is a major opportunistic pathogen and is widespread in different parts of the world. CMV infection is a major cause of death in immune-suppressed patients who received bone marrow, hematopoietic stem cell, or solid organ transplants (34–37). Pharmaceutical treatment of CMV infection includes the use of foscarnet, cidofovir, ganciclovir, and its prodrug valganciclovir. Valganciclovir is the L-valyl ester of ganciclovir that has improved oral bioavailability. Both ganciclovir and valganciclovir are used as first-line therapy for CMV infection in humans (38). All drugs target the CMV DNA polymerase pUL54 to inhibit viral DNA replication. Ganciclovir acts as a nucleoside analogue and needs to be initially phosphorylated by UL97, a CMV protein kinase (4, 6, 7). Cidofovir does not require this initial phosphorylation step and acts as nucleotide analogue by itself. Foscarnet has a different mode of action by acting as a pyrophosphate analogue (39). Two viral proteins involved in resistance against the aforementioned drugs are UL97 and UL54. Mutations in *UL97* lead to reduced phosphorylation of ganciclovir and hence decreased drug activity (40, 41). About 90% of all ganciclovir resistant cases detected so far have been attributed to mutations in *UL97* (3). Commonly found mutations in *UL97* were mapped to codons 460, 520, and 590–607 (3, 7, 10–12, 42). Choosing antiviral regimen relies on the diagnostic screening of ganciclovir-resistant mutant CMV strains in human subjects. In this study, we report a genotyping method for the identification of H520Q and C603W mutations in the *UL97* gene of CMV. These two mutations confer

Fig. 3. HRMA for C603W. (a) Melting peaks of unlabeled probes and PCR fragment flanking codon 603 of the *UL97* gene. WT and C603W genotypes are indicated in the probe region. (b) Normalized melting peaks for genotyping detection of C603W mutation. The melting peaks are shown after normalization. (c) Normalized difference curves obtained by subtracting each peak from the 100% C603W peak.

CMV ganciclovir resistant (42,43). Our study showed that HRMA coupled with unlabeled DNA probes is sensitive and reliable in detecting mutant CMV DNA in experimental settings, and can be tested in clinical settings for the detection of ganciclovir-resistant CMV strains. Compared to laborious phenotypic screening by culture-based assays, our protocol provides a fast diagnosis of viral genotype within hours (44). Although current protocol only detects H520Q and C603W mutants, modified protocols with similar experimental conditions may be used to detect other known drug resistance related mutations in viral isolates or patient clinical samples.

CONCLUSION

HRMA with unlabeled probe could serve as a cost-effective, high-throughput, closed-tube genotyping method for the detection of H520Q and C603W mutations in CMV infections.

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