

Rapid detection of codon 460 mutations in the UL97 gene of ganciclovir-resistant cytomegalovirus clinical isolates by real-time PCR using molecular beacons

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Abstract

A rapid real-time polymerase chain reaction (PCR) assay using molecular beacons has been developed for the simultaneous detection of wild-type and mutant strains of cytomegaloviruses (CMV) with respect to codon 460 of the UL97 gene has been developed. The molecular beacons were designed to complement the wild-type codon 460 or the mutant sequence arising from a single base-pair difference (point mutation). Discrimination between wild-type and mutant templates was demonstrated as the beacons did not generate fluorescence with their respective mismatch targets but only with those that they were designed to perfectly anneal with. Samples that harbor mixed populations of CMV could also be readily recognized. Applied to a small number of clinical samples, the retrospective screening by this assay are in general concordance with that obtained by PCR-RFLP. Using molecular beacons strategy, codon 460 mutation was detected in ten out of the total number of 40 samples, whereas the latter method identified nine samples as containing the mutation. The discrepant result arose from the genotyping of one clinical sample as mixed (containing both wild-type and mutant CMV strains) by molecular beacons but as wild-type by PCR-RFLP, suggesting that this real-time strategy is possibly more sensitive for mutation analysis.

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1. Introduction

Prolonged antiviral prophylaxis or therapy is often associated with the emergence of resistant viruses [9]. Ganciclovir-resistant strains of cytomegalovirus (CMV) arise from mutations, mainly point mutations and also deletion of entire codons, in the UL97 or DNA polymerase gene, or both viral genes concurrently [7,8,12,16,17]. The UL97 gene encodes a protein kinase and a consequence of single or multiple amino acid changes to the protein is impaired monophosphorylation of ganciclovir leading to inability form ganciclovir triphosphate, the active form of the drug [5]. To date, the majority (94%) of all

ganciclovir-resistant clinical CMV isolates contain mutations are clustered at three sites within the codon 400–707 region of UL97, with the majority of mutations occurring at codons 460, 594 and 595 [11].

A variety of molecular laboratory assays for diagnostic screening of ganciclovir-resistant CMV isolates have been developed, replacing phenotypic assays such as plaque reduction assay (PRA), that are frequently time consuming and laborious, and not amenable to automation. For instance, owing to the slow growth of the virus, a typical PRA for CMV resistance to ganciclovir requires at least three weeks before results can be reported. This laboratory diagnostic method is therefore not useful for timely management of patients with CMV infection. To date, molecular assays that have been developed for identification of CMV mutations known to confer ganciclovir resistance frequently involve conventional polymerase chain reaction (PCR) followed by restriction enzyme digestion and gel electrophoresis [6] and/or DNA sequencing [3].

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The introduction of real-time PCR amplification that utilizes fluorogenic reporter probes has allowed for rapid and convenient sequence variation detection. Because hybridization of these probes to their complementary target is highly specific, real-time PCR techniques have already been demonstrated for the genotyping of acquired genetic alterations in human diseases and infectious agents [4,13]. In the current study, we investigated the use of a real-time PCR platform using molecular beacons for the specific detection of antiviral drug resistance. The focus of our study was a point mutation (ATG to GTG) in codon 460 of the UL97 CMV gene. This frequently occurring mutation results in an amino acid substitution (methionine to valine) and has previously been shown by marker transfer studies to confer ganciclovir resistance in CMV [6]. Here we propose a real-time PCR strategy for the simultaneous detection and genotyping of CMV DNA for the wild type and mutant strains of clinical isolates of the virus.

2. Materials and methods

2.1. Specimens and DNA isolation

DNA extraction was performed directly from blood samples using a modified phenol-chloroform method, and spectrophotometrically quantified as previously described. The 40 samples were from a cohort of pediatric solid organ transplant patients admitted to the National University Hospital, Singapore. Prior to coding for unbiased reexamination, all samples were analyzed for the presence of CMV by quantitative conventional PCR, as previously described [2]. This was followed by codon 460 mutation analysis via PCR-restriction fragment length polymorphism (that is, conventional nested PCR, *Nla*III restriction enzyme digestion and polyacrylamide gel electrophoresis), also as previously described [6]. Included in the study, as a wild-type positive control, is the laboratory CMV strain AD169 (American Type Culture Collection, Manassas, VA, USA).

2.2. Real-time PCR identification of wild-type or mutant CMV strains at codon 460 of the UL97 gene and human β -actin gene using molecular beacons

The PCR primers and molecular beacons were synthesized commercially (Proligo SAS, France). PCR primers and molecular beacons) to amplify and probe a portion of the UL97 CMV gene region flanking codon 460, based on the published strain AD169 reference sequence (EMBL Accession number X17403), were designed using Beacon Designer software (Premier Biosoft International, Palo Alto, CA, USA). The PCR product generated by these primers is 149 bp long. Based on criteria first described by Tyagi and Kramer [18], the molecular beacons consisted of probe sequences 26 nucleotides long that anneal either to the wild-type or to mutant sequences, 7-nucleotide arm sequences,

Table 1
Sequences of primers and molecular beacons

Name	Sequence ^a
Primer	
Forward	5'-GGCCGACGCTATCAAATTTC-3'
Reverse	5'-GGATAGGGCTCGCTGAGG-3'
Molecular beacon	
MB-Wild-type 460	5'-(Texas Red) <i>CGCGATCGACATTACACCCAT</i> - <i>GAACGTGCTCATGATCGCG</i> (DABCYL)-3'
MB-Mutant 460	5'-(6-FAM) <i>CGCGATCGACATTACACCCC*</i> T- <i>GAACGTGCTCATGATCGCG</i> (DABCYL)-3'

* indicates site of mutation.

^a Italicised sequences indicate stem sequences.

and the fluorophores Texas Red (sulforhodamine101 sulfonyl chloride) and FAM (6-carboxy-fluorescein) covalently linked to the 5' ends of the wild-type and mutant beacons, respectively, and the quencher DABCYL (4-[4'-dimethylaminophenylazo]benzoic acid) linked to the 3' ends of both beacons. The sequences of the primers and molecular beacons are summarized in Table 1. Primer and molecular beacon sequences for human β -actin gene were previously described (Technical Note no. 2701, Bio-Rad Laboratories, Inc.); the molecular beacon for β -actin was labeled with the fluorophore TET (tetrachloro 6-carboxy-fluorescein) and quencher DABCYL at the 5' and 3' ends, respectively.

Using the iCycler iQ detection system (Bio-Rad Laboratories, Inc, USA), PCR conditions were optimized by varying molecular beacon (100–500 nM) and primer (100–1000 nM), and the annealing temperature (50–60 °C). Optimal duplex PCR reactions contained 25 μ l of 2 \times QuantiTect Probe PCR Master Mix (QIAGEN GmbH, Germany), 100 nM of forward primers for CMV UL97 and β -actin genes, 250 nM of the corresponding reverse primers, 200 nM of molecular beacons for the wild-type or mutant sequence and β -actin, and 1 μ g of total DNA in a 50 μ l final volume. Moderately asymmetric primer concentrations used allowed for improved signal generation as more strands complementary to the molecular beacon probes were amplified. The thermal cycling program consisted of 15 min at 95 °C to activate the HotStarTaq DNA polymerase, followed by 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C. Fluorescence was monitored during the 60 °C annealing phases.

3. Results

Nucleic acids isolated from 40 blood samples were subject to real-time PCR molecular beacon analysis for the presence of the A to G mutation in codon 460 of the UL97 CMV gene. Each sample was simultaneously analyzed in two separate reaction wells, each well containing PCR reaction mixture with either the wild-type beacon or the mutant beacon duplexed with primers and molecular beacon for β -globin. Fluorescence was measured during every cycle

of the PCR using the appropriate excitation and emission filters for FAM, Texas Red and TET, respectively. Validity of the duplex PCR reactions for each blood sample was confirmed by an increase in fluorescence following real-time PCR for β -globin.

Comparison of the multiple amplification curves obtained following real-time PCR for wild-type or mutant CMV strains was made after normalization of the fluorescence for each molecular beacon as previously described [15]. Fig. 1 shows the amplification curves obtained, after normalization of fluorescence, with PCR amplifications using both molecular beacons of three different patient samples that harbor wild-type strains of CMV (sample a), both wild-type and mutant strains (sample b) or mutant strains alone (sample c) with respect to codon 460 of the UL97 gene. The results in Figs. 1a and b indicate that PCR amplification of CMV in each sample produced an increase in the fluorescence of only the molecular beacon that was complementary to the intended target DNA. For instance, in Fig. 1a, there was increase in fluorescence when wild-type beacon was tested against samples a and b (containing wild-type CMV and a mixed population, respectively) but not against sample c (which contained no wild-type CMV). When the mutant beacon was tested against the same samples, only samples b and c (which contained the mixed population and mutant CMV, respectively), displayed increase in fluorescence (Fig. 1b). Simultaneous fluorescence of both molecular beacons in sample b indicated the presence in sample c of both

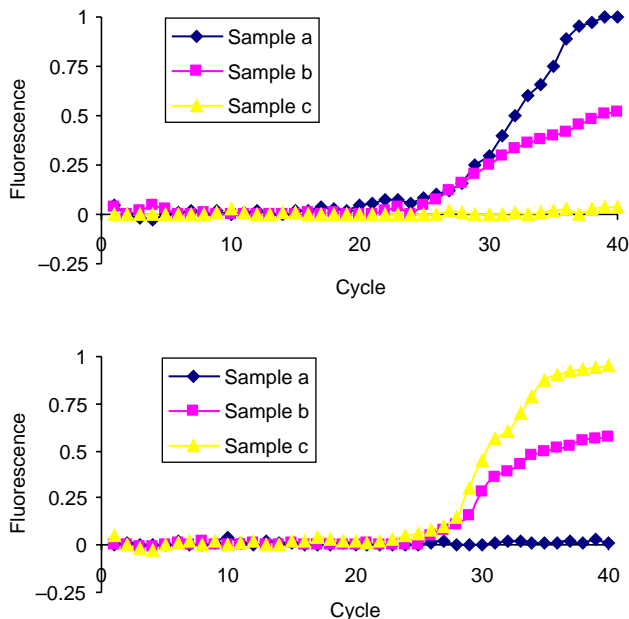


Fig. 1. Real-time PCR amplification plots of three different viral genotypes with respect to codon 460 of the UL9 CMV gene: sample a (wild-type CMV only), sample b (both wild-type and mutant CMV, sample c (mutant CMV only). All samples were probed with both wild-type and mutant molecular beacons (Fig. 1a and b, respectively). Normalized fluorescence of both the wild-type and mutant beacons is plotted on the y-axis and the number of PCR cycles on the x-axis.

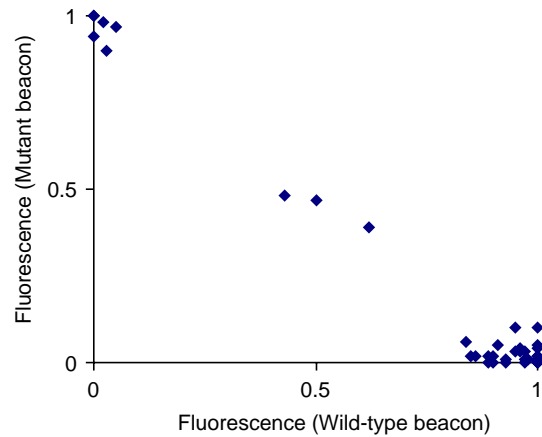


Fig. 2. Scatter plot of 40 clinical samples based on fluorescence values obtained at the last PCR cycle when analyzed by real-time PCR using both wild-type and mutant molecular beacons. The three clusters obtained correspond to the three possible genotypes.

wild-type and mutant strains of the virus. Thus the genotypes of different combinations of CMV genomes could be correctly predicted by this strategy. It was also observed that samples negative for CMV DNA as well as negative (nuclease-free water) controls did not generate any fluorescence for either beacon (data not shown).

Analysis of the scatter plot of fluorescence values received in the last PCR cycle for each clinical sample tested against both wild-type and mutant molecular beacons (Fig. 2) show three distinct clusters, reflecting the three possible combinations of wild-type and mutant CMV strains in any given sample with CMV DNA.

Table 2 summarizes the results obtained by both PCR-RFLP and real-time PCR analysis by molecular beacons for

Table 2
Comparison of two DNA-based methods (PCR-RFLP and real-time PCR using molecular beacons) for codon 460 UL97 CMV gene mutation analysis

	PCR-RFLP	Molecular beacons PCR
A. Clinical samples		
Wild-type strain only	31	30
Mixed (both mutant and wild type strains)	3	4
Mutant strain only	6	6
Total number	40	40
	Genotype detected	Genotype detected
B. Artificial mixtures (% CMV AD169 wild-type to mutant strains, each combination assayed in triplicates)		
100:0	Wild-type (3/3)	Wild-type (3/3)
95:5	Wild-type (3/3)	Mixed (2/3)
		Wild-type (1/3)
90:10	Mixed (3/3)	Mixed (3/3)
80:20	Mixed (3/3)	Mixed (3/3)
70:30	Mixed (3/3)	Mixed (3/3)
0:100	Mutant (3/3)	Mutant (3/3)

the detection of wild-type and mutant strains in clinical samples. With one exception, the results obtained by molecular beacon analysis show general concordance with the other molecular approach for the clinical samples tested with the exception of one sample which was genotyped as mixture of wild-type and mutant strains by molecular beacons but as wild-type only by PCR-RFLP (Table 2A).

To further evaluate the ability of the molecular beacon assay to discriminate genotype artificial mixtures of different percentages of mutant (patient) and wild-type (AD169) strains of the virus of known copy numbers were prepared and assayed in triplicates. The results (Table 2B) are comparable to PCR-RFLP and indicate that a sample with approximately 10% or more of the mutant strains against a background population of wild-type virus can also be correctly clustered in the wild-type/mutant group (as per Fig. 2). However, molecular beacon assay was able to identify artificial mixtures at a ratio of 95:5 wild-type to mutant strains in two out of the triplicate samples whereas all samples were genotyped as wild-type using PCR-RFLP.

4. Discussion

A rapid real-time PCR assay using molecular beacons for the simultaneous detection of wild-type and mutant CMV viruses with respect to codon 460 of the UL97 gene has been developed. The molecular beacons were designed to complement the wild-type codon 460 or the mutant sequence arising from ATG to GTG change (methionine to valine amino acid substitution). We elected to perform the detection of wild-type and mutant CMV strains in each sample simultaneously but in separate reaction tubes in order to avoid competition and other problems inherent in more complex multiplex PCR assays [10]. Each reaction tube therefore is a duplex PCR with amplification and detection of wild-type or mutant CMV strains and coamplification and detection of human DNA with β -globin primers and molecular. The latter serves as a control for both sample adequacy and PCR amplification.

Discrimination between wild-type and mutant templates was demonstrated as the beacons did not generate fluorescence with their respective mismatch targets but only with those that they were designed to perfectly anneal with (Fig. 1). In fact, samples that harbor mixed populations of wild-type and mutant viruses could also be recognized by examining of the amplification curve (Fig. 1) or by a scatter plot (Fig. 2). A separate cluster is observed, distinguishable from the clusters of wild-type and mutant genotypes when the fluorescence values of the last PCR cycle obtained using either wild-type or mutant molecular beacon were plotted.

Applied to a small number of clinical samples, the retrospective mutation screening results generated by the molecular beacons real-time PCR assays are in general concordance with that obtained by PCR-RFLP. Using molecular beacons strategy, codon 460 mutation was

detected in ten out of the total number of 40 samples, whereas the latter method identified nine samples as containing the mutation.

The discrepant result arose from the genotyping of one of the clinical sample as mixed (containing both wild-type and mutant CMV Strains) by molecular beacons but as wild-type by PCR-RFLP. It has been reported that PCR-RFLP is able to detect mutant CMV templates when the percentage of mutants in the viral population reaches 10% [6]. In a separate experiment using artificial mixtures of wild-type and mutant CMV templates of varying percentages (Table 2), we demonstrated that the molecular beacons used in this study were able to correctly and reproducibly genotype samples that contained 10% or more mutant templates against a wild-type background. However, we observed that two out of three samples in the triplicates containing mixtures of 5% mutant CMV DNA templates could also be unambiguously genotyped by this method. The improved sensitivity of real-time over conventional PCR for the detection of viruses in routine and research laboratories has been documented and attributed mainly to improved detection by fluorogenic chemistries such as molecular beacons as well as advances in instrumentation [1,13,14]. Our data is also in support of this observation, and indicate that the molecular beacons assay is possibly more sensitive for mutation analysis of codon 460 mutations than PCR-RFLP. As real-time PCR also allows for quantitation of the copy number or amount of starting template, an area for further investigation with this new technology is the determination of the exact percentage of mutants in the viral population in an isolate that is correspondent to the appearance of clinical resistance to ganciclovir.

In conclusion, the results obtained in this study show that molecular beacons can be successfully applied for mutation analysis. In contrast to conventional PCR that entails visual or densitometric analysis of ethidium-bromide stained gel electrophoretic patterns, a real-time PCR strategy allows closed-tube, automated detection of single-base pair substitutions without the need for post-amplification processing. As the majority of ganciclovir-resistant clinical isolates of CMV contain diagnostically useful point mutations in the UL97 gene, the development of new beacons in a similar screening strategy or in a multiplex PCR format with the use of different report fluorophores is warranted.

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