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Detection of Common Disease-Causing Mutations in Mitochondrial DNA (Mitochondrial Encephalomyopathy, Lactic Acidosis with Stroke-Like Episodes *MTTL1* 3243 A>G and Myoclonic Epilepsy Associated with Ragged-Red Fibers *MTTK* 8344A>G) by Real-Time Polymerase Chain Reaction

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The 3243A>G mutation in the *MTTL1* ($tRNA^{Leu}$) gene and the 8344A>G mutation in the *MTTK* ($tRNA^{Lys}$) gene are the most common mutations found in mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes and myoclonic epilepsy associated with ragged-red fibers, respectively. These mitochondrial DNA mutations are usually detected by conventional polymerase chain reaction followed by restriction enzyme digestion and gel electrophoresis. We developed a LightCycler real-time polymerase chain reaction assay to detect these two mutations based on fluorescence resonance energy transfer technology and melting curve analysis. Primers and fluorescence-labeled hybridization probes were designed so that the sensor probe spans the mutation site. The observed melting temperatures differed in the mutant and wild-type DNA by 9°C for the *MTTL1* gene and 6°C for the *MTTK* gene. This method correctly identified all 10 samples that were 3243A>G mutation-positive, all 4 samples that were 8344A>G mutation-positive, and all 30 samples that were negative for both mutations, as previously identified by traditional gel-based methods. This LightCycler assay is a rapid and reliable technique for molecular diagnosis of these mitochondrial gene mutations. ***(J Mol Diagn 2006, 8:277–281; DOI: 10.2353/jmoldx.2006.050066)***

Mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS) and myoclonic epilepsy associated with ragged-red fibers (MERRF) are diseases caused by mutations in mitochondrial DNA (mtDNA). The 3243A>G mutation in the *MTTL1* ($tRNA^{Leu}$) gene and the 8344A>G mutation in the *MTTK* ($tRNA^{Lys}$) gene of mtDNA account for about 80% of MELAS and MERRF cases, respectively.[1,2] In clinical laboratories, these mtDNA mutations have often been detected using polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis by gel electrophoresis. Although this method is effective, it is relatively time consuming and introduces the risk of cross contamination by PCR products.

Detection of mutations in mtDNA can be accomplished by performing continuous PCR amplification and melting curve analysis using fluorescent hybridization probes on a LightCycler. Two fluorescently labeled, sequence-specific probes, one of which spans the mutation site, are used for each of these assays. When the mutation-spanning sensor probe and an anchor probe are annealed in tandem to the target mtDNA, fluorescence resonance energy transfer results in increased fluorescent energy that is detectable by the LightCycler. After amplification by PCR, melting curve analysis of the product is accomplished by heating to dissociate the sensor probe from its target, which leads to a decrease in fluorescence. The temperature at which the probe dissociates depends on whether a mismatch is present, with a mismatch yielding a lower melting temperature (T_m).[3,4]

We have developed novel PCR assays to detect the two common mtDNA mutations (3243A>G and 8344A>G) in MELAS and MERRF using fluorescence resonance energy transfer technology and melting curve analysis on a real-time PCR platform.[5,6] The assays were analytically validated by optimization of amplification conditions and evaluation of performance characteristics

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Table 1. Primers and Probes

	MELAS *MTTL1*	MERRF *MTTK*
F	5′-CCCTGTACGAAAGGACAA-3′	5′-CTACGGTCAATGCTCTGAAA-3′
R	5′-CAATGAGGAGTAGGAGGT-3′	5′-GTGATGAGGAATAGTGTA-3′
S	5′-GGCCCTGCCATCTTAACAAA-3′	5′-AAGATTAAGAGAACCAACACCTCT-3′
A	5′-GTTCTTGGGTGGGTGTGGGT-3′	5′-TACAGTGAAATGCCCCAACTAAATACTACCGT-3′

Mutation position is underlined. Donor probes (anchor probe in MELAS; sensor probe in MERRF) are labeled with fluorescein at the 3′ end; acceptor probes (sensor probe in MELAS; anchor probe in MERRF) are labeled with LC Red640 at the 5′ end and phosphorylated on the 3′ end to prevent extension during PCR. F, forward primer; R, reverse primer; S, sensor probe; A, anchor probe.

on known positive and negative samples, as defined by conventional PCR and RFLP methods.

Materials and Methods

Sample Preparation

Thirteen cases of clinically confirmed MELAS ($n = 10$) or MERRF ($n = 3$) and 30 unaffected controls were identified from the records of the University of North Carolina Hospital and the Ohio State University Molecular Laboratories. Genotypes were determined by traditional PCR and RFLP methods.[7,8] DNA from the NA11906 lymphoblastoid cell line, derived from a patient known to carry the MERRF *MTTK* 8344A>G mutation (Coriell Institute, Camden, NJ), was also included. The study was performed in compliance with local and federal regulations and under the supervision of our Institutional Review Board.

mtDNA was extracted from blood using the MagNA Pure LC with DNA Isolation kit I reagents, according to the manufacturer's protocol (Roche Diagnostics, Indianapolis, IN) or by manual methods.[9]

Primers, Probes, and Amplification

Primer and probe sets were designed based on the Human Mitochondrial DNA Cambridge Reference Sequence (GenBank no. NC_001807) and guidelines set forth by Roche Diagnostics,[4] with the assistance of "The LightCycler Probe Design Software" (version 1.0; Roche Diagnostics). Primers and probes were synthesized by TIB Molbiol (Berlin, Germany). Several sets of primers and probes were tested for their ability to detect the mutations, including two pairs of primers and three sets of probes for *MTTL1* 3243A>G and two pairs of primers and one set of probes for *MTTK* 8344A>G. The pairs that gave the best results for each locus are shown in Table 1.

Probes were designed so that the sensor probes span the mutation sites, with the probe for MELAS designed to be in the antisense orientation and to match the *MTTL1* mutant sequence perfectly (Figure 1A), leading to more stable hybridization to the mutant sequence. In contrast, the sensor probe for the MERRF 8344A>G mutation was designed to be in the sense orientation and to match the normal sequence perfectly (Figure 1B). The gaps between anchor and sensor probes were four nucleotides for MELAS and one for MERRF.

PCR conditions (primer, probe, and magnesium concentrations and annealing temperatures) and the melting curve analysis programs were optimized to distinguish known mutant samples from known normal ones. Asymmetric PCR was used to produce excessive complementary strand target for probe hybridization (ie, fivefold more forward than reverse primer for MELAS and fivefold more reverse than forward primer for MERRF). Except for primer concentrations, the reaction conditions and thermocycler programs were identical for both assays, which were performed using a first generation LightCycler (Roche Diagnostics). The 10-μl reaction contained 2 μl of template DNA, 2 mmol/L $MgCl_2$, 0.3 μmol/L each of sensor and anchor probes, 1 μl of FastStart DNA Master Hybridization Probes (Roche Diagnostics), and primers at 0.6 μmol/L (forward) and 0.1 μmol/L (reverse) targeting *MTTL1* or at 0.1 μmol/L (forward) and 0.6 μmol/L (reverse) targeting *MTTK*. Amplification conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 0 seconds, annealing at 57°C for 10 seconds, and extension at 72°C for 20 seconds, with 20°C/second transition time. Samples were genotyped by melting curve analysis. Briefly, this analysis was performed 1) by denaturation at 95°C for 30 seconds, 2) by hybridization to favor binding of probes to both mutant and normal targets at 58°C for 60 seconds and 49°C for 60 seconds, respectively (transition rate of

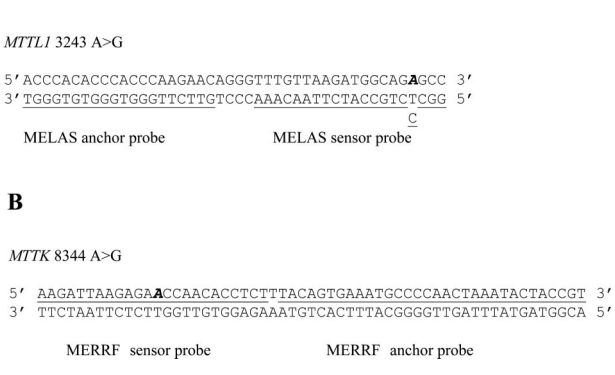

Figure 1. Mutation detection by real-time PCR and melting curve analysis using hybridization probes targeting normal or mutant DNA. **A:** Sequence of the MELAS *MTTL1* region. **B:** Sequence of the MERRF *MTTK* region. The sites of the mutations are indicated in bold, and the sequences corresponding to the probes are underlined. Note that the sensor probe for *MTTL1* is in the antisense orientation and is complementary to the mutant sequence, whereas the sensor probe for *MTTK* is in the sense orientation and is complementary to the normal sequence.

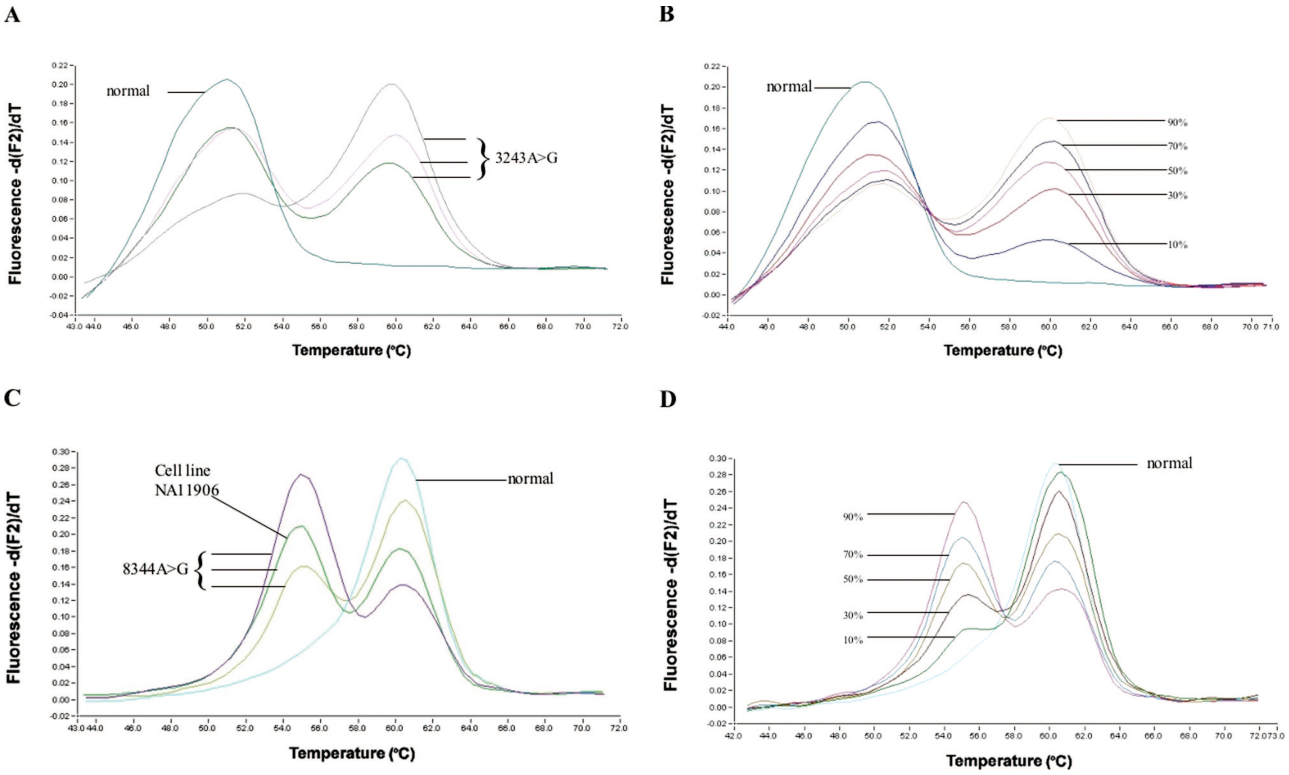

Figure 2. Melting curves for MELAS *MTTL1* 3243A>G normal ($n = 1$) and mutant ($n = 3$) samples (**A**) and a dilution series with MELAS *MTTL1* 3243A>G and normal DNA (**B**). Melting temperatures are 60°C for mutant DNA and 51°C for normal. Melting curves for MERRF *MTTK* 8344A>G normal ($n = 1$) and mutant ($n = 3$) samples (**C**) and a dilution series with MERRF *MTTK* 8344A>G and normal DNA (**D**). Melting temperatures are 55°C for mutant DNA and 61°C for normal. The percentages in **B** and **D** indicate the relative amounts of a mutant patient sample mixed in with a normal DNA sample.

20°C/second), and finally 3) by dissociation of reporter probe from template by slowly raising the temperature to 75°C (transition rate of 0.4°C/second) with stepwise acquisition of fluorescence. The data were converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature (−dF/dT) against temperature by the LightCycler Data Analysis Software. The entire amplification and melting curve program takes about 45 minutes.

Because of heteroplasmy, ie, the existence of both normal and mutant mitochondrial DNA, the mutant mtDNA in patient tissues is usually seen in the presence of normal mtDNA.[10,11] Mutant samples from patients are, therefore, expected to have two peaks, the amplitudes of which depend on the degree of heteroplasmy in the tissue sampled.

Results

Thirteen known positive samples (10 for the MELAS *MTTL1* 3243A>G mutation and 3 for the MERRF *MTTK* 8344A>G mutation) and 30 samples negative for both mutations were analyzed by the LightCycler assays. Representative melting curves are shown in Figure 2, A and C. Melting profiles from affected individuals showed two peaks: for *MTTL1*, the melting temperatures were 60°C (mutant) and 51°C (normal); and for *MTTK*, they were 61°C (normal) and 55°C (mutant). An additional DNA sample from a cell line (NA11906) that has the 8344A>G mutation was analyzed by the real-time PCR assay, and the expected result was obtained (Figure 2C). All results were concordant between the real-time PCR and the conventional PCR and RFLP methods (Table 2).

To evaluate the effect of varying ratios of mutant to normal mtDNA, we made dilutions of mtDNA from patients with known mutations in 10% increments into an mtDNA sample from a normal individual. The exact mutant/normal mtDNA ratio in these mixtures was unknown because the extent of heteroplasmy in the patient samples was not determined by another method. Although the mutation detection assays were not designed to be quantitative, the dilution study results show that the primer and probe sets used for both MERRF *MTTK* and MELAS *MTTL1* analysis can resolve differences of 10% in the levels of mutant mtDNA in a normal background (Figure 2, B and D). Mutations in patient samples could be detected at levels as low as 10% for either 3243A>G or 8344A>G in a mixture with a normal sample.

Table 2. Results of MELAS and MERRF Mutations Detected by Both Conventional and LightCycler Methods

LightCycler PCR	MELAS 3243 A>G		MERRF 8344 A>G	
Conventional PCR	Positive	Negative	Positive	Negative
Positive	10	0	3	0
Negative	0	30	0	30

Discussion

MELAS and MERRF are rare mitochondrial diseases, and the heterogeneous clinical manifestations sometimes make the clinical diagnosis difficult. Detection of disease-causing mutations will help to confirm the diagnoses. Conventional PCR-RFLP followed by gel electrophoresis is the method most commonly used for detecting these mtDNA mutations. The conventional PCR method is effective but is relatively labor intensive, and results are sometimes difficult to interpret because of the presence of faint bands on the gel. We have developed novel assays for the common MELAS and MERRF mutations using real-time PCR followed by melting curve analysis on the LightCycler. Compared with conventional PCR methods, the LightCycler method is more rapid and relatively easy to interpret. The LightCycler offers a continuous, closed system to amplify and detect mutation with minimal risk of cross contamination.

For the MELAS 3243 A>G mutation, the sensor probe was designed to match the mutant sequence perfectly, leading to more stable hybridization to the mutant sequence. As expected, the mutant exhibited a higher T_m than the wild-type sample (60 versus 51°C, respectively). In contrast, the sensor probe for the MERRF 8344A>G mutation was designed to perfectly match the normal sequence; accordingly, the wild-type sample exhibited a higher T_m than the mutant sample (61 versus 55°C).

All known-positive patient samples (10 for the MELAS *MTTL1* 3243A>G mutation and 3 for the MERRF *MTTK* 8344A>G mutation) and 30 samples negative for both mutations analyzed by the LightCycler assays yielded concordant results between the real-time PCR and the conventional PCR-RFLP methods. Thus, the new assays appear to perform as well as conventional methods. The heights of melting-curve peaks above the baseline of 0.00 units of fluorescence were measured for mutant and normal DNA, and the fraction of mutant DNA in each sample was estimated as $height_{mutant}/(height_{mutant} + height_{normal})$. The reproducibility of these ratios was determined in six replicates of a sample from a known MELAS patient and five replicates of a known MERRF patient. The mean ratios and coefficients of variation (CVs) were 0.66 (CV = 10.6%) and 0.52 (CV = 3.8%), respectively, which shows that the results are highly reproducible.

Because of heteroplasmy, quantitation of the relative amounts of normal and mutant mtDNA would be desirable. Several quantitative real-time PCR methods have been reported, including the use of Molecular Beacons, TaqMan probes, or SYBR Green technologies.[11–13] Our method was not designed to be quantitative because there are scant data to suggest that melting peak size is accurate for quantitative measurement,[3] but our assay has adequate sensitivity to differentiate 10% increments of patient DNA diluted into normal human DNA samples and to detect as little as 10% mutant mtDNA in both MELAS and MERRF patient samples (Figure 2, B and D). The sensitivity of the assay is actually greater than 10%, given that the patient samples used for the dilution series had significant fractions of normal mitochondrial DNA. Estimates based on relative peak heights are that the starting samples contained 52% (MERRF) and 66% (MELAS) mutant molecules; these estimates lead to the conclusion that the tests are sensitive to 5 to 7% heteroplasmy or less. The clinical significance of variations in the fraction of mutant mtDNA in blood is not clear, given that mutation load varies by tissue type and that clinical symptoms are expected to track most closely with certain tissue types, such as skeletal muscle.[10,14] We do not include estimates of the percentage of heteroplasmy in clinical reports. There are other, relatively labor-intensive methods that can be used to quantitate the extent of heteroplasmy.[15–17]

In summary, compared with conventional PCR and RFLP analysis, real-time PCR assays are less time and labor intensive, relatively easy to interpret, and offer very low risk of cross contamination. The method that we have described will be especially useful for laboratories that are already equipped with a LightCycler. These novel real-time PCR assays provide a rapid and reliable technique for molecular confirmation of clinical and biochemical finding in MELAS and MERRF patients.

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