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Assessing Heteroplasmic Load in Leber's Hereditary Optic Neuropathy Mutation 3460G→A/MT-ND1 with A Real-Time PCR Quantitative Approach

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To quantify the amount of the 3460G→A/ND1 point mutation responsible for Leber's hereditary optic neuropathy, we developed a quantitative real-time polymerase chain reaction method based on the SYBR Green assay and a new approach using the TaqMan assay. Both methods were based on the amplification refractory mutation system, comparing the heteroplasmic load quantified by restriction fragment length polymorphism in 15 Leber's hereditary optic neuropathy family members, with the results obtained using quantitative real-time polymerase chain reaction methods. The comparative evaluation of mitochondrial DNA (mtDNA) heteroplasmy from blood samples showed significant correlation between restriction fragment length polymorphism analysis, real-time SYBR Green assay, and TaqMan assay. We validated the last method by measuring experimental samples composed by a known proportion of cloned plasmids containing either the wild-type or mutant sequence, giving a correlation coefficient of 0.999 ($P < 0.0001$). The real-time amplification refractory mutation system polymerase chain reaction by TaqMan assay provides a rapid, reliable, sensitive, reproducible, and one-step quantitative method to detect heteroplasmic mutant mtDNA. This method allows the quantitation of a broad range of mutational load (up to 100%, down to 0.01%) on the basis of *in vitro* calibration, thus rendering the TaqMan assay suitable for the diagnostic analysis of heteroplasmic load in mtDNA-related disorders. *(J Mol Diagn 2007, 9:538–545; DOI: 10.2353/jmoldx.2007.060183)*

Mitochondria, the main source of energy within cells, contain their own genome. The mitochondrial DNA (mtDNA), which is a double-stranded circular intronless DNA, contains genes encoding subunits of the respiratory chain as well as genes for tRNAs and rRNAs. Mutations in mtDNA are responsible for mitochondrial encephalomyopathies, a group of maternally inherited disorders characterized by impaired energy production. Mutations in nuclear DNA also account for mitochondrial disorders, being mostly of the subunits of the mitochondrial respiratory chain complexes encoded by the nuclear genome; the intergenomic signaling and regulatory role of nuclear proteins on mitochondrial function are further aspects of nuclear involvement in mitochondrial dysfunction.

Leber's hereditary optic neuropathy (LHON; Online Mendelian Inheritance of Man no. 535000) is a maternally inherited disorder characterized by a primary degeneration of the retinal ganglion cells followed by optic nerve atrophy.[1] Central vision is mostly affected because of the preferential death of the small nerve fibers of the papillomacular bundle.[2] Several mitochondrial mutations are proposed to be involved in disease development, but most cases are associated with one of three mtDNA point mutations, 3460G→A/MT-ND1, 11778G→A/MT-ND4, and 14484T→C/MT-ND6, which are responsible for approximately 90% of LHON cases worldwide. These three very frequent mtDNA point mutations are considered primary, and they affect genes coding for different subunits of complex I with modest or subtle biochemical signatures in complex I function. The 3460G→A mutation affects the ND1 subunit, and this mutation has been consistently found to result in a 60 to 80% reduction in complex I-specific activity in a variety of tissues such as patient platelets, fibroblasts, and lymphoblasts.[3]

According to the peculiar rules of mitochondrial genetics, LHON is maternally inherited, and most LHON families carry the mtDNA mutation in the homoplasmic condition, but only a subset of individuals becomes blind

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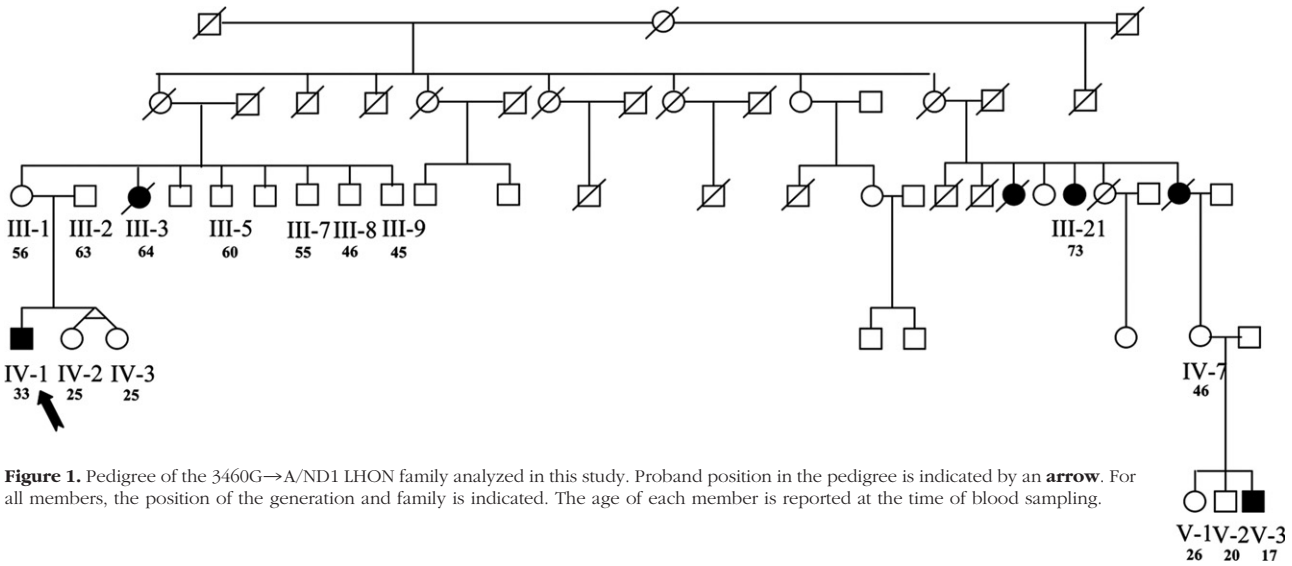

Figure 1. Pedigree of the 3460G→A/ND1 LHON family analyzed in this study. Proband position in the pedigree is indicated by an **arrow**. For all members, the position of the generation and family is indicated. The age of each member is reported at the time of blood sampling.

(incomplete penetrance). These individuals are frequently grouped in some pedigree branches.[4] Moreover, LHON mostly affects young males, which cannot be explained solely on the basis of the mtDNA mutations.[1] Thus, LHON differs from other mitochondrial diseases because it is associated with a homoplasmic mtDNA mutation, it has a male prevalence, and it shows an incomplete and variable penetrance. The existence of further genetic determinants, such as nuclear modifying genes[5] possibly on chromosome X, has been largely debated.[6] The most compelling evidence for a modifying role comes from the association of mtDNA haplogroup J with the 11778G→A and 14484T→C, which possibly increases LHON penetrance. On the contrary, there is no explanation for why the 3460G→A mutation is distributed in all haplogroups at frequencies similar to the control population.[7,8] Most probably this is because of the biochemical severity of this mutation and the capability of clinical expression without the modifying intervention of mtDNA haplogroups. In fact, this mutation also has the lowest male/female ratio and is most frequently found heteroplasmic, as in the case of more severe mtDNA mutations.[1] Thus, the availability of a reliable quantitative method for evaluation of heteroplasmy is particularly relevant for the 3460G→A mutation to study its somatic and germline segregation in patients and families.

The traditional approach in determining heteroplasmic load in mtDNA point mutation-related disorders is the polymerase chain reaction (PCR) amplification of the DNA fragment containing the mutation, followed by restriction fragment length polymorphism (RFLP) analysis and subsequent detection through ethidium bromide-stained agarose gel or polyacrylamide separation in the presence of radiolabeled deoxynucleotide in the PCR. The detection limit for RFLP-ethidium bromide is approximately 5 to 10%, whereas that for the radioactive method is between 2 and 5%.[9]

It is clear that a more sensitive method is required to detect lower amounts of mutation in pedigrees. A reproducible method that avoids the use of radioactively labeled nucleotides, a more sensitive approach that could lower the detection limit, and a specific method that allows a clear quantification of both mtDNA populations are the main characteristics of the method that should be developed for our purposes.

In the present study, we developed a real-time quantitative PCR technique to quantify the mutational load of the 3460G→A mutation. The method is based on an "amplification refractory mutation system" (ARMS),[10] and it was first applied on a quantitative assay in the presence of SYBR Green to set up primer specificity. After this approach, we designed a labeled probe for TaqMan assay to increase the specificity of the quantitative PCR. We showed some differences between the use of a fluorescent intercalator such as SYBR Green and the use of a TaqMan probe of heteroplasmic mtDNA mutants. The results demonstrate that the real-time assay is a rapid, sensitive, and reliable method that can replace RFLP analysis in detection and quantification.

Materials and Methods

Patients, DNA Sources, and DNA Isolation

Venous blood samples were obtained, after informed consent and institutional approval of the Department of Neurological Sciences of Bologna University, from a male LHON proband homoplasmic for the 3460G→A mutation and from further maternally related individuals of both healthy or affected carriers of the 3460G→A/MT-ND1 mutation (Figure 1). In total, 15 family members (eight males and seven females) were included in this study. Only two males (IV-1 and V-3) and two females (III-3 and III-21) presented with visual loss with the typical hallmarks of LHON.

The pedigree has been previously studied as pedigree 4[8]; it has also been evaluated spectroscopically[11] and biochemically (as family 4).[12] In all of the previous studies, the DNA from the same blood withdrawals used in

Table 1. Real-Time PCR Primers

Amplification product	Nucleotide position	Oligonucleotide sequence	Amplicon size (bp)
Mutant molecules	3291 to 3311	5′-TCCTCTTCTTAACAACATACC-3′	188
	3478 to 3460	5′-TGGTGAAGAGTTTTATGGT-3′	
Wild-type molecules	3291 to 3311	5′-TCCTCTTCTTAACAACATACC-3′	187
	3477 to 3460	5′-GGTGAAGAGTTTTATGGC-3′	
Total mtDNA	3291 to 3311	5′-TCCTCTTCTTAACAACATACC-3′	227
	3517 to 3499	5′-AGAGGGTGATGGTAGATGT-3′	

these experiments has been evaluated by PCR/RFLP. Total DNA was isolated from peripheral blood by using proteinase K digestion followed by standard phenol-chloroform extraction and ethanol precipitation.[12]

Quantitative Detection of mtDNA with a 3460G→A/ND1 Point Mutation: Primers for ARMS

For the amplification refractory method approach, a set of PCR primers with a sequence matched to the mutated mtDNA at the 3′ end was used for selective amplification of the mutated mtDNA. Another set of primers was aimed to amplify selectively wild-type molecules, whereas a third set of primers was designed to amplify total mtDNA (wild-type plus mutated molecules). The primers were designed using Primer Express software (Applied Biosystems, Foster City, CA), and Table 1 reports their amplification targets and sequence with nucleotide positions referred to the mtDNA sequence.[13] Primer specificity has been tested by gradient PCR (Eppendorf-5 Prime, Inc., Boulder, CO) to achieve the best yield and specificity at the most suitable annealing temperature (60°C) to set up a real-time PCR assay.

Quantitative Assay Using SYBR Green

The total amount of master mixture contained SYBR Green dye (Applied Biosystems) with AmpliTaq Gold DNA polymerase, dNTPs, and dUTP, and $MgCl_2$ was used according to manufacturer's protocols. Reactions also contained 100 nmol/L each of the forward and reverse primers for amplification of mutant and wild-type mtDNA and 200 nmol/L each of the forward and reverse primers for total mtDNA amplification, a range of 5 to 100 ng of total DNA in a final volume of 25 μl. Real-time PCR conditions were 2 minutes at 50°C and 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C and annealing/extension for 60 seconds at 60°C. The PCR products were quantified with an automatic sequence detection system (ABI Prism 7700; Applied Biosystems) at each step of amplification using the SDS software (version 1.0; Applied Biosystems).

Quantification Using TaqMan Assay

An alternative approach to quantify heteroplasmic load is represented by the TaqMan assay.[14] The nucleotide sequence of the minor groove binder (MGB) TaqMan probe was 5′-FAM-TATATACAACTACGCAAAGGC-3′ (3394 to 3414). The melting temperature of 71°C for this probe is ideal for use with the sets of primers with the annealing temperature of 60°C, designed by us according to the manufacturer's protocol (Primer Express; Applied Biosystems), which suggests an annealing temperature for the TaqMan MGB probe at least 10°C higher than the primers annealing temperature.

PCR started at 95°C for 15 seconds, followed by 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. The reaction mixture (25 μl) contained template DNA (5 ng), TaqMan Universal PCR Master Mix (2× composed of DNA polymerase AmpliTaq Gold), AmpErase UNG (uracil *N*-glycosylase), dNTPs with dUTP, and appropriate $MgCl_2$ concentrations according to the Applied Biosystems data sheet, 200 nmol/L TaqMan probe, and 200 nmol/L of each of the forward and reverse primers for quantifying total mtDNA or 100 nmol/L of each primer for quantifying mutant and wild-type mtDNA. The PCR products were quantified the same way as SYBR Green amplicons.

Assay Calibration

For mtDNA quantification by PCR analysis, we generated calibration curves with serial dilutions of the target sequence PCR products. Three different mtDNA amplicons were produced and inserted in a pGEM-T Easy Vector (Promega, Charbonnières, France), thus creating three different plasmids. One plasmid contained 3460G (wild-type), whereas another contained 3460A (mutant), both encompassing nucleotides 3291 to 3478. These plasmids were used for the calibration of wild-type and mutant mtDNA populations. The third plasmid was created by inserting an mtDNA sequence from nucleotides 3291 to 3517, and this plasmid was used as a calibrator for total mtDNA (mutant + wild-type).

Plasmid DNA was isolated and purified with the Fast Plasmid Mini kit (Eppendorf) according to the manufacturer's protocol. The plasmid copy number was determined by dividing the total DNA concentration by the weight of each plasmid molecule. Both mutant and wild-type plasmid vectors had the same size (3015 and 3014 bp, respectively); thus, the weight of double-stranded mtDNA molecule (ie, for the mutant plasmid) was estimated as follows: $[(3.015\ \text{kb} + \text{amplicon kb})\text{MWt}]/A$, where MWt denotes the molecular weight of 1 kb of double-stranded DNA (6.6×10^5 g/mol), and A denotes Avogadro's number (6.02×10^{23} molecules/mol).[15]

Table 2. Heteroplasmic Load Obtained with PCR/RFLP, Real-Time PCR Using SYBR Green, and Real-Time PCR Using TaqMan MGB Probe

Sample	% PCR/RFLP	% SYBR Green (error range)*	% TaqMan (error range)*
III-1	56	64.7 (57.9 to 71.5)	89.2 (88.2 to 90.2)
III-2	0	0 (0 to 0)	0 (0 to 0)
III-3	100	88.7 (80.8 to 96.7)	100 (100 to 100)
III-5	22	5.5 (4.8 to 6.2)	7 (5.2 to 8.8)
III-7	19	5.2 (4.9 to 5.6)	46.8 (44.6 to 49)
III-8	17	2 (0.6 to 3.4)	21 (20.1 to 21.9)
III-9	100	90 (90 to 90)	100 (100 to 100)
III-21	100	91 (89.6 to 91.4)	100 (100 to 100)
IV-1	100	85 (77.9 to 92.1)	100 (100 to 100)
IV-2	100	99.5 (98.8 to 100.2)	100 (100 to 100)
IV-3	100	75 (67.5 to 82.1)	100 (100 to 100)
IV-7	66	57 (54.2 to 59.8)	74.5 (73.7 to 75.3)
V-1	74	85 (77.7 to 93.3)	90.3 (89.3 to 91.3)
V-2	100	60 (60 to 60)	100 (100 to 100)
V-3	100	81.2 (79.3 to 81.3)	99.8 (99.4 to 100.2)

*Mean and error range (±1 SD) are obtained from three independent experiments.

Regarding relative quantification, Ct (threshold cycle) of target sequences (mutant or wild-type) were related to Ct of total sequence (mutant + wild-type) used as a normalizer. The $2^{-\Delta\Delta Ct}$ values were obtained by serial dilutions of mutant and wild-type plasmid DNA. Data were obtained by averaging three independent experiments.

RFLP Analysis

The RFLP quantitative analysis was performed using the protocol previously described.[11]

Results

Development of a Method for Quantifying the Amount of 3460G→A Point Mutation in mtDNA

Because LHON mutation at nt3460 could be present in low percentages in patients, we developed a method to detect and quantify small amounts of mutant mtDNA. Under the strict PCR conditions described in the previous section, only three sets of primers were selective and specific enough to amplify the target mtDNA region 3460 according to the universal parameters of Applied Biosystems reagents. The amplified DNA fragment was detected by electrophoresis on agarose gel, and it was confirmed to be the corresponding mtDNA region by sequencing (data not shown). The absence of amplified band from ρ^0 cell line DNA (devoid of mtDNA) subjected to PCR cycling with the three sets of primers excluded nuclear pseudogene amplification.

In the experimental design, we selected allele-specific primers modified only at the 3′ end without adding any mismatch near the 3′ end. Such allele-specific primers have been tested, together with other sets of primers, by using gradient PCR to choose the best sets of primers giving a high amount of specific products working at the highest annealing temperature. The purpose of this kind of choice is to design a TaqMan-based quantitative real-time PCR method by using SYBR Green assay to set up PCR conditions. Moreover, the TaqMan probe had to be designed to be annealed at a temperature of over 70°C, at least 10°C higher than the annealing temperature of the primers.

Quantification of Heteroplasmy Using SYBR Green

Regarding quantitative analysis, we first detected the optimal concentrations of primers according to the universal protocol using fluorogenic SYBR Green. The optimum concentration of 100 nmol/L provided a low Ct and high ΔRn (defined as the magnitude of the fluorescence signal generated during the PCR at each time point) when using the sets of primers for the amplification of wild-type and mutated molecules. The optimum concentration for the total mtDNA amplification was 200 nmol/L. These optimized primer concentrations amplified specific amplicons of the mtDNA 3460 region. To assess the efficiency of real-time SYBR Green assay, calibration curves were generated from duplicate amplification of three independent serial dilutions of the cloned plasmid mtDNA 3460 region PCR products. These curves were obtained from Ct values plotted against the logarithm of plasmid copy number. There was a good correlation between the cycle number and mtDNA copy number with correlation coefficients (slopes) of −3.486 for mutated and total copies of primers and −3.719 for wild-type primers. The corresponding amplification efficiencies, 94 and 86%, calculated on the basis of the formula $\text{Eff} = 10^{(-1/\text{slope})} - 1$,[16] were in an acceptable range in favor of the specificity of amplicons obtained in real-time PCR with the selected copies of primers according to standard SYBR Green assay.

This optimized SYBR Green assay was applied to reanalyze samples to quantify 3460G→A in mtDNA in the individuals of the pedigree. The mean Ct values of amplification by total primers were used as normalizer, thus giving ΔCt, whereas the ΔCt of mutant and wild-type

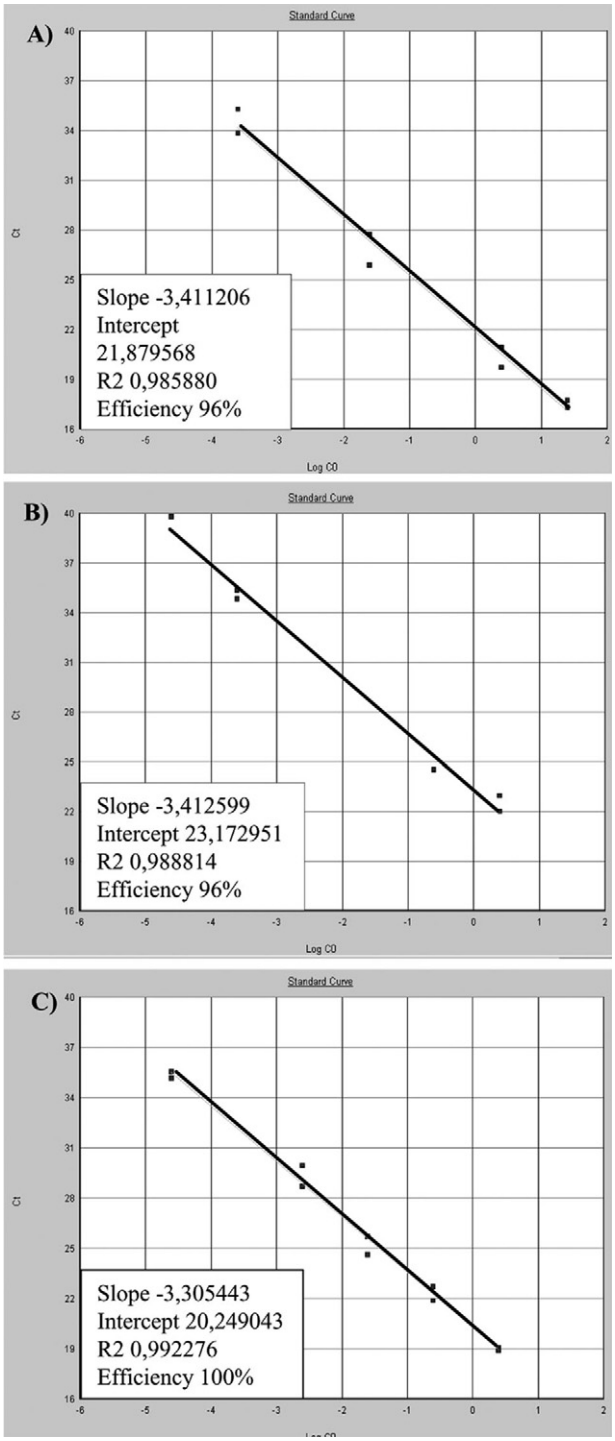

Figure 2. Calibration curves of mtDNA amplification obtained by serial dilutions of plasmids using TaqMan MGB probe. **A:** Calibration curve of mutant mtDNA; **B:** calibration curve of wild-type mtDNA; and **C:** calibration curve of total mtDNA. Slopes of each calibration curve with the corresponding calculated efficiency and correlation coefficients (R^2) are reported for each set of primers.

plasmids (at a concentration of 2.6×10^{14} copies/ml) was used as a calibrator ($\Delta\Delta$Ct).[17] Results are reported in Table 2. The correlation coefficient (R^2) for RFLP versus real-time PCR proportion of mutant mtDNA was 0.942 ($P < 0.0001$).

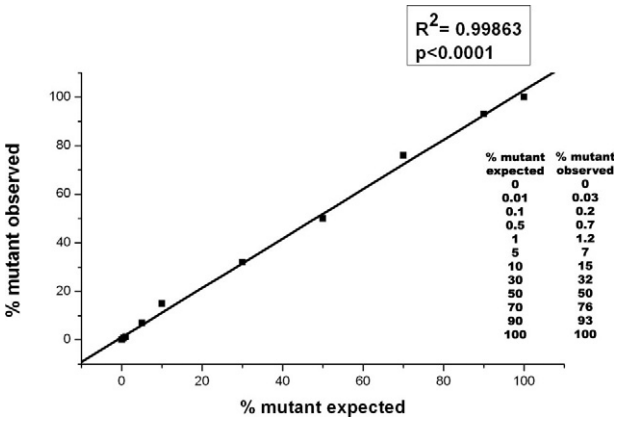

Figure 3. Correlation of expected and observed percentages of 3460G→A mutant mtDNA by real-time ARMS-PCR analysis, using TaqMan assay, of 12 samples containing various proportions of mutant mtDNA. Equation for the line: $y = 1.02x + 1.06\%$.

Quantification of Heteroplasmy Using TaqMan Probe

After the SYBR Green approach, we tested primer amplification protocols and designed a labeled probe to amplify samples by TaqMan assay. The probe was labeled with 6-carboxyfluorescein at the 5′ end with a melting temperature of approximately 71°C, 10°C higher than the primer melting temperature, and it was designed as an MGB probe, which binds to the minor groove of the double-stranded DNA. Calibration curves showed good correlation between the cycle number and mtDNA copy number with correlation coefficients of −3.411, −3.412, and −3.305 for mutant, wild-type, and total copies of primers, respectively, demonstrating a good efficiency (96 to 100%) of amplification reaction using tested primers according to the selected conditions in the SYBR Green assay (Figure 2).

For method validation, we mixed known amounts of the wild-type and mutant mtDNA to obtain 12 samples containing various proportions of mutant mtDNA. Results of TaqMan assay PCR are shown in Figure 3. The correlation coefficient for the observed versus expected proportion of mutant was 0.99863. A proportion of mutant mtDNA as low as 0.01% was detected by this method.

We re-analyzed our samples by real-time TaqMan assay to quantify 3460G→A in mtDNA of LHON patients. The Ct values of amplification by total primers were used as a normalizer (ΔCt), and a starting concentration of 2.6×10^{14} copies/ml of plasmids harboring the 3460 region mtDNA was used as a calibrator ($\Delta\Delta$Ct).[17] Results are reported in Table 2. The correlation coefficient (R^2) for RFLP versus TaqMan assay proportion of mutant mtDNA was 0.949 ($P < 0.0001$).

Discussion

Point mutations can be present in mtDNA either in a homoplasmic (all mtDNA molecules carry the mutation) or heteroplasmic (coexistence of mutant and wild-type mtDNA molecules) fashion. The conventional approach

for the quantitation of heteroplasmic mtDNA mutations involves PCR/RFLP analysis usually performed after the "hot last cycle" to avoid the formation of heteroduplexes affecting quantitation after digestion.[18] This method is time-consuming, including PCR amplification, introduction of radiolabeled deoxynucleotides, digestion with restriction endonuclease, polyacrylamide gel electrophoresis, and quantitation through radioactive signal intensity. Moreover, incomplete digestion with restriction endonuclease could affect the final result.

The most reliable alternative approach to quantify heteroplasmy is represented by real-time PCR. This method has been successfully applied in quantitation of total mtDNA[19,20] and mtDNA deletions.[21] Quantitative analysis of heteroplasmic mtDNA mutation has been performed by several groups using SYBR Green and ARMS amplification,[9] hydrolysis probes,[22] or Molecular Beacons.[23] Furthermore, new quantitative real-time PCR approaches involved the use of specific TaqMan probes to discriminate wild-type and mutant mtDNA molecules.[24,25]

The application of different methods to quantify the mutational load in mtDNA disorders also includes methods based on pyrosequencing and SnapShot technology.[26,27] Heteroplasmic load in families harboring the 3460G→A has also been successfully investigated by using the primer extension technology.[28] Fastness and reliability are the main characteristics requested for new methods, thus implying the use of single-tube tests or reproducible and standardized methods similar to radiolabeled PCR/RFLP.

The ARMS technique to amplify selectively wild-type and mutant molecules has been extensively used, thus contributing to the development of newer applications. The choice of using appropriate primers and selective amplification protocols does not eliminate the inconvenience of nonspecific amplifications, thus limiting the dynamic range of ARMS allele-specific real-time PCR assay because of the late-cycle amplification of the nontarget allele.[29] By using ARMS real-time PCR in the presence of SYBR Green, we met this inconvenience by amplifying in nonspecific mode the mutant plasmid using wild-type-specific primers. Even if our nonspecific amplification occurred and shifted 12 cycles after the specific amplifications, we did not observe the same nonspecific amplification in the presence of TaqMan probe, by using the same set of primers in the presence of the same target molecules. We designed primers for the TaqMan assay, considering that additional mismatched nucleotides before the 3′ end of the primer have an inhibitory effect on PCR efficiency and act to delay the generation of both specific and nonspecific PCR products; this can be useful in enabling a PCR to be stopped at a certain time point (ie, 30 cycles), where specific PCR products have been generated but nonspecific PCR has not yet occurred.[30] This can be easily achieved in setting up a quantitative real-time PCR method by using SYBR Green. We did not design such primers, and nonspecific amplification has been observed in our SYBR Green assay, in which nonspecific molecules have been amplified by wild-type-specific primers after 30 cycles (Ct = 30), whereas the specific product had a Ct of 18.

In the comparison of quantitative real-time PCR methods with the conventional radioactive PCR/RFLP, it is remarkable that all of the homoplasmic samples assayed with the latter method did not result in homoplasmy when quantified by means of SYBR Green (Table 2). One possible explanation can be found in the RFLP method itself. When choosing a restriction endonuclease to digest selectively wild-type or mutant molecules, it should be noted that the efficiency of the cut is ensured to be approximately 100%, and different conditions can also affect digestion, thus generating incomplete digested products and consequent overestimation of the uncut molecule.

In our PCR/RFLP assay, we digested the wild-type molecules, and incomplete digestion overestimated mutant molecules. This can be a plausible explanation, besides the fact that even the proband resulted in heteroplasmy. A more exhaustive explanation exists in the fact that by using the SYBR Green technique associated with ARMS amplification, the nonspecific amplification of mutant molecules with wild-type primers resulted in an underestimation of "true" wild-type molecules. In fact, we could have reported as wild-type molecules, in a complex mix like our samples, a mixture of specific wild-type amplified molecules plus nonspecific mutant molecules, overestimating the population of total wild-type molecules amplified using wild-type primers. We did not encounter the same inconvenience by using the same primers and technique in the presence of TaqMan probe, probably because of the specificity of the reaction that further delayed the appearance of the nonspecific signal.

Quantitative real-time PCR approaches for heteroplasmy of different mtDNA point mutations have been previously reported. Bai and Wong[9] used ARMS real-time PCR in the presence of SYBR Green to quantify heteroplasmy of mtDNA 3243A→G mutation, showing good correlation between the real-time PCR quantitation and PCR/RFLP analysis. Nomiyama et al[22] quantified low amounts of the same 3243A→G mutation in peripheral blood of diabetic patients by using ARMS real-time PCR in the presence of TaqMan probe. They set up the calibration of the method to quantify low amounts of the mutation (less than 1%). Singh and colleagues[24] applied a quantitative real-time PCR method to quantify the amount of 3243A→G mutation discriminating wild-type and mutant molecules by the means of specific TaqMan probes, comparing the result obtained with the pyrosequencing method and fluorescent PCR/RFLP. The three methods showed detection limits for mutant molecules less than 1%, thus showing high sensitivity.

We decided to develop such a quantitative method to evaluate heteroplasmy for one of the primary mutations responsible for LHON. We focused our efforts to set up a reproducible method for the follow up of mutational loads in LHON pedigrees, thus allowing us to follow up LHON carriers along time. The method we set up showed good reproducibility and high sensitivity in comparison with the conventional radioactive PCR/RFLP method, allowing the

detection of a broad range of mutational load (from 0.01 to 100%), on the basis of *in vitro* calibration (Figure 3).

The first approach was to design specific primers for specific amplification of the target sequence, followed by SYBR Green binding and reporting of the PCR products. A successful result in this assay was a good correlation between RFLP analysis and SYBR Green assay, even if heteroplasmic loads detected using SYBR Green were lower than the ones obtained by PCR/RFLP for the aforementioned reasons.

The same primers used for SYBR Green experiment were used in the presence of the TaqMan probe, showing a good correlation between percentage of TaqMan assay and RFLP analysis. All homoplasmic samples resulting from PCR/RFLP analysis were confirmed by real-time TaqMan assay, except for sample V-3, which was almost completely homoplasmic (99.8%).

With the very same PCR conditions and primers used, we showed that in both approaches used in this experiment, the use of specific primers is essential for the specificity of the reaction: only one mismatch at the 3′ end is sufficient for quantitation in the presence of TaqMan probe, as demonstrated also by the work of Nomiyama and colleagues.[22] The need of additional mismatched nucleotides is essential for the allele-specific reaction in the presence of SYBR Green, rendering more feasible the modification of PCR condition for the maintenance of primer specificity, as demonstrated by Bai and Wong.[9] In conclusion, both quantitative real-time PCR techniques are fast and reliable, but the choice of the allele-specific primers is essential for the reliable quantitation in both methods.

We believe that the use of real-time quantitative ARMS-PCR in the presence of TaqMan probe is a fast, precise, and reliable method for the follow-up of the mutational load along time of multiple tissues from different individuals of LHON pedigrees, thus allowing a continuous monitoring of the genotype-phenotype correlation within the same individuals belonging to a specific pedigree.

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