Mutation analyses in Korean patients with MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes)

Han-Wook Yoo, Gu-Hwan Kim and Tae-Sung Ko

The mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) is inherited maternally, in which the MTTL1*MELAS 3243 mutation has been most commonly found as a heteroplasmy of A to G point mutation in the $tRNA^{Leu(UUR)}$ gene. The MTTL1*MELAS 3271 mutation is known to be the second common mutation, though clinical features of both mutations are not remarkably different. Recently, a variety of minor mutations have been reported in patients with MELAS. In this study, major efforts have been made to investigate the allele frequency of major three mutations including MTTL1*MELAS 3243, 3252, 3271 in 10 Korean families with MELAS probands. The PCR and subsequent direct sequencing of the PCR product in the regions spanning these three mutation sites were employed to identify the mutation in each proband. All family members have been screened for the presence of these three mutations by PCR-RFLP assay using *Apa* I, *Acc* I and *Bfr* I restriction enzymes. The MTTL1*MELAS 3243 mutation was most commonly found (7 out of 10 families tested) followed by the MTTL1*MELAS 3271 which was identified in 1 out of 10 families. In the remaining 2 families, none of three mutations were found, indicating the presence of a either nuclear mutation or yet unidentified mitochondrial DNA mutation in these families.

Keywords: MELAS, Mutation

INTRODUCTION

Once the human mitochondrial genome had been characterized and sequenced in 1981 (Anderson *et al.*, 1981), it became a clear candidate for causing diseases with maternal inheritance pattern. Mitochondrial disorders, inherited maternally, result in defects predominantly in muscle and/or the central nervous system with various clinical features. Mitochondrial encephalomyopathies show some common clinical features such as dementia, short stature, sensory neural hearing loss and lactic acidosis (Di Mauro *et al.*, 1985). These heterogeneous diseases are categorized into various sub-groups depending on their specific clinical features. The clinical characteristics of MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) are episodic vomiting, seizures, and recurrent cerebral insults resembling strokes and causing hemiparesis or cortical blindness (Montagna *et al.*, 1988). In patients with MELAS, a preferential vascular involvement is believed to be the main characteristic clinical features of stroke-like episode (Hasegawa *et al.*, 1991). A vast majority of people with the clinical features of MELAS have heteroplasmic A to G point mutation in the dihydrouridine loop of the $tRNA^{Leu(UUR)}$ gene at nt. 3243(MTTL1*MELAS 3243) (Goto *et al.*, 1990) and a small portion of individuals with MELAS are known to be carrying a heteroplasmic T to C point mutation at nt. 3271 in the terminal nucleotide pair of the anticodon stem of the $tRNA^{Leu(UUR)}$ gene(MTTL1*MELAS 3271) (Goto *et al.*, 1991). A third mutation was identified infrequently in the $tRNA^{Leu(UUR)}$ gene at nt. 3252 of mitochondrial DNA (mt.DNA) as a heteroplasmic G to A transition (MTTL1*MELAS 3252) in patients with MELAS (Morten *et al.*, 1993).

Identification of the molecular lesions in mitochondrial diseases is important in clarifying the mode of inheritance. In addition, heteroplasmy adds a degree of complexity to the transmission of mitochondrial DNA mutations within families because of the mitochondrial bottle neck.

In this study, efforts have been made to determine the frequency of each of these mutations in Korean patients with MELAS who were diagnosed on the basis of the clinical diagnostic criteria set by Ciafaloni *et al.*(Ciafaloni *et al.*, 1992).

Department of Pediatrics, Asan Medical Center, Ulsan University College of Medicine, Asan Research Institute for Life Sciences, Seoul, Korea
Correspondence : Han-Wook Yoo, Department of Pediatrics, Asan Medical Center, Ulsan University College of Medicine, #388-1 Poongnap-Dong, Songpa-Gu, Seoul 138-040, Korea
Tel: 82-2-224-3374, Fax: 82-2-473-3725, E-mail: hwyoo@www.amc.seoul.kr

MATERIALS AND METHODS

Clinical diagnostic criteria

The clinical diagnosis of MELAS has been made on the basis of (1) stroke-like episodes (with CT or MRI evidence of focal brain abnormalities), (2) lactic acidosis, ragged red fiber, or both; and (3) at least two of the following local or generalized seizures, dementia, recurrent headache and vomiting (Ciafaloni *et al.*, 1992).

Patients

The patients included in this study were from 10 unrelated Korean families. The mitochondrial DNA analysis was undertaken in 27 subjects including 10 probands and 17 related family members.

Total genomic DNA isolation

The total genomic DNA was isolated from peripheral leukocytes from the patients and their appropriate family members. After treatment with 0.5% sodium dodecyl sulphate and proteinase K, genomic DNA was extracted using phenol and chloroform and precipitated with cold ethanol according to standard procedures (Aldridge *et al.*, 1984).

Construction of Primers

Unique oligonucleotides for the PCR and sequencing the human mitochondrial genome were synthesized. They were designed to cover the well known mutation sites for MELAS, nt. 3243, 3252, 3271: the nucleotide sequence of sense primer for nt. 3243, 3252, 3271, located on mitochondrial genome nucleotides 3153-3172, was 5'-TTC ACA AAG CGC CTT CCC CC-3', the antisense primer for nt. 3243, located on mitochondrial genome nucleotides 3551-3531, was 5'-GCG ATG GTG AGA GCT AAG GTC-3': the antisense primer for nt. 3252, located on mitochondrial genome nucleotides 3274-3253, was 5'-TAA AGT TTT AAG TTT TAG TCG A-3': the antisense primer for nt. 3271, located on nt. 3301-3272, was 5'-TAA GAA GAG GAA TTG AAC CTC TGA CCT TAA-3'(Anderson *et al.*, 1981).

Amplification of total genomic DNA obtained from lymphocytes

Using each set of primers, PCR was carried out. After an initial denaturation of template DNA, amplification was performed for 30 cycles and consisted of denaturation at 94°C for 1 min, annealing for 45 sec at 54°C (MTTL1*MELAS 3243), 56°C (MTTL1*MELAS 3252), 54°C (MTTL1*MELAS 3271), and extension at 72°C for 1 min. For each reaction, the 100 μL amplification mixture contained 100 ng of total genomic DNA, 1 μmol/L each of sense and antisense primers, 50 mmol/L KCL, 10 mmol/L Tris HC1, pH 8.8, 1.5 mmol/L $MgC1_2$, 0.l% triton X-100, 50 μmol/L dNTPs, and 2.5 units of *Taq* DNA polymerase(Promega Biotec. Madison, WI, USA). The reaction was run in a thermocycler (Perkin-Elmer Cetus, Norwalk, CT, USA).

Direct sequencing of double stranded PCR product with/without purification

PCR product (10 μL of each) obtained from each proband was electrophoresed on 1.5% agarose gel to examine whether the targeted template was specifically amplified. When a single specific product was amplified, double strtanded DNA sequencing was performed directly without purification. Otherwise, the fragments of the expected sizes were excised from the gel and further purification was accomplished using the Jetsorb kit (Genomed, Germany). The original amount of template for individual sequencing reaction ranged from 5 to 10 μL of each PCR product. Double stranded DNA sequencing was performed in both direction using sense and antisense primers, and Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH, USA) according to manufacturer's instructions with the following modifications. Sequenase DNA polymerase and inorganic pyrophosphatase were mixed together (1 volume of each) and diluted 6 volumes of glycerol enzyme dilution buffer (20 mmol/L Tris · HCl, pH 7.5, 2 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 50% glycerol). The labelling reaction using [α-^{35}S] dATP was extended to 10 min at room temperature. The reaction mixture was electrophoresed on 8% denaturing polyacrylamide gel in 1 × glycerol tolerant gel buffer (20 × buffer is 216 g Tris base, 72 g taurine, 4 g $Na_2EDTA \cdot 2H_20$ in 1 L H20) (Sanger *et al.*, 1977).

Restriction enzyme analysis in familiy members

Once the mutation was identified in the proband of individual family, amplified PCR products from appropriate family members were digested with *Apa* I enzyme at 37°C for the mutation MTTL1*MELAS 3243, with *Acc* I at 37°C for the mutation MTTL1*MELAS 3252, and with *Bfr* I enzyme at 37°C for the mutation MTTL1*MELAS 3271.

RESULTS

Identification of the MTTL1*MELAS 3243 mutation

This mutation was most commonly found in MELAS patients. It was identified in 7 probands in 7 out of 10 unrelated families (70%) with clinically diagnosed MELAS by direct sequencing the mutated region of mitochondrial DNA. They had a heteroplasmic A to G point mutation at nt. 3243 in the $tRNA^{Leu(UUR)}$ gene (Fig. 1).

Restriction enzyme analysis with *Apa* I in the family members of each proband

The PCR-RFLP assay was performed in 13 related subjects from 7 families with proband carrying the MTTL1*MELAS 3243 mutation. Among the 13 subjects tested, 11 subjects have been confirmed to be a heteroplasmic carrier of the MTTL1*MELAS 3243 mutation since the 399 bp PCR product has been partially cleaved by *Apa* I restriction enzyme, generating 305 and 94 bp fragments (Fig. 2).

Identification of the MTTL1*MELAS 3271 mutation

This mutation was identified in one proband out of 10 probands (10%) by direct sequencing the mutated region. The proband had a homoplasmic T to C point mutation at nt 3271 in the terminal nucleotide pair of the anticodon stem of the $tRNA^{Leu(UUR)}$ gene (Fig. 3).

Restriciton enzyme analysis with *Bfr* I in the family members of each proband

The PCR-RFLP assay was performed in 2 related subjects (mother, a sibling) of the proband. Both of them turned out to carry a heteroplasmic MTTL1*MELAS 3271 mutation since the 148 bp PCR product has been partially digested by *Bfr* I enzyme into 120 and 28 fragments (Fig. 4).

Identification of the MTTLI*MELAS 3252 mutation

This mutation has been screened by PCR-RFLP assay using *Acc* I digestion or direct sequencing of the mutated region. None of probands tested showed this kind of mutation.

Frequency of each mutation in 10 unrelated families with MELAS

Among 10 families with a MELAS proband, who have been screened for the MTTL1*MELAS 3243, 3252, 3271 mutations, 7 probands were carrying the MTTL1*MELAS 3243, one proband carrying the MTTL1*MELAS 3271, and none having the MTTL1*MELAS 3252 mutation.

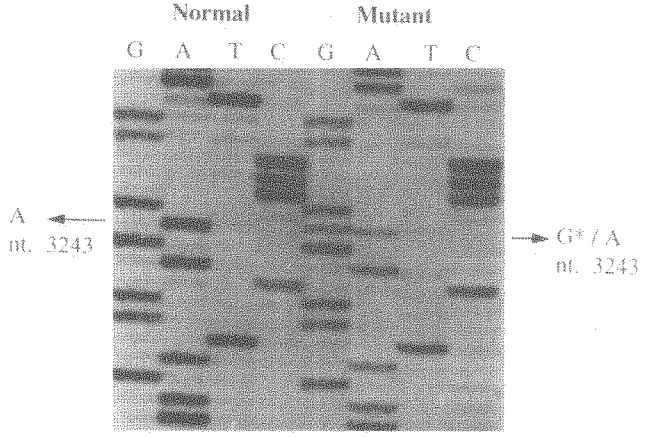

Fig. 1. Identification of the MTTL1*MELAS3243: a heteroplasmic A to G point mutation at nt 3243 in the $tRNA^{Leu(UUR)}$ gene.

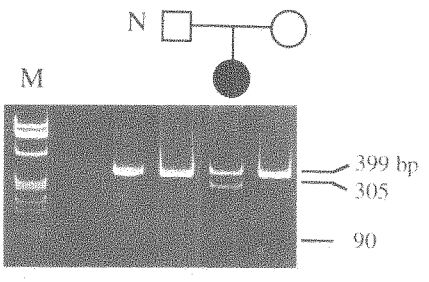

Fig. 2. PCR-RFLP analysis with *Apa* I enzyme reveals partial cleavage of the 399 bp PCR product, indicating that the proband is heteroplasmic for the MTTL1*MELAS 3243 mutation. M: Φχ 174 *Hae* III-digested DNA marker.

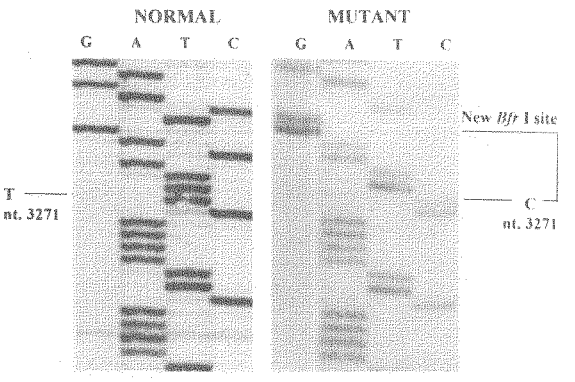

Fig. 3. Identification of the MTTL1*MELAS3271: a homoplasmic T to C point mutation at nt 3271 in the $tRNA^{Leu(UUR)}$ gene.

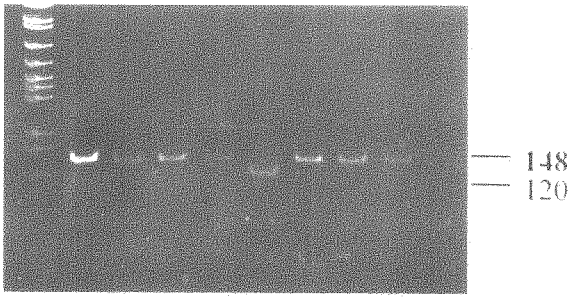

Fig. 4. PCR-RFLP analysis with *Bfr* I enzyme reveals complete digestion of the 148 bp PCR product, indicationg that the proband is homoplasmic for the MTTL1*MELAS3271 mutation. M: Φχ 174 *Hae* III-digested DNA marker.

DISCUSSION

In this study, we found that MTTL1*MELAS 3243 was the most frequent mutation in Korean patients with MELAS as a heteroplasmy state, although we did not quantitate the ratio of heteroplasmy for the mutated mitochondria. Approximately 80% of individuals with the clinical characteristics of MELAS are known to carry a heteroplasmic A to G point mutation in the dihydrouridine loop of the $tRNA^{Leu(UUR)}$ gene at bp 3243 (MTTL1*MELAS 3243) in other studies (Goto *et al.*, 1992; Moraes *et al.*, 1992). The MTTL1*MELAS 3243 mutation can be associated with a wide variety of clinical presentations. Patients carrying this mutation may have different combinations of encephalomyopathy, blood vessel involvement, opthalmoplegia, dementia, sensory neural hearing loss, cardiac conduction block, hypertrophic cardiac myopathy, renal tubular dysfunction and endocrinologic abnormalities including diabetes mellitus (Ciafaloni *et al.*, 1992; Van den Ouweland *et al.*, 1992). This mutation not only alters the dihydrouridine loop of the $tRNA^{Leu(UUR)}$ gene but also changes a conserved nucleotide at the binding site for a nuclear DNA encoded transcription termination factor (Hess *et al.*, 1991). The transcription termination factor truncates H-strand transcription after the 16S rRNA gene has been traversed, thus maintaining the high ratio of rRNA:mRNA ratio in mitochondria. This reduces the efficacy of transcription termination so that it can interfere with processing of 16S rRNA and the adjacent $tRNA^{Leu(UUR)}$ or increase the production of the termination factor that can bind to other site in the D-loop and impair L-strand transcription (Hess *et al.*, 1991; Kaufmann *et al.*, 1996). Alternatively, the MTTL1*MELAS 3243 mutation may directly impair protein synthesis by interfering with polypeptide chain elongation and has no effect on termination factor function (Chomyn *et al.*, 1992). In rapidly dividing cells such as in hematopoietic lineages, this mutation may segregate to extremely low levels, making DNA diagnosis from peripheral blood difficult. Heteroplasmy (the presence of both normal and mutant mt.DNA in a single individual) is present in the vast majority of mitochondrial disease, so that the proportion of mutant mt.DNA varies from 0 to 100%. The level of mutant mt.DNA also varies in different tissues and changes with time (Poulton *et al.*, 1993; Huang *et al.*, 1996; Marchington *et al.*, 1997). This might be the reason why the relatively low frequency of this mutation was found in this study. Not only the molecular defect when using the skeletal muscle but also the ratio of heteroplasmy of mutant mt. DNA needs to be further investigated.

An additional 7.5-10% of MELAS patients have been known to carry the MTTL1*MELAS 3271 in the terminal nucleotide pair of the anticodon stem of $tRNA^{Leu(UUR)}$ gene, which is located out of the binding region of the transcription termination factor (Goto *et al.*, 1991; Tokunaga *et al.*, 1993). These patients are known to have same clinical, biological features and pathologic findings as in those with the MTTL1* MELAS 3243 mutation (Goto *et al.*, 1992). In our study, the MTTL1*MELAS 3271 mutation was found only in one family with MELAS out of 10 families (10%).

The third mutation has been rarely reported in MELAS patients. This mutation is a heteroplasmic G to A transition at nt. 3252 of the $tRNA^{Leu(UUR)}$ gene, which is located in dihydrouridine loop (Morten *et al.*, 1993). In our study, we were unable to identify this mutation by nucleotide sequencing and restriction analysis. Among 10 families diagnosed clinically on the basis of aforementioned criteria, we identified mutation in mitochondrial DNA of 8 families. The remaining cases appear to be caused by either nuclear DNA mutation or other as yet unidentified mt.DNA mutation. Recently, a variety of rare mutations have been identified in patients with MELAS (Campos *et al.*, 1995; Manfredi *et al.*, 1995; Li *et al.*, 1996; Taylor *et al.*, 1996; Shaag *et al.*, 1997). In addition, it has to be pointed out that it is very hard to predict the natural course of asymptomatic heteroplasmic carriers of the mutation in related family members, though the recurrence rate is presumed to be even higher than in autosomal dominantly inherited disorders. It is prerequisite to examine the heteroplasmy ratio of the mutated mitochondrial DNA in specific target tissues in order to correlate it with clinical features.

ACKNOWLEDGEMENT

This research project has been partly supported by the Grant from the Asan Research Institute for Life Sciences.

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