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A maternally inherited diabetes and deafness patient with the 12S rRNA m.1555A>G and the ND1 m.3308T>C mutations associated with multiple mitochondrial deletions

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ABSTRACT

Maternally inherited diabetes and deafness (MIDD) is a mitochondrial syndrome characterized by the onset of sensorineural hearing loss and diabetes in adults. Some patients may have other additional clinical features common in mitochondrial disorders such as pigmentary retinopathy, ptosis, cardiomyopathy, myopathy and renal affections. We report a 40-year-old Tunisian patient presenting maternally inherited type 2 diabetes and deafness (MIDD). A molecular genetic analysis was conducted in the patient and his twin sister, but no reported mutations in the $tRNA^{Leu(UUR)}$ and $tRNA^{Glu}$ genes were found, especially the two mitochondrial m.3243A>G and the m.14709T>C mutations in muscle and blood leukocytes. The results showed the presence of the mitochondrial NADH deshydrogenase 1 (ND1) homoplasmic m.3308T>C mutation the 2 tested tissues (blood leukocytes and skeletal muscle) of the proband and in the patient's sister blood leukocytes. In addition, we identified the mitochondrial 12S rRNA m.1555A>G mutation in muscle and blood leukocytes. The Long-range PCR amplification revealed the presence of multiple deletions of the mitochondrial DNA extracted from the patient's skeletal muscle removing several tRNA and protein-coding genes. Our study reported a Tunisian patient with clinical features of MIDD in whom we detected the 12S rRNA m.1555A>G and the ND1 m.3308T>C mutations with mitochondrial multiple deletions.

1. Introduction

Mitochondrial diseases include a group of disorders including diabetes mellitus, hearing impairment, renal failure and cardiovascular diseases caused by different mutations in the mtDNA. Maternally inherited diabetes and neurosensory deafness, also designated as MIDD, is a mitochondrial disease which is often associated to a mutation in the $tRNA^{Leu(UUR)}$ gene of the mtDNA in position 3243 and which is accounts for 0.5–2.8% of diabetic patients [1–3].

The second common mitochondrial mutation associated with MIDD was the m.14709T>C mutation in the $tRNA^{Glu}$ reported in several studies [4–6]. Some other mitochondrial disorders are reported in MIDD such as myopathies, and macular dystrophy [7,8].

Mitochondrial mutations were also reported in both syndromic and nonsyndromic forms of mitochondrial sensorineural hearing loss [9] especially in the 12S rRNA and in the tRNA genes. The m.1555A>G mutation in the 12S rRNA is one of the most mutation associated with aminoglycoside-induced and nonsyndromic deafness in several families from different ethnic backgrounds [10,11].

Besides, mtDNA deletions were found in several mitochondrial disorders such as maternally transmitted diabetes [12], MIDD [13], wolfram syndrome [14], endocrinopathies of early onset and deafness [15].

In this study, we report a Tunisian patient with a clinical maternally inherited diabetes and deafness (MIDD). The mitochondrial DNA genetic study in the proband revealed the presence of m.1555A>G mutation in two tested tissues (blood leucocytes and skeletal muscle) as well as multiple mitochondrial deletions in the DNA extracted from the skeletal muscle.

2. Materials and methods

2.1. Patient

The studied patient is a 40-year-old man suffering from maternally inherited type 2 diabetes and deafness (MIDD). His twin sister is affected with diabetes, dumbness and deafness.

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2.2. Controls

In addition, 200 Tunisian healthy individuals from the same ethnocultural group were tested as controls. These controls should have no personal or family history of diabetes or deafness or any other disorder. All individuals (patient and controls) provided informed consent.

2.3. Methods

2.3.1. DNA extraction from blood leucocytes and muscle biopsy

After getting informed consent from the patient, total DNA was extracted from peripheral blood using standard procedures [16]. In addition, DNA was extracted from skeletal muscle according to the protocol established by FastPure™ DNA Kit Cat. # 9191 (TAKARA).

2.3.2. Screening of the m.3243A>G mutation in the $tRNA^{Leu(UUR)}$ gene in blood leucocytes and in muscle biopsy

The mitochondrial m.3243A>G mutation was analyzed by PCR-RFLP. The PCR amplification and the PCR-RFLP analysis with the *ApaI* restriction enzyme (BioLabs) were performed as described previously [17].

2.3.3. Mutational screening of the mitochondrial $tRNA^{Glu}$ and cytochrome b gene in blood leucocytes and in muscle biopsy

PCR amplification of 382 bp of the mitochondrial $tRNA^{Glu}$ and cytochrome b genes was performed using the following primers: 5′-ACCACACCGCTAACAATCA-3′ and 5′-TTGATGAAAAGGCGGTTGA-3′ in a total volume of 50 μL containing 150 ng of genomic DNA, 8 pmol of each primer, 200 μM of each dNTP, 30 mM KCl, and 2 U of Taq DNA polymerase (Qiagen). The conditions for the PCR reaction were as follows: 94 °C for 3 min followed by 30 cycles (94 °C for 30 s; 62 °C for 30 s, and 72 °C for 30 s) and a final extension at 72 °C for 5 min in a thermal cycler (GenAmp PCR System 9700; Applied Biosystem).

2.3.4. Screening of the mitochondrial m.1555A>G mutation in the mitochondrial 12S rRNA gene in blood leucocytes and in muscle biopsy

The mitochondrial 12S rRNA gene was amplified using a couple of primers corresponding to the mitochondrial genome at positions 1133–1152 and 1752–1705. Screening of the m.1555A>G mutation was carried out using PCR-RFLP. PCR amplification of a 339 bp DNA fragment of the 12S rRNA gene containing the mutation was performed using primers as previously reported [18]. After PCR amplification, PCR products were digested the restriction endonuclease *HaeIII* (fermentas). In the wild allele, digestion resulted in two fragments of 218 bp and 121 bp. The m.1555A>G mutation created a novel restriction *HaeIII* site and the digestion resulted in three fragments of 218, 91 and 30 bp.

2.3.5. Long-range PCR amplification in blood leucocytes and in muscle biopsy

Long-range PCR was performed using Long PCR Enzyme Mix (# K0182) (Fermentas) using different combinations of primers (Table 1). The PCR amplification was performed using the Long PCR Enzyme Mix. The conditions for the PCR reaction were as follows: initial denaturation at 93 °C for 3 min, followed by 10 cycles: 30 s at 93 °C, 30 s at 58,5 °C and 12 min at 68 °C and then 25 cycles at 93 °C for 30 s, 58,5 °C for 30 s, 68 °C for 12 min and 10 s, and a final extension at 68 °C for 11 min. PCR products were separated on a 0.8% agarose gel.

2.3.6. Direct sequencing

Direct sequencing of PCR products was performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM/PE Biosystems) and the products were resolved on ABI PRISM 3100-Avant.

Table 1
Primers used for the Long-range PCR amplifications of mtDNA.

Size	Primers	Sequences	Nucleotidic positions
10162 bp	M9F	5′GAGGCCTAACCCCTGTCTTT 3′	5835–5854
	M22R	5′ AGCTTTGGGTGCTAATGGTG 3′	15997–15978
8089 bp	M12F	5′ ACGAGTACACCGACTACGGC 3′	7908–7927
	M22R	5′AGCTTTGGGTGCTAATGGTG 3′	15997–15978

2.3.7. The sequence alignment and the pathogenicity prediction

The sequence alignment of the mitochondrial NADH dehydrogenase 1 was performed using the ClustalW program (http://align.-genome.jp/sit-bin/clustalw). The assessment of the possible impact of an amino acid substitution on the three-dimensional protein structure and the possible effect of the mtDNA change on protein function was performed using PolyPhen-2 program (prediction of functional effects of human nsSNPs) (http://genetics.bwh.harvard.edu/pph2/). PolyPhen structurally analyzes an amino acid polymorphism and predicts whether an amino acid change is likely to be deleterious to protein function [19,20].

3. Results

We started the molecular study by a mutational screening of the mitochondrial genes associated with maternally inherited diabetes. In fact, we screened the $tRNA^{Leu(UUR)}$ and the $tRNA^{Glu}$ genes in the studied patient and his twin sister. The results revealed the absence of reported mutations especially the two mitochondrial m.3243A>G and m.14709T>C mutations. However, the direct sequencing of the fragment containing the entire $tRNA^{Leu(UUR)}$ gene and parts of its flanking MT-ND1 showed the presence of the m.3308T>C mutation in homoplasmic form in the DNA extracted from blood leukocytes and skeletal muscle of the proband and his sister's blood leukocytes (Fig. 2A). In addition, this m.3308T>C variation was absent in the 200 healthy Tunisian individuals.

The ND1 m.3308T>C mutation substitutes the first amino acid, translation-initiating methionine with a threonine at position 1 (M1T) which is a highly evolutionary conserved amino acid residue of the mtDNA encoded ND1 peptide (Fig. 2B). In fact, PolyPhen analysis showed that this mutation was predicted to be probably damaging with a score of 0.992 (Fig. 2C).

In addition, PCR-RFLP analysis of the fragment of the mitochondrial 12S rRNA gene in the DNA the extracted blood leukocytes and skeletal muscle of the studied patient showed the presence of the homoplasmic m.1555A>G mutation (Fig. 1A). This mutation was also present in blood leukocytes of his sister but it was absent in the 200 healthy Tunisian individuals. The presence of this mutation was confirmed by direct sequencing (Fig. 1B).

Besides, the direct sequencing analysis of the mitochondrial $tRNA^{Glu}$ and the cytochrome b flanking gene, showed the presence of the homoplasmic m.14766C>T (T7I) and the m.14769A>G (N8S) variations in the cytochrome b gene in the patient's blood leucocytes and muscle.

Since maternally transmitted diabetes and deafness could be associated to mitochondrial deletions, we performed a Long-range PCR amplification of the two fragments of 10.162 kb and 8.089 kb in the DNA extracted from blood leukocytes and the skeletal muscle of the studied patient. Results showed the expected fragments of 10.162 kb, and 8.089 kb suggesting the absence of mitochondrial deletions in blood leukocytes (Fig. 3). However, the Long-range PCR amplification of the 8.089 kb in the DNA extracted from the

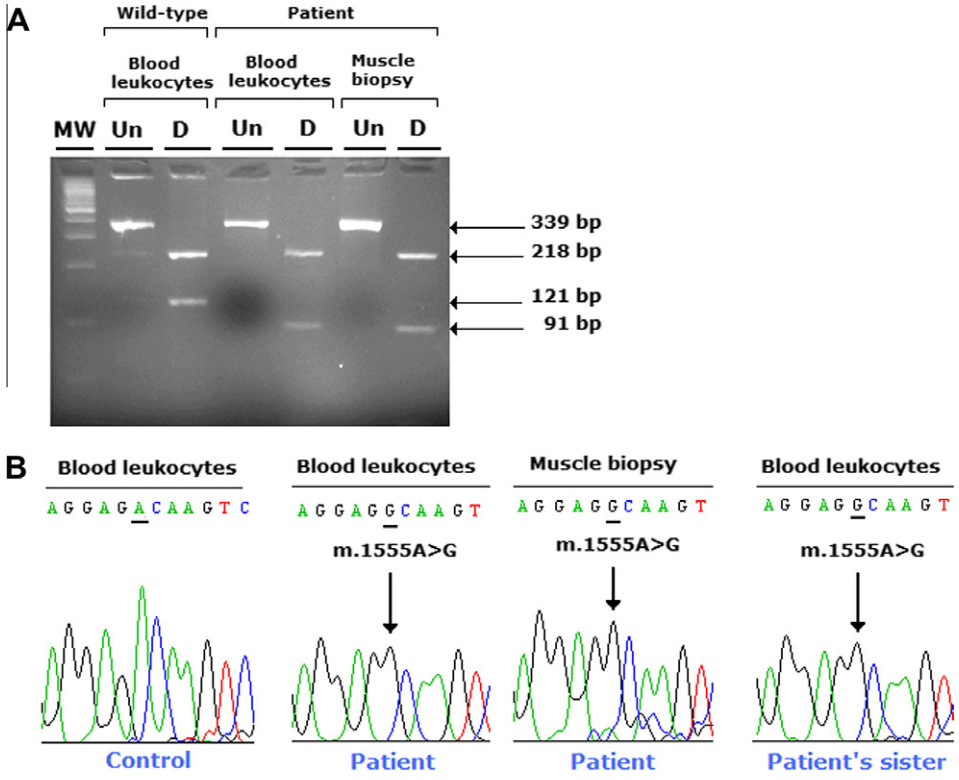

Fig. 1. (A) Results of PCR-RFLP analysis of DNA: A 339 bp PCR fragment is digested with *HaeIII*. The wild-type mtDNA is cleaved into two fragments of 218 and 121 bp in length, whereas PCR product containing the m.1555A>G mutation is cleaved into three fragments, 218, 91, and 30 bp in length. MW: DNA Ladder 100 bp; Un: undigested PCR products; D: digestion with *HaeIII*. (B) Sequence chromatograms from normal individual and affected patients with the m.1555A>G mutation in the mitochondrial 12S rRNA gene in the Blood leukocytes and in Muscle biopsy.

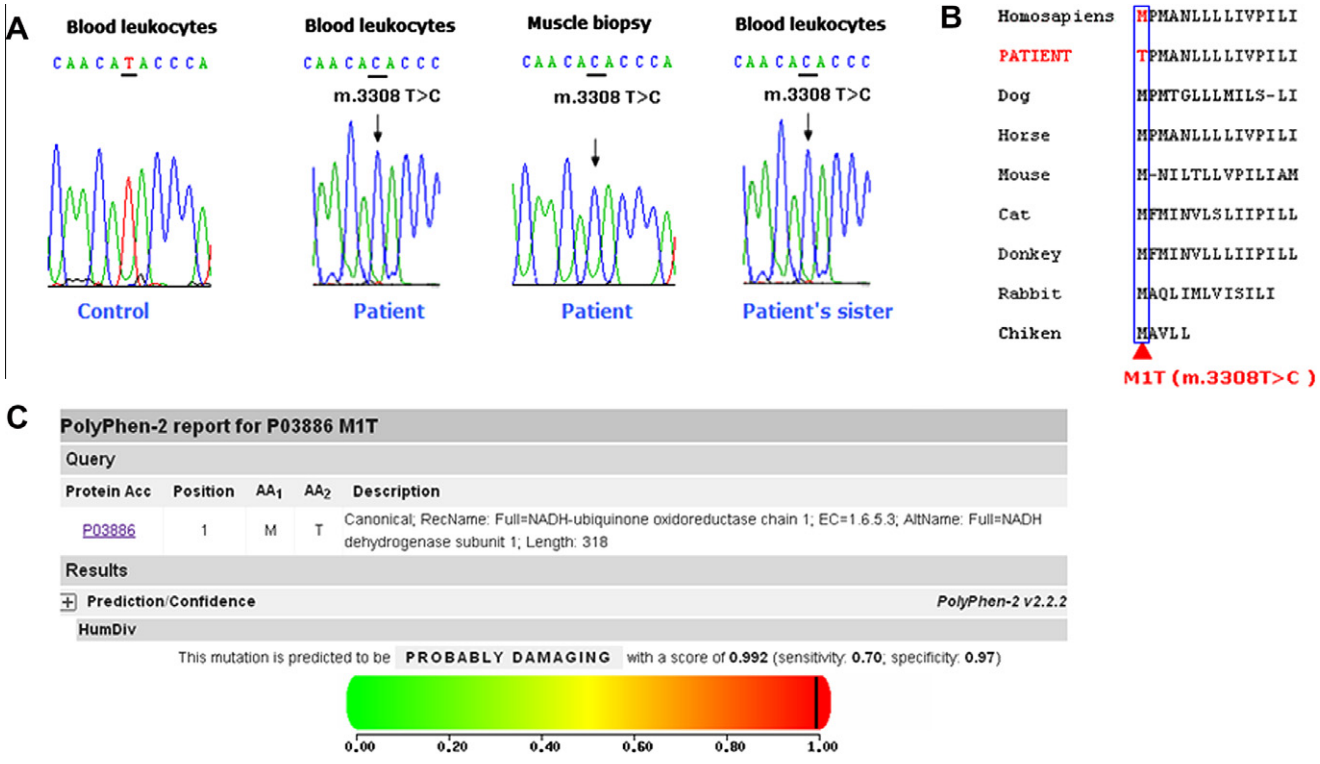

Fig. 2. (A) Sequence chromatograms from normal individual and affected patients with the m.3308T>C (M1T) mutation in the mitochondrial NADH dehydrogenase 1 gene in the Blood leukocytes and in Muscle biopsy. (B) Alignment of the ND1 protein in different species showing the high conservation of the amino acid 1. (C) Results of the PolyPhen analysis predicting the probably damaging impact of the M1T substitution on the ND1 protein with a score of 0.992.

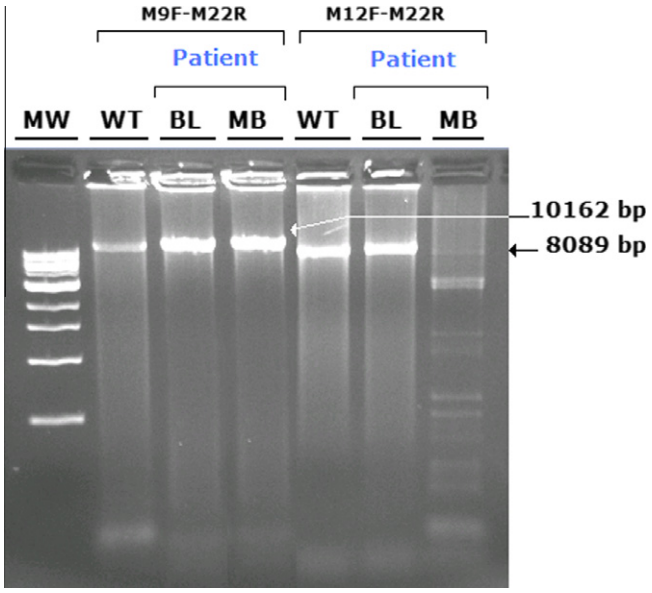

Fig. 3. Long-range PCR amplification in the DNA extracted from the patient's blood Leukocytes and skeletal muscle. PCR was used to amplify 2 different fragments of 10162 bp and 8089 bp and PCR products were separated by electrophoresis in a 0.8% agarose gel with ethidium bromide. MW: 1 kb DNA Ladder 250–10,000 bp (Fermentas SMO318); WT: wild type; Blood Leukocytes (BL) and in Muscle Biopsy (MB).

proband's skeletal muscle showed the presence of several fragments suggesting the presence of heteroplasmic multiple mitochondrial deletions (Fig. 3). These deletions removed several mitochondrial tRNA and protein-coding genes.

4. Discussion

We performed a mitochondrial DNA screening in a 40-year-old patient presenting with MIDD and in his twin sister affected with diabetes, dumbness and deafness. The results revealed the presence of the 12S rRNA m.1555A>G and the ND1 m.3308T>C mutations associated with multiple mitochondrial deletions. The mitochondrial genetic analysis showed that the m.3243A>G was absent in blood leucocytes and skeletal muscle of the proband. This variation was the most common mutation described in diabetic patients from different populations [21–23,5]. It was also reported in patients with diabetes and deafness [24,25]. However, other studies reported its absence in patients with diabetes and deafness [26,27].

In addition, the results showed the absence of the m.14709T>C mutation in the mitochondrial $tRNA^{Glu}$ in the 2 tested tissues of the studied patient. However, this variation was identified in a patient with adult-onset type 2 diabetes and severe myopathy with a hearing impairment [28]. Moreover, the m.14709T>C mutation was reported in several studies in patients with diabetes associated or not with other symptoms such as myopathy [29,5,6].

The PCR-RFLP analysis showed the presence of the homoplasmic m.1555A>G mutation in the mitochondrial 12S rRNA gene in the 2 tested tissues of the studied patient and in blood leukocytes of his twin sister. This mutation was usually reported in patients with nonsyndromic hearing loss [30–32,10] but our results showed its first presence in patient with MIDD. The 12S rRNA m.1555A>G mutation was reported with high prevalence (5.3%) in sensorineural deafness patients in Indonesia but it was absent in patients with deafness and type 2 diabetes from the same population [27]. Some other studies reported the presence of the m.1555A>G mutation in cardiomyopathy [33], Leber's hereditary optic neuropathy [34] and encephalomyopathies [35].

Our results showed also the presence of the homoplasmic m.3308T>C mutation in the NADH dehydrogenase 1 gene in blood leukocytes and skeletal muscle of the studied patient and in his sister's blood leukocytes. The m.3308T>C mutation was absent in 200 healthy controls. The mitochondrial m.3308T>C mutation leads to a methionine with a threonine substitution at position 1 (M1T) which is a highly evolutionary conserved amino acid residue of the mtDNA encoded ND1 peptide. Thus, it affects the ND1 protein, an important mitochondrial enzyme and a component of complex I, which is responsible for electron transfer from NADH to ubiquinone. Besides, it was reported that mutations in ND1 gene were related to mitochondrial diseases such as diabetes and cardiomyopathy [36–38]. The m.3308T>C mutation was described in patients with MELAS [39,40]. The m.3308T>C mutation located in the last three nucleotides adjacent to the 3′ end of the $tRNA^{Leu(UUR)}$ gene, caused a significant decrease both in the amount of ND1 mRNA and co-transcribed $tRNA^{Leu(UUR)}$ in mutant cells [41].

In addition, Long-range PCR amplification in the studied patient with MIDD showed multiple deletions in the mitochondrial DNA extracted from skeletal muscle. In previous studies, mitochondrial DNA deletions were reported in maternally inherited diabetes [12], MIDD [13] and wolfram syndrome [14,42]. It was also reported that a large mitochondrial DNA deletion was detected in the absence of the m.3243A>G mitochondrial DNA mutation in a 17-year-old patient with diabetes, deafness, cataract, and maculopathy [42].

In conclusion, our study reported, the first description of the m.1555A>G mutation in the mitochondrial 12S rRNA mutation in a patient with MIDD in association with ND1 m.3308T>C mutation and multiple mitochondrial deletions in DNA extracted from skeletal muscle.

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