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The first concurrent detection of mitochondrial DNA m.3243A>G mutation, deletion, and depletion in a family with mitochondrial diabetes

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Abstract

Background: Mitochondrial diabetes (MD) is a rare monogenic form of diabetes and divided into type l and type 2. It is characterized by a strong familial clustering of diabetes with the presence of maternal transmission in conjunction with bilateral hearing impairment in most of the carriers. The most common form of MD is associated with the m.3243A>G mutation in the mitochondrial *MT-TL1*, but there are also association with a range of other point mutations, deletion, and depletion in mtDNA.
Methods: The mitochondrial genome anomalies were investigated in a family with clinical features of MD, which includes a proband presenting severe MD conditions including cardiomyopathy, retinopathy, and psychomotor retardation.
Results: By investigating the patient's blood leukocytes and skeletal muscle, we identified the m.3243A>G mutation in heteroplasmic state. This mutation was absent in the rest of the family members. In addition, our analysis revealed in the proband a large mtDNA heteroplasmic deletion (~1 kb) and a reduction in mtDNA copy number.
Conclusion: Our study points out, for the first time, a severe phenotypic expression of the m.3243A>G point mutation in association with mtDNA deletion and depletion in MD.

KEYWORDS
m.3243A>G mutation, mitochondrial diabetes, mitochondrial DNA, mtDNA deletion, mtDNA depletion

1 | INTRODUCTION

Mitochondria are present in most eukaryotic cells and deeply involved in various important cell functions, especially energy production in the form of ATP (Adenosine triphosphate) (Wescott, Kao, Lederer, & Boyman, 2019). Mitochondria carry their own circular DNA, which exists in multiple copies per cell and its inheritance is only maternal (Stewart & Larsson, 2014). Several mutations in mitochondrial DNA (mtDNA) have been indicated as cause of human mitochondrial diseases associated with the defects in oxidative production of energy (Sharma & Sampath, 2019).

Previous reports demonstrated that defects in mtDNA function can lead to mitochondrial diabetes (Sarhangi et al., 2017;

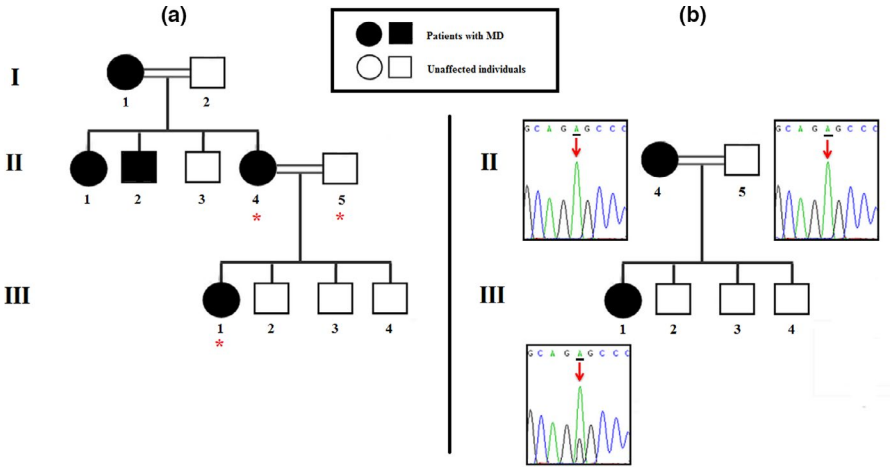

FIGURE 1 (a) Pedigree of Family A with mitochondrial diabetes. Asterisks indicate the individuals from whom DNA samples were obtained and tested. Generations are indicated on the left in Roman numerals and the numbers under the individuals represent identification numbers for each generation. (b) Sequence chromatograms showing the presence of the m.3243A>G mutation in the mitochondrial *MT-TL1* in patient III.1

Tabebi et al., 2017). Mitochondrial diabetes (MD) is defined as a mitochondrial disease accompanied with chronic hyperglycemia characterized by impaired secretion of insulin, insulin resistance, or combined defects (Ng, Taylor, & Schaefer, 2017). It is a rare monogenic form of diabetes with a frequency of 1% (Li, Slone, Fei, & Huang, 2019) and classified into type l and type 2 (Tabebi et al., 2017). MD is characterized by a strong familial clustering of diabetes with a maternal transmission in conjunction with bilateral hearing impairment in most of the carriers (Mezghani et al., 2013) and also with several other effects such as retinopathy, neuropathy, and nephropathy (Sheetz & King, 2002; Tabebi et al., 2015, 2017).

In many cases, MD was associated with the m.3243A>G mutation in mtDNA, which is located in *MT-TL1* (OMIM 590050) gene coding mitochondrial tRNALeu$^{(UUR)}$. MD has also been associated with a range of other point mutations in mitochondrial genes such as mt-tRNA genes, (*MT-TI* [OMIM 590045], *MT-TS1* [OMIM 590080], and *MT-TK* [OMIM 590060]) and mt-proteins genes (*MT-ND1* [OMIM 516000], *MT-ND4* [OMIM 516003], *MT-COX2* [OMIM 516040], and *MT-COX3* [OMIM 516050]) (Maassen, Janssen, & 't Hart, L.M., 2005; Mezghani et al., 2010; Tabebi et al., 2017; Wilson et al., 2004). In addition, deletion (Mezghani et al., 2013) and depletion (Guillausseau et al., 2001) have also been described in patients with MD.

Here, we investigate the mitochondrial genome anomalies in a family with MD by performing Sanger sequencing for a whole mitochondrial genome screening, a qPCR for mitochondrial copy number analysis, and Long-range PCR for the detection of large deletions.

2 | MATERIAL AND METHODS

2.1 | Patients

In this report, we studied a Tunisian family with clinical features suggestive of mitochondrial diabetes type 2. The main diagnosis criterion was the maternal transmission of diabetes within generations with a bilateral hearing impairment in most of the carriers according to the WHO (World Health Organization) criteria.

The patient (III.1) is an underweight (BMI, 16.4 kg/m^2), 38-year-old female suffering from diabetes since the age of 32. The patient suffered from chronic bilateral hearing impairment as well, and the audiogram showed that the defect preferentially affected high-frequency perception. The cardiopulmonary auscultation and the neurological examination were abnormal where the proband presented a psychomotor retardation and global- and axial hypotonia. The brain MRI examination showed a significant cortical atrophy. The electroretinography (ERG) revealed electrical signs of retinopathy. Dilated mitochondrial cardiomyopathy with left ventricular hypertrophy was also found. Laboratory investigations revealed elevated blood lactate level of 2.9 mmol/L (normal value <2 mmol/L) and positive staining for ragged red fibers on muscle biopsy characteristic of mitochondrial cytopathy.

Family history was notable in that her mother had insulin-requiring diabetes while her father was a healthy individual without any family history of diabetes (Figure 1a). Her three siblings were all males and healthy. She recalled that her maternal grandmother and one of the maternal aunts had diabetes with deafness, whereas her uncle had only diabetes without deafness.

2.2 | Controls

One hundred Tunisian healthy individuals from the same ethnocultural group were tested as controls. These controls have no personal or family history of diabetes or any other disorder.

All individuals (patient and controls) provided informed consent, in accordance with the ethics committee of Hedi Chaker Hospital (Sfax, Tunisia).

2.3 | Methods

2.3.1 | DNA extraction

Total DNA was extracted from peripheral blood leukocytes using phenol–chloroform standard procedures (Lewin & Stewart-Haynes, 1992) and from muscular biopsy, only for patient III.1, using «FastPure™ DNA Kit Cat.# 9191» (TAKARA) according to the manufacturer's recommendations.

2.3.2 | Whole mitochondrial DNA screening

PCR amplification and sequencing of the mitochondrial genes

The entire mitochondrial DNA was amplified using 24 overlapping pairs of primers as described elsewhere (Rieder, Taylor, Tobe, & Nickerson, 1998). After the PCR amplification, each PCR product was purified using NucleoSpin (MACHEREY-NAGEL) and sequenced with ABI PRISM 3100-Avant automated DNA sequencer using the BigDye Terminator Cycle Sequencing reaction kit v1.1. The resulting sequences were compared with the updated Cambridge sequence (GenBank accession number: NC_012920). The blast homology searches were performed using the programs available at the National Center for Biotechnology Information website and compared with the wild-type sequence. Areas containing putative novel variations were amplified and sequenced again on both strands to exclude possible PCR artifacts.

Screening of the m.3243A>G mutation in the tRNALeu$^{(UUR)}$ gene using PCR-RFLP and quantification of the heteroplasmy rates

PCR restriction fragment-length polymorphism analysis was used to find the m.3243A>G mutation in blood leukocytes or muscular biopsy. The PCR amplification and the PCR-RFLP analysis with the *ApaI* restriction enzyme (BioLabs) were performed as described elsewhere (Bannwarth, Procaccio, & Paquis-Flucklinger, 2005; Mkaouar-Rebai et al., 2007). The m.3243A>G mutation creates an *ApaI* restriction site and the digestion results in two fragments of 234 and 294 bp, whereas in the absence of this mutation, a 528 bp fragment corresponding to the PCR product will be observed. For the quantification of the heteroplasmic rates of the m.3243A>G mutation, we analyzed the digested fragments with the Gel DOC™-XR (Bio-RAD).

2.3.3 | Long-range PCR amplification in blood leukocytes or muscular biopsy

Long-range PCR was performed to detect mtDNA deletions using Long PCR Enzyme Mix (#K0182) (Fermentas). To amplify the fragment of 10.162 kb, we use a couple of primers corresponding to mitochondrial genome positions 5835–5854 and 15997–15978. The conditions for the PCR reaction and the primer sequences were described previously by Tabebi et al. (2017).

2.3.4 | Relative quantification of the mtDNA

For the determination of mtDNA content in the whole blood samples, quantitative PCRs were performed in duplicates in 48-well reaction plates (Applied Biosystems). Each reaction (final volume 10 µl) contained 10-ng DNA, 5 µl of Power SYBR-Green PCR Master Mix (TAKARA), and 0.2 µM each of forward and reverse primers. *ND4* (NADH dehydrogenase subunit 4), a mitochondrial-encoded gene, was amplified (ND4-F: CGCACTAATTTACACTCA, ND4-R: GCTAGTCATATTAAGTTGTTG) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) (OMIM 138400), a nuclear-encoded gene, was used as normalizing control (GAPDH-F: CCCTGTCCAGTTAATTTC, GAPDH-R: CACCCTTTAGGGAGAAAA). The selection of the target gene (*ND4,* mitochondrial gene) is described in detail in the Supplementary Material.

The PCR condition was 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s, and primer extension at 72°C for 15 s. The melting curve analysis was provided by the Dissociation Curve software to ensure the assay specificity. The slope of standard curve indicated high real-time PCR efficiency rates, 1.08 for *ND4*, and 1.19 for *GAPDH*, in the investigated range from 0.2 to 200 ng DNA input ($n = 4$) with high linearity (Pearson correlation coefficient $r = 0.99$).

We then determined the mtDNA copy number relative to nuclear DNA (nDNA) using the method as described by Venegas, Wang, Dimmock, & Wong, (2011). The data are expressed as relative quantification of the mtDNA on nuclear DNA copy number (mtDNA/nDNA). The quantitative differences were statistically analyzed by Student's *t* test, where *p* values lower than .05 were considered significant.

3 | RESULTS

3.1 | Whole mitochondrial DNA screening

In the present study, we performed a sequence analysis of the whole mtDNA in a Tunisian family presenting three generations of maternally inherited diabetes (Figure 1a). The whole mitochondrial genome screening revealed the presence of several reported substitutions in the D-loop region and the coding genes (Table 1). Among these variations, the

TABLE 1 Prediction of punctual mitochondrial variations detected in the studied family members and the phenotypic association

Patients	Locus	Nucleotide change	Homo/heteroplasmic change	AA change	Phenotypic association
III.1	MT-HV1	m.16224T>C	Homoplasmic		LHON
III.1		m.16311T>C	Homoplasmic		Encephalomyopathy, cardiomyopathy, deafness, MELAS, LHON
II.4, III.1		**m.16519T>C**	Homoplasmic		LHON, encephalomyopathy, cardiomyopathy, deafness, MIDD
II.4, III.1	MT-ND1	**m.3480A>G**	Heteroplasmic		LHON, MIDD
III.1	MT-ND6	m.14167C>T	Homoplasmic	E169E	LHON
II.4, II.5, III.1		m.14212T>C	Homoplasmic	V154V	—
II.4, III.1		**m.14257A>G**	Homoplasmic	P139P	Diabetes
II.4, II.5, III.1	MT-HV2	m.73A>G	Homoplasmic		—
II.4, II.5, III.1		m.87A>T	Heteroplasmic		—
II.4, II.5, III.1		m.263A>G	Homoplasmic		—
II.4, III.1		**insC311-315**	Homoplasmic		—
III.1	MT-TL1	**m.3243A>G**	Heteroplasmic		Diabetes, deafness, MELAS, LHON, MIDD, encephalomyopathy, cardiomyopathy

Abbreviations: LHON, Leber hereditary optic neuropathy; MELAS, myopathy-encephalopathy-lactic acidosis-stroke-like episodes; MIDD: maternally inherited diabetes deafness.

The bold values are the mitochondrial mutations associated with mitochondrial diabetes.

additional C in the 311–315 track and the m.16519T>C in the D-loop region, m.3480A>G in *MT-ND1* and m.14257A>G in *MT-ND6* (OMIM 516006) were found in the proband (III.1). They are maternally inherited and associated with diabetes and deafness according to the MITOMAP database (mitochondrial database).

Additionally, the patient (III.1) exclusively presented the heteroplasmic m.3243A>G mutation in the *MT-TL1* (Figure 1b). This mutation was absent in the other family members (her mother II.4 and her father II.5) indicating that the mutation is de novo (Figure 1b). This transition creates an *ApaI* restriction site which is absent in the PCR product of wild-type DNA. The PCR-RFLP analysis in leukocyte revealed the absence of the mutation in the patient's parents which is in accordance with the sequencing results (Figure 2a). In addition, the digested fragment profile in the patient quantified with the Gel DOC^{TM}-XR (Bio-RAD) analyzer showed that the m.3243A>G mutation was present in the patient with high heteroplasmic loads: 54.7% in muscular tissue and 25% in leukocytes (Figure 2b).

3.2 | mtDNA deletion and depletion analysis

Patient III.1 presented a severe phenotype comparing to her family members and we speculated MD could be associated with mitochondrial deletions. To test this hypothesis, we performed a Long-range PCR amplification of the 10.162 kb fragment which contains the major arc of mtDNA. The DNA was extracted from the blood leukocytes of studied individuals (II.4, II.5, and III.1) and muscle tissue of the patient (III.1) carrying the m.3243A>G mutation. Amplification results of the proband (III.1) showed the presence of two distinctive fragments, 10.162 kb and ~9.0 kb, in both tissues indicating the presence of heteroplasmic mitochondrial deletion (Figure 3a). These deletions remove several mitochondrial tRNA and protein-coding genes. However, the long-range PCR amplification in the family members (II.4 and II.5) showed only the expected fragment of 10.162 kb indicating the absence of mitochondrial deletions in their blood leukocytes in the mtDNA-tested region (Figure 3a).

The relative quantification of mtDNA copy number using mtDNA/nDNA in the leukocytes of individuals II.4, II.5, or III.1 with qPCR revealed a significant variation in this ratio compared to controls (Figure 3b). The results showed that the proband with the m.3243A>G mutation had low levels of mtDNA compared to controls (mean = 75.07% ± 5.39, $t = 4.625$; $p = .04$), which suggests a depletion in the blood leukocytes. However, the mean amount of the relative mtDNA copy number had increased significantly in the leukocytes of the mother (II.4) and of the father (II.5) (p=.04): 112%±2.685 and 121.5%±4.790, respectively.

4 | DISCUSSION

In the present study, we performed a mutational screening of the whole mitochondrial DNA in a Tunisian family with a history of MD. The mtDNA sequencing showed the presence

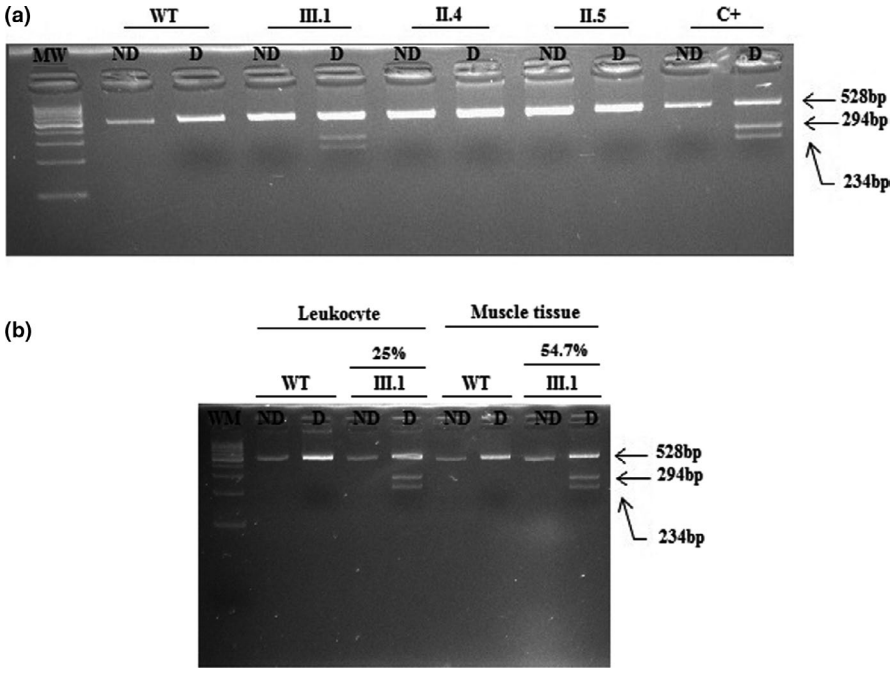

FIGURE 2 Screening of the m.3243A>G mutation by PCR-RFLP: A 528 bp PCR fragment is digested with *ApaI*. The wild-type PCR product does not contain *ApaI* restriction site, whereas PCR product containing the m.3243A>G mutation is cleaved into two fragments of 234 and 294 bp in length. (a) Digestion in the leukocytes of patients II.4, II.5, III.1, and normal individual (WT), and positive control of the mutation (C+); (b) Digestion in leukocyte and muscular tissue of patient III.1 and normal individual (WT). (C+, positive control of the mutation 3243A>G; D, digested PCR product; MW, 100-bp marker; ND, not digested PCR product; WT, wild-type individual)

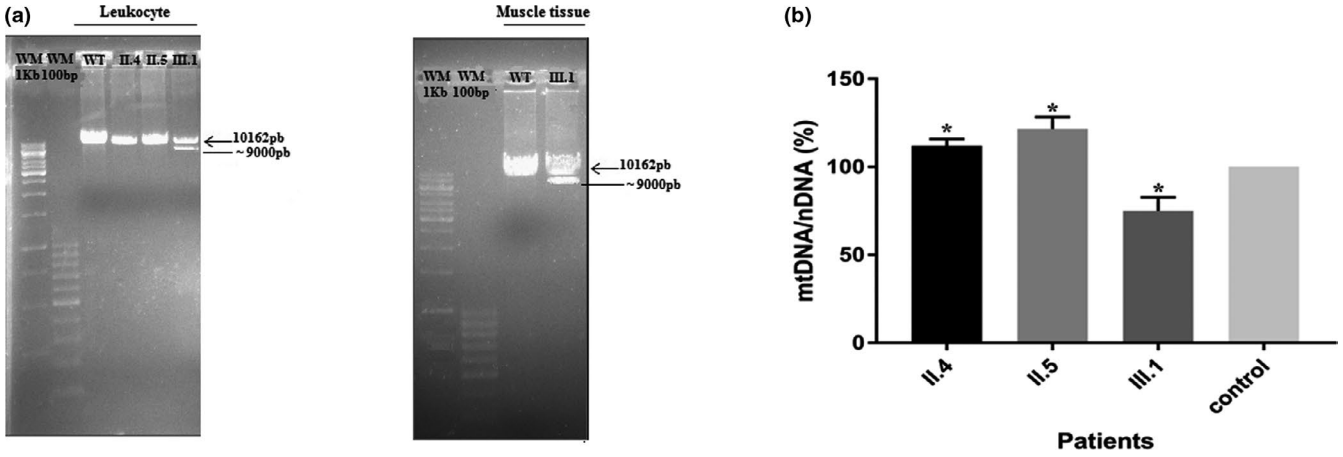

FIGURE 3 (a) Long-range PCR was used to amplify a fragment of 10.162 kb and PCR products were separated by electrophoresis in a 0.8% agarose gel with ethidium bromide: Long-range PCR amplification in the DNA extracted from the patients II.4, II.5, III.1, and normal individual (WT) blood leukocytes (left) and long-range PCR amplification in the DNA extracted from the patient III.1 and normal individual (WT) muscular tissue (right). (MW, 1 kb DNA Ladder 250–10,000 bp (Fermentas SMO318) (lane 1); MW, 100-bp marker (line 2), WT, wild-type). (b) Relative quantification of the mtDNA on nuclear DNA copy number (mtDNA/nDNA) in peripheral blood cells. Total DNA isolated from whole blood has been quantified by real-time quantitative PCR analysis using the GAPDH as an endogenous control. The PCR data were analyzed using a comparative Ct method and values are here expressed as the percentage of mtDNA/nDNA copy number. The 100% is indicating the range observed in the control. The significant differences detected by one-way ANOVA test are indicated in the figure (*$p < .05$)

of several reported substitutions including the m.3243A>G mutation in the *MT-TL1*. This mutation was present only in the patient III.1 at heteroplasmic state in the blood leukocytes and the muscular tissue. This patient also presented ~1.0 kb mtDNA deletion and mitochondrial copy number decrease.

The m.3243A>G mutation in the mitochondrial *tRNALeu*$^{(UUR)}$ was previously reported as the most prevalent mutation in mitochondrial diabetes in several diabetic population worldwide with a prevalence varying from 0.1% to 10% (Maassen et al., 2004). In Tunisian diabetic population, this mutation was only reported in 1.07% (Bouhaha, Abid Kamoun, Elgaaied & Ennafaa, 2010 which was similar to the values reported in Japanese (Suzuki et al., 2003), German (Klemm et al., 2001), and French diabetic populations (Guillausseau et al., 2001). In early onset (6.40 years) diabetes, the m.3243A>G mutation was found in 3% of patients while in late-onset (>40 years) diabetes only 0.3% of patients carry this mutation (Ng et al., 2000).

The m.3243A>G has also been associated with various severe mitochondrial diseases and syndromes. Indeed, it has been identified in approximately 80% of MELAS (Myopathy-Encephalopathy-Lactic Acidosis-Stroke like episodes) patients (Deschauer, Wieser, Neudecker, Lindner, & Zierz, 1999; Mezghani et al., 2011) and in about 38% of them with cardiac involvement (Berardo, Musumeci, & Toscano, 2011). In addition, this mutation has been reported in deafness (Murphy, Turnbull, Walker, & Hattersley, 2008), myopathy (Kärppä et al., 2005), Leigh syndrome (Carelli & Chan, 2014), ophthalmoplegia associated with epilepsy (Mancuso et al., 2014), cardiomyopathy (Alila et al., 2016), and ocular and renal affections (Löwik, Hol, Steenbergen, Wetzels, & Heuvel, 2005; Michaelides et al., 2008).

Several previous cohort studies reported maternal inheritance of the m.3243A>G mutation (Frederiksen et al., 2006) but only rarely at sporadic presentation, in contrast to other mitochondrial mutations (Cree, Samuels, & Chinnery, 2009; de Laat et al., 2016). Furthermore, m.3243A>G at a de novo state was observed in the cases with more severe phenotype, which is in accordance with the presentation of the proband of our studied family (de Laat et al., 2016). This proband carried the de novo m.3243A>G mutation at heteroplasmic state with rate of 25% in the blood leukocytes and much higher 54.7% in the skeletal muscle. As in Table 2, the results are in accordance with a previous description of heteroplasmic rate of the m.3243A>G mutation in blood leukocytes (18%–30%) and in the muscle biopsy (70%–89%) of patients with MD. These heteroplasmic rates were lower than those observed in patients with MELAS syndrome but they both lead to a very severe phenotype in patients presenting mitochondrial diseases including deafness, weakness, myopathy, and so on (Table 2).

The complex phenotype associated with m.3243A>G mutation was explained in several studies by its effect on the oxidative phosphorylation and ATP production. Not surprisingly, the m.3243A>G mutation was described to be responsible for reduced ATP production and impaired oxidative phosphorylation due to the respiratory chain deficiency (Alila et al., 2016). Furthermore, cellular studies investigated that the m.3243A>G mutant revealed deficiencies in RNA processing (Rossmanith & Karwan, 1998), aminoacylation (Park, Davidson, & King, 2003), posttranscriptional tRNA modification (Helm, Florentz, Chomyn, & Attardi, 1999), and translation.

In addition to the m.3243A>G pathogenic mutation, we found four other maternally inherited mitochondrial variations in the proband III.1, which were previously described to be associated with diabetes and deafness. These reported substitutions are in the *MT-ND1* (1) and *MT-ND6* (1) coding genes or in the noncoding displacement D-loop region (2) (Table 1). The two variations m.3480A>G (p.K58=) and m.14257A>G (p.K6=) found in the *MT-ND1* and *MT-ND6*, respectively, were previously described as potential diabetogenic mtDNA defect markers (Li et al., 2014; Yu, Yu, Liu, Wang & Tang, 2004). Furthermore, the mononucleotide repeat (poly-C) between 303 and 315 nucleotides (D310) in the D-loop has been recently identified as a hotspot mutation and often associated with severe mitochondrial diseases and human neoplasia including breast cancer (Yu et al., 2008). This homopolymeric C stretch is part of the conserved sequence block II within the D-loop and is involved in the formation of the RNA-DNA hybrid leading to the initiation of mtDNA heavy-strand replication (Schwartz, Alazzouzi, & Perucho, 2006). On the other hand, the 16519T>C SNP in the D-loop region was correlated

TABLE 2 An overview of de novo reports of the m.3243A>G mutation

Phenotype	Sex/age of onset (year)	Heteroplasmic level (%)			Special features	Report
		Muscle	Blood	Buccal saliva		
MELAS	M/21	89	36	—	Deafness, weakness	Yamamoto, 1996
MD	—	79	10	—	—	
MELAS	M/2	70	30	—	Epilepsy, weakness, psychomotor delay	Campos et al. (1996)
MELAS	M/54	39	6	—	Weakness, deafness, diabetes	Deschauer et al. (1999)
MD	F/8	—	18	55	Deafness, hypertension, proteinuria	Maassen, Biberoglu, 't Hart, Bakker & de Knijff, 2002
MELAS	M/34	82	—	40	Encephalopathy, deafness, epilepsy, myopathy	de Laat et al. (2016)
MD	F/32	**54.7**	**25**	—	Deafness, cardiomyopathy, encephalopathy, retinopathy, psychomotor delay	**This study**

Abbreviations: F, female; M, male; MD, mitochondrial diabetes; MELAS, myopathy, encephalopathy, lactic acidosis, stroke-like episodes.

The bold values are our results from this study.

significantly with diabetes mellitus (Navaglia et al., 2006). This variant is thought to alter the transcription levels of mitochondrial proteins involved in oxidative phosphorylation, which, if occurring in the β cells, may lead to β-cell failure (Navaglia et al., 2006; Simmons, Suponitsky-Kroyter, & Selak, 2005). These mitochondrial point mutations together with the pathogenic m.3243A>G mutation could at least partially explain the severe phenotype observed in the proband III.1. Additional mtDNA investigation revealed that this patient also presented a heteroplasmic mtDNA deletion of ~1.0 kb, which was absent in the other family members examined. The mtDNA deletion is a rare anomaly in mitochondrial diabetes, associated with severe phenotypes such as dilated cardiomyopathy and neurological effects (Ballinger et al., 1992; Mezghani et al., 2013; Rigoli, Salpietro, Caruso, Chiarenza, & Barberi, 1999). The severity of phenotypes associated with mtDNA deletions was variable and linked to the loss of function of mitochondrial genes (Chen, 2013). To our knowledge, this is the first report that analyzes the co-occurrence of the mitochondrial deletion with the pathogenic mutation m.3243A> G.

The relative quantification of mtDNA/nDNA showed a significant decrease in the leukocyte mtDNA copy number of patient III.1 presenting the m.3243A>G. Decrease in mtDNA has been often observed in mitochondrial disorders as well as cell lines harboring m.3243A>G mutation, indicating that mtDNA depletion can be a common secondary phenomenon and also that the cause of mtDNA depletion is not always due to mutation of a nuclear gene (Pyle et al., 2007). Interestingly, subjects II.4 and II.5 who do not carry the m.3243A>G mutation presented an increase in the mtDNA content in their leukocytes. This could be explained by the increased expression of the mitochondrial transcription factor A (TFAM, OMIM 600438), which plays a key role in the regulation of mtDNA replication as described recently (Farge & Falkenberg, 2019).

5 | CONCLUSIONS

The whole mitochondrial DNA analysis in a Tunisian family with a history of MD revealed the m.3243A>G mutation (*MT-TL1*) in a de novo heteroplasmic state in the proband. Together with the high heteroplasmic rate of this mutation, we also found the ~1.0 kb deletion in the mtDNA and the depletion of the mitochondrial content. It is likely that the co-occurrence of these mitochondrial mutations explains the severity of the MD phenotype in the proband compared to other family members, namely diabetes and deafness associated with cardiomyopathy, encephalopathy, retinopathy, and psychomotor delay.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Mouna Tabebi: performed the molecular biology experiment and data mining for the genetic data and wrote the manuscript. Wajdi Safi, Mohamed Abid, and Mouna Mnif: provided clinical samples. Rahma Felhi and Olfa Alila Fersi: performed the molecular biology experiment. Leila Keskes: supplied biological materials. Faiza Fakhfakh: supervised and proofed the manuscript.

DATA AVAILABILITY STATEMENT

Additional supporting information may be found online in the Supporting Information section.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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