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A novel MT-CO1 m.6498C>A variation associated with the m.7444G>A mutation in the mitochondrial COI/tRNA$^{Ser(UCN)}$ genes in a patient with hearing impairment, diabetes and congenital visual loss

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ABSTRACT

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of dysfunction of the mitochondrial respiratory chain. Sensorineural hearing loss (SNHL) has been described in association to different mitochondrial multisystem syndromes, often involving the central nervous system, neuromuscular, or endocrine organs. In this study, we described a Tunisian young girl with hearing impairment, congenital visual loss and maternally inherited diabetes. No mutation was found in the mitochondrial tRNA$^{Leu(UUR)}$ and the 12S rRNA genes. However, we detected the m.7444G>A mutation in the mitochondrial COI/tRNA$^{Ser(UCN)}$ genes. This mutation eliminates the termination codon of the MT-CO1 gene and extends the COI polypeptide by three amino acids (Lys–Gln–Lys) to the C-terminal. The whole mitochondrial genome screening revealed the presence of a novel mutation m.6498C>A (L199I) in the mitochondrial DNA-encoded subunit I of the cytochrome c oxidase (COX). This "probably damaging" transversion affects a highly conserved domain and it was absent in 200 Tunisian controls. The studied patient was classified under the haplogroup H2a.

1. Introduction

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of dysfunction of the mitochondrial respiratory chain. This dysfunction could affect the cochlea which is highly sensible to these mitochondrial disruptions and this is the reason why sensorineural hearing loss is a symptom frequently present in mitochondrial diseases, both in syndromic and isolated forms [1].

In fact, sensorineural hearing loss (SNHL) has been described in association to different mitochondrial multisystem syndromes, often involving the central nervous system, neuromuscular or endocrine organs [2]. However, in other patients, the hearing impairment can be isolated often due to mutations in the 12S rRNA gene (MT-RNR1) or in the mitochondrial tRNA$^{Ser(UCN)}$ gene (MT-TS1). In fact, these 2 genes were found to be hot-spots for mutations associated with non-syndromic and aminoglycoside-induced hearing loss [2–4].

Of these, the 12S rRNA gene mutations are the most important causes of HL worldwide [5–7]. Furthermore, sequence variants identified in tRNA$^{Ser(UCN)}$ and COI/precursor of tRNA$^{Ser(UCN)}$ genes, including m.7444G>A, m.7445A>G, m.7472insC, m.7510T>C and m.7511T>C were described in both, SNHL and aminoglycoside-induce hearing impairment [8–12].

Mitochondrial mutations associated to non-syndromic deafness are often homoplasmic or at high levels of heteroplasmy, suggesting that high threshold of the mutated mtDNA must accumulate for pathogenicity. Variable phenotypic expression of these mtDNA mutations requires the contribution of other factors such as nuclear modifier genes, environmental factors, or other mitochondrial mutations.

Besides to mutations in the 12S rRNA and the tRNA$^{Ser(UCN)}$ genes, mutations in the mitochondrial tRNA$^{Leu(UUR)}$ gene were also reported with syndromic deafness. Indeed, the m.3243A>G is also one of the important causes of maternally inherited diabetes and deafness [13,14]. Additionally, the m.3243A>T, m.3264T>C and m.3291T>C mutations in this tRNA gene have also been associated with syndromic deafness [15,16].

In the present study, we reported the m.7444G>A mutation in the mitochondrial COI/tRNA$^{Ser(UCN)}$ genes and a novel m.6498C>A mutation in the MT-CO1 gene in a Tunisian patient with hearing impairment, maternally inherited diabetes and congenital visual loss.

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2. Patient and methods

2.1. Patient

The proband is a 14 years-old girl born by caesarian section after an uneventful pregnancy. She had a history of type 2 diabetes since her mother and maternal aunt were diabetic. However, she has a sister (17 years-old) and a brother (16 years-old) who are healthy.

Her birth weight was 3700 g, her height was 49 cm with a head circumference of 37 cm and an Apgar index of 7/9. She had a congenital visual loss and she showed a normal psychomotor development during the first years of life.

At the age of 7 years, she showed a polyuria polydipsia syndrome associated with an asthenia. Her BMI (Body Mass Index) was 22.5 (normal values 13.5 and 17.9) with a weight of 47 kg and a height of 146 cm at this age. The cardiopulmonary auscultation and the neurological examination were normal with the absence of sensory-motor deficits.

The biological analysis revealed a hyperglycemia (11.2 mmol/l) associated with glucosuria and a normal complete blood count. Then, the patient was treated by subcutaneous doses of insulin (Actrapid® and Insulatard®). The evolution was marked by rapid normalization of blood glucose and no episodes of hypoglycemia were observed.

Besides, the audiogram showed a sensorineural hearing loss and the renal echo-Doppler demonstrated a moderate dilatation of the right kidney excretory cavities.

This multisystem disorder suggests a mitochondrial disease especially since the diabetes was maternally inherited.

In addition, 200 Tunisian healthy individuals were tested as controls. These controls should have no personal or family history of any disorder. Informed consent was obtained from all the individuals with the approval of the appropriate Ethic Committee before being enrolled in the genetic study.

3. Methods

3.1. DNA extraction

Peripheral blood samples were obtained from the patient and total DNA was extracted from peripheral blood leucocytes using phenol–chloroform standard procedures [17]. Blood samples from the family' members were not available.

3.2. Mutational analysis of the mitochondrial 12S rRNA and $tRNA^{Ser(UCN)}$ genes

DNA fragments spanning the entire 12S rRNA and $tRNA^{Ser(UCN)}$ genes were amplified by PCR using oligodeoxynucleotides corresponding to the mtDNA at positions (545–564) and (1752–1705) for the first gene, and (7398–7417) and (7633–7614) for the second one. The PCR reactions were performed as previously reported [18].

3.3. Mutational analysis of the mitochondrial $tRNA^{Leu(UUR)}$ gene

The screening of the mitochondrial m.3243A>G mutation in the $tRNA^{Leu(UUR)}$ gene was carried out by PCR-RFLP with the restriction endonuclease *ApaI* (BioLabs) as previously described [19].

3.4. PCR amplification and sequencing of the mitochondrial genome

The whole mitochondrial genome was amplified using 24 overlapping pairs of primers as described elsewhere [20]. PCR products were then purified using NucleoSpin (MACHEREY-NAGEL) and sequenced with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM/Biosystems).

The blast homology searches were performed using the program available at the National Center for Biotechnology Information Web site in comparison with the updated consensus Cambridge sequence (GenBank Accession NC_012920). Regions containing putative novel variations were amplified and sequenced again on both strands to exclude that they were PCR artifacts.

3.5. Sequence alignment

The sequence alignment of the mitochondrial COX1 gene was performed using the Clustal W program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Sequences from the species were obtained from NCBI.

3.6. The pathogenicity prediction of the MT-CO1 mutated protein

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 program (Polymorphism Phenotyping) (http://coot.embl.de/PolyPhen/). PolyPhen structurally analyzes an amino acid polymorphism and predicts whether that amino acid change is likely to be deleterious to protein function.

PolyPhen uses the predicted hydrophobic and transmembrane (PHAT) matrix score to evaluate the possible functional effect of a substitution in the transmembrane region.

3.7. Mitochondrial haplogroup analysis

After sequencing the entire mitochondrial DNA, we performed mitochondrial haplogrouping analysis using MitoTool database (http://www.mitotool.org/genome.html) and we confirmed our result using classifications detailed in previous reports [21].

4. Results

We described a 14 years-old girl with a multisystem disorders affecting ocular, neurosensoriel and endocrine organs. Faced to the hearing impairment and the maternally inherited diabetes observed in this patient, we first tested the $tRNA^{Leu(UUR)}$ gene which is a hot spot for pathogenic mutations associated with mitochondrial diseases with various clinical features especially the MIDD (Maternally Diabetes and Deafness) often caused by the m.3243A>G. This mutation was screened by PCR-RFLP with the *ApaI* restriction enzyme but our results showed its absence in homoplasmic and heteroplamic form in DNA extracted from the blood leucocytes of the studied patient.

We also screened the mitochondrial 12S rRNA gene mutations since they were reported as the most important causes of mitochondrial hearing loss in many populations but the results showed the absence of pathogenic mutation.

Then, we studied the $tRNA^{Ser(UCN)}$ gene since many mitochondrial mutations associated to syndromic and non-syndromic hearing loss were reported in this gene. The results showed the presence of the homoplasmic m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ genes (Fig. 1A). This mutation eliminates the termination codon of the cytochrome c oxidase subunit I (COXI) gene, extending the COI polypeptide by three amino acids. Thus, it causes a read-through of the stop condon AGA of the COI message on the H strand of mtDNA, thereby adding three amino acids (Lys–Gln–Lys) to the C-terminal of the polypeptide (Fig. 1B).

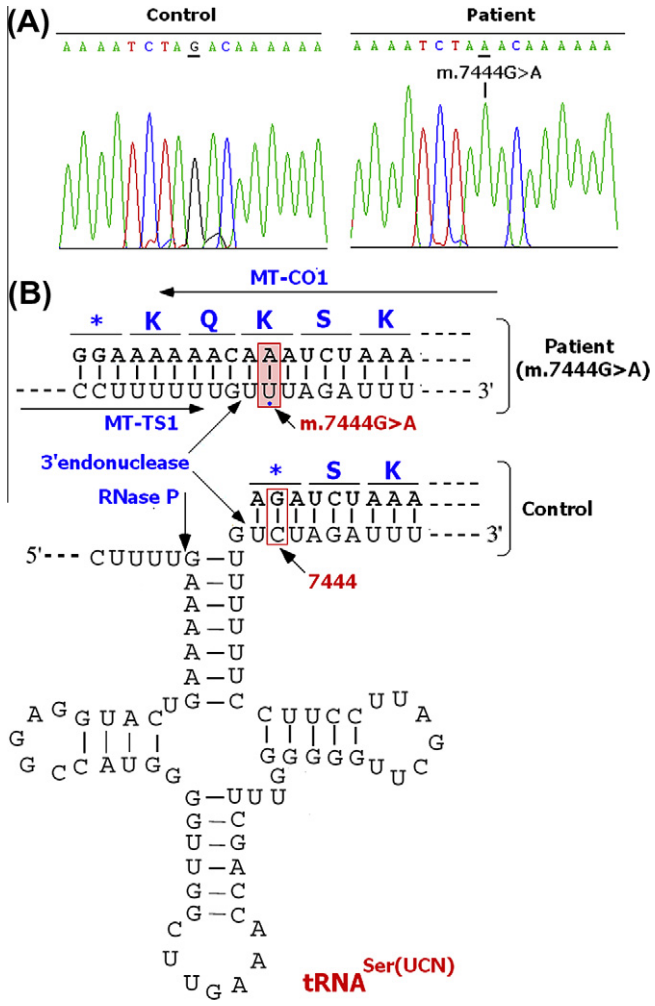

Fig. 1. (A) Sequence chromatograms showing the presence of the m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ genes in the studied patient and its absence in a control subject. (B) Location of the m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ genes showing the extension of the mitochondrial COI polypeptide resulting from this mutation in the patient.

In addition, we performed a screening of the whole mtDNA in this patient. The results showed the presence of a novel m.6498C>A variation in the mitochondrial COI gene (Fig. 2A) which was absent in 200 Tunisian healthy individuals. This variation substitutes the leucine residue at position 199 to isoleucine. The affected aminoacid is located in a highly conserved domain of the COI polypeptide in many species (Fig. 2B). Besides, PolyPhen-2 analysis predicted that this variant is "probably damaging" with scores of 0.990 and 0.997 on HumDiv and HumVar models, respectively (Fig. 3A) and showed that the Leu199 is located in the core of the mitochondrial COI protein (Fig. 3B).

The whole mitochondrial genome screening revealed also the presence of reported polymorphisms in the D-loop region, the ribosomal and transfert RNAs but also the coding genes (Table 1). The detected missense substitutions, especially the 4216T>C (Y304H), the 5460G>A (A331T), the 8932C>T (P136S) and the 13276A>G (M314V) variations were reported in the literature in patients with LHON, Alzheimer's disease, prostate cancer and unaffected individuals (www.mitomap.org).

All the mitochondrial variants detected in the studied patient allowed us to classify her under the haplogroup H2a.

5. Discussion

In the present study, we performed the clinical, genetic, and molecular characterization of a Tunisian girl with hearing impairment, congenital visual loss and a maternally inherited diabetes.

The mutational analysis of the $tRNA^{Leu(UUR)}$ and the 12S rRNA genes associated respectively with MIDD and hearing loss showed the absence of any pathogenic mutation especially the m.3243A>G and the m.1555A>G mutation.

The 3243A>G mutation is known to cause mitochondrial encephalomyopathy, lactic acidosis, and stroke-like symptoms (MELAS) [13] and it is also one of the important causes of maternally inherited diabetes and deafness [14]. The primary defect of this mutation was an inefficient aminoacylation of the $tRNA^{Leu(UUR)}$ [22]. This mutation also affected the processing of the longer RNA precursors [23] and the base post transcriptional modification of the $tRNA^{Leu(UUR)}$ [24]. However, this mutation seems to be too rare in patients with NSHL, MIDD or MELAS in the Tunisian population [18,25].

The m.1555A>G mutation in the mitochondrial 12S rRNA gene is the most common mutation associated with non-syndromic hearing loss. It was reported in many families of different ethnic origins [5,26,27,4], with a prevalence of 0.5–2.4% in European patients, 7% in Chinese patients [28,29] and 0.5–1% in Tunisian population [18].

We also studied the mitochondrial $tRNA^{Ser(UCN)}$ gene since many mutations associated to syndromic and non syndromic hearing loss were reported in this gene [12,8,10]. The PCR-RFLP analysis and a direct sequencing of this gene showed the presence of the m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ gene (Fig. 1A). This substitution results in a read-through of the stop codon AGA of the COI protein on the heavy strand of mtDNA, by adding three amino acids (K–Q–K) to the C-terminal of the polypeptide (Fig. 1B) [30,31]. Alternatively, the m.7444G>A mutation, like the m.7445G>A transition is adjacent to the site of 3′end endonucleolytic processing of L-strand RNA precursor, spanning $tRNA^{Ser(UCN)}$ and ND6 mRNA. Thus, it leads to the processing defect of the $tRNA^{Ser(UCN)}$ precursor by decreasing the steady-state level of this RNA, as well as, decreasing stability of cotranscripted ND6 mRNA precursor [32]. Indeed, it has been demonstrated that the biochemical defects caused by m.7444G>A mutation are likely below the proposed threshold level to support a normal respiratory phenotype [33].

The m.7444G>A mitochondrial DNA mutation was found in only a few cases worldwide, alone or in cosegregation with other mitochondrial DNA mutations. In fact, it was found in Caucasian and Asian pedigrees with nonsyndromic and aminoglycoside-induced hearing loss [34,8]. This mutation often occurs nearly or completely in homoplasmic form, indicating a high threshold for pathogenicity.

In fact, the homoplasmic m.7444G>A mutation was found in association with the homoplasmic m.1555A>G MT-RNR1 mutation in a 3-generation Chinese family with aminoglycoside-induced sensorineural hearing loss. The dose and age at the time of drug administration seemed to be correlated with the severity of the hearing loss [30].

Besides, in 2007, the m.7444G>A transition was detected in 7 of 1542 Han Chinese individuals with aminoglycoside ototoxicity or nonsyndromic sensorineural hearing loss. All the 7 patients were treated by aminoglycosides between 1 and 3 years of age and began suffering hearing loss within 3 months. Two of these patients had both the m.7444G>A and the m.1555A>G mutations [8]. Family histories suggested very low penetrance for the 7444G>A mutation alone. In contrast, there were several members of the 2 families with both m.7444G>A and m.1555A>G who had sensori-

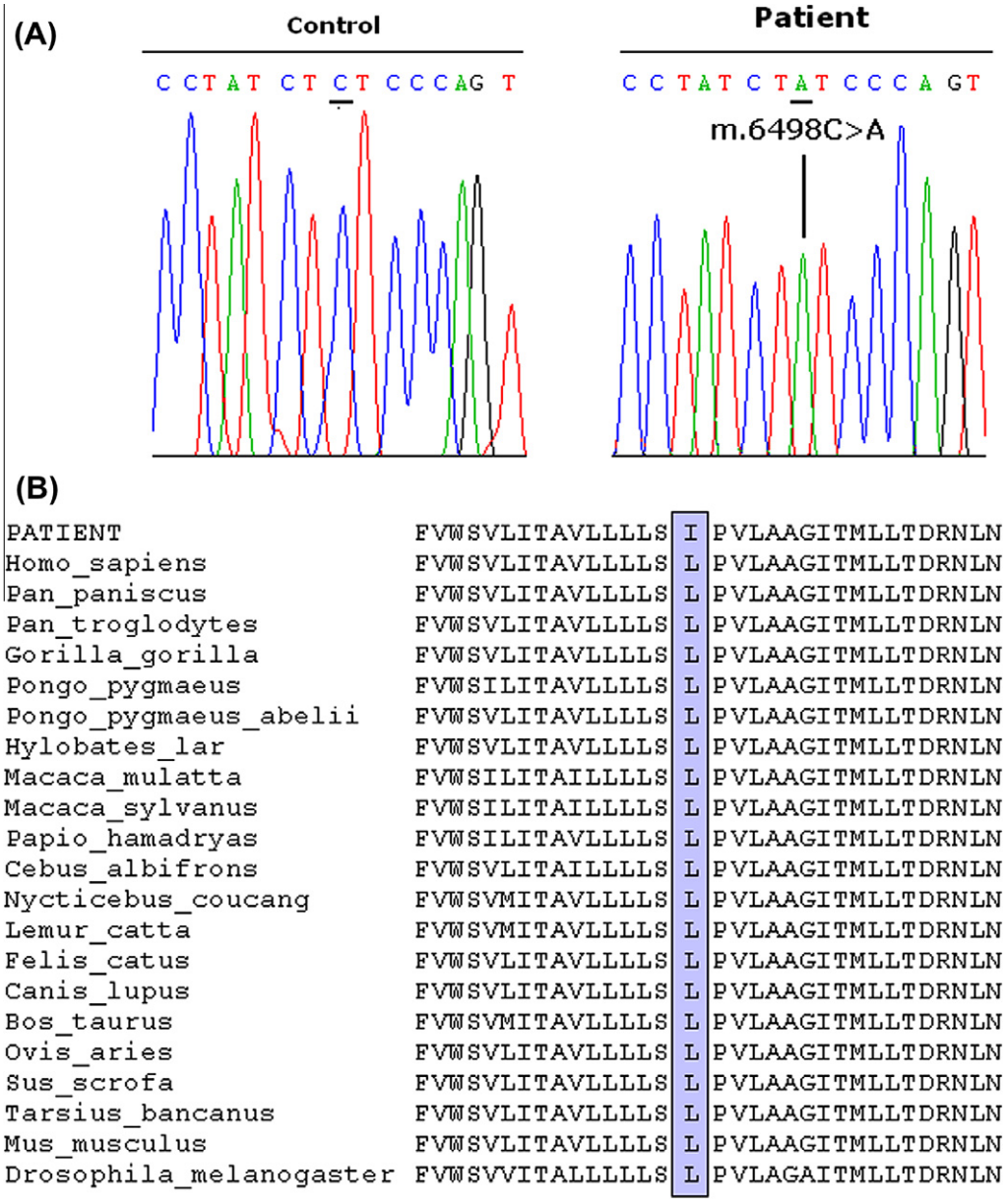

Fig. 2. (A) Sequencing electropherogram showing the presence of the novel m.6498C>A mutation in the mitochondrial COI gene in the studied patient and its absence in a control subject. (B) Sequence alignment of the mitochondrial COI gene in different species. The leucine at position 199 is highly conserved throughout evolution.

neural hearing loss without aminoglycoside exposure, indicating a higher penetrance of hearing loss in those with 2 mutations [8].

In addition, the m.7444G>A mutation was found in 2 patients harboring LHON mutations: the MT-ND1 m.3460A>G mutation in one case and the MT-ND6 m.14484A>G in the other case. Thus, the m.7444G>A mutation could be probably a secondary LHON mutation associated with visual loss [35].

These results suggest that m.7444G>A itself is not sufficient to produce a clinical phenotype and additional modifier factors are required for pathogenic manifestation of m.7444G>A substitution. In fact, mitochondrial and/or nuclear modifier gene(s) or aminoglycoside(s) may play a role in the phenotypic expression of the deafness associated to the m.7444G>A mutation.

Since the clinical investigation showed that the studied patient was not treated with aminoglycosides, we thought to look for mitochondrial modifier genes. Thus, we performed a screening of the whole mitochondrial genome which showed the presence of reported polymorphisms (Table 1). The detected missense variations were described in the literature in patients with LHON, Alzheimer's disease, prostate cancer and unaffected individuals (www.mitomap.org), so they seem not to be associated to the disorders in our studied patient.

The screening of the whole mtDNA showed also the presence of a novel m.6498C>A (L199I) substitution in a highly conserved domain of the mitochondrial COI gene (Fig. 2B) which was absent in 200 Tunisian healthy individuals. PolyPhen-2 analysis predicted that this substitution is "probably damaging" (Fig. 3A) and showed that the affected Leu199 is located in the core of the mitochondrial COI protein (Fig. 3B).

The affected cytochrome c oxidase subunit I is 1 of 3 mitochondrial DNA encoded subunits (MT-CO1, MT-CO2, MT-CO3) of the respiratory complex IV. This complex is the terminal enzyme in the respiratory chain, located in the inner membrane of mitochondria and bacteria. It catalyzes the reduction of dioxygen to water and pumps an additional proton across the membrane for each proton consumed in the reaction. The resulting electro-chemical

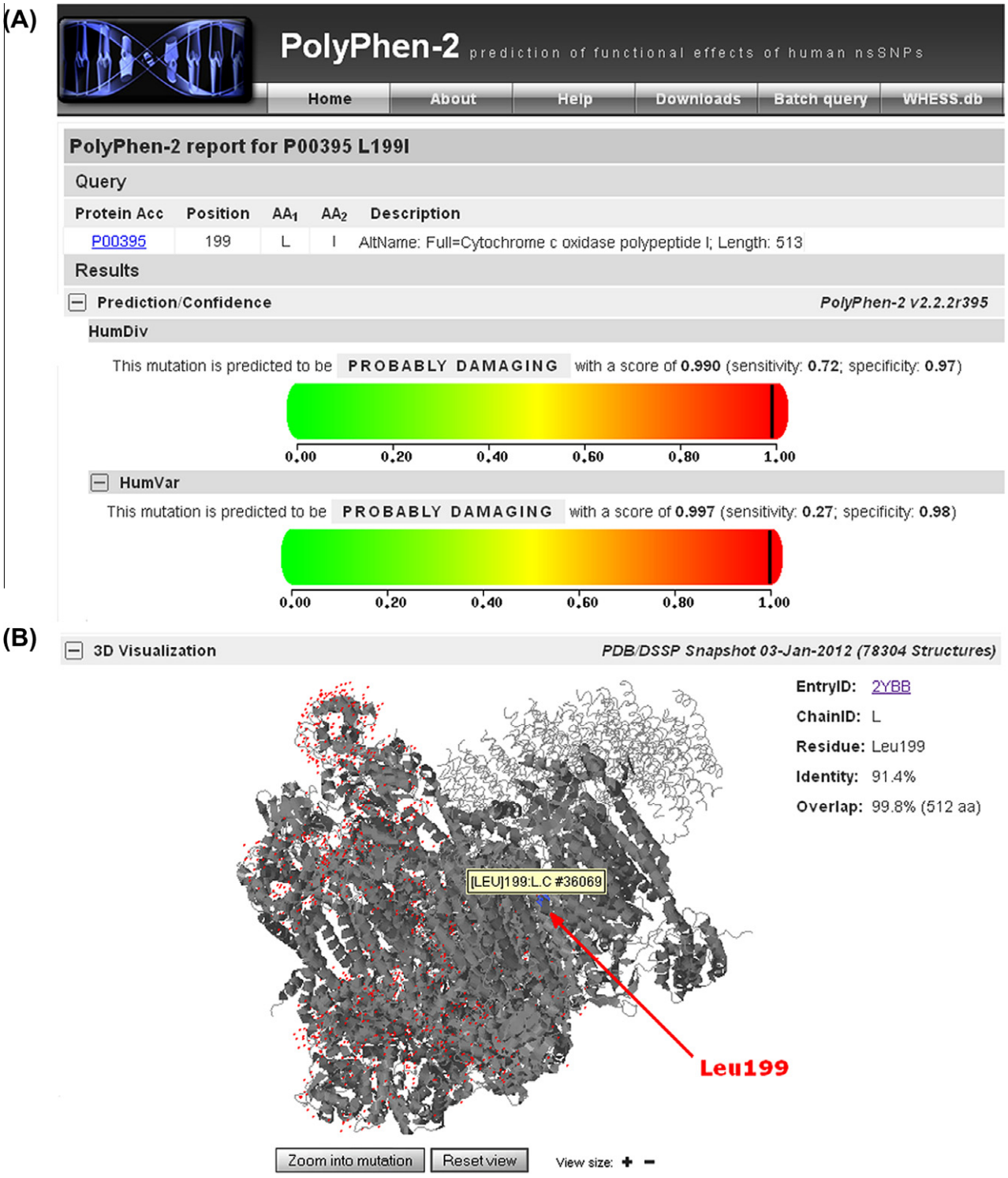

Fig. 3. (A) Results of the PolyPhen-2 analysis predicting the pathogenicity the p.199L>I substitution on the mitochondrial COXI protein. (B) Location of the Leu199 residue in the 3D structure of the mitochondrial COXI protein.

gradient is used elsewhere, for instance in the synthesis of ATP. Deficiency of cytochrome c oxidase (COX) causes a clinically heterogeneous variety of neuromuscular and non-neuromuscular disorders in childhood and adulthood and is one of the most frequent causes of mitochondrial defects [36]. The clinical phenotypes include stroke, cardioencephalomyopathy, hepatic failure and Leigh's syndrome [37].

Thus, the novel m.6498C>A (L199I) mutation found in the cytochrome c oxidase subunit I could reduce the activity of oxidative phosphorylation complex IV. Besides, the m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ genes causes a defect in the processing of the L-strand RNA precursor leading to mitochondrial dysfunctions. Thus, the association of these two mutations may affect the interaction of the COXI protein with

Table 1
Mitochondrial variants detected in the studied patient with deafness, diabetes and congenital visual loss.

Locus	Nucleotide change	Position	Aminoacid change
MT-HV2/MT-DLOOP	A>G	63	NCS
MT-HV2/MT-DLOOP	G>A	185	NCS
MT-HV2/MT-DLOOP	A>G	189	NCS
MT-HV2/MT-DLOOP	A>G	200	NCS
MT-HV2/MT-DLOOP	A>G	263	NCS
MT-CBS2/MT-HV2/MT-DLOOP	insC	309	NCS
MT-CBS2/MT-HV2/MT-DLOOP	insC	315	NCS
MT-RNR1	A>G	750	NCS
MT-RNR1	A>G	1438	NCS
MT-RNR2	A>G	2706	NCS
MT-ND1	T>C	3396	Syn
MT-ND1	T>C	4216	Y>H
MT-ND2	A>G	4769	Syn
MT-ND2	G>A	5460	A>T
MT-TA	C>T	5601	NCS
MT-CO1	**C>A**	**6498**	**199L>I**
MT-CO1	**G>A**	**7444**	**STOP>K**
MT-ATP6	A>G	8701	T>A
MT-ATP6	A>G	8860	T>A
MT-ATP6	C>T	8932	P>S
MT-CO3	T>C	9540	Syn
MT-ND4	T>C	10873	Syn
MT-ND4	T>C	10915	Syn
MT-ND4	G>A	11914	Syn
MT-ND5	C>T	12705	Syn
MT-ND5	A>G	13276	M>V
MT-TT	delT	15940	NCS
MT-HV1/MT-DLOOP	C>T	16209	NCS
MT-HV1/MT-DLOOP	C>T	16223	NCS
MT-HV1/MT-DLOOP	T>C	16311	NCS

NCS, non-coding sequence; novel variation is highlighted and written in bold.

the other subunits which could alter the mitochondrial respiratory chain.

To our knowledge, the present study reported the first case with the m.7444G>A mutation in the mitochondrial COI/precursor of $tRNA^{Ser(UCN)}$ genes in Tunisia and Africa. The described proband suffers from multisystemic disorders affecting ocular, neurosensoriel and endocrine organs which could be caused by the association of the two reported mutations.

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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