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A novel mutation MT-COIII m.9267G>C and MT-COI m.5913G>A mutation in mitochondrial genes in a Tunisian family with maternally inherited diabetes and deafness (MIDD) associated with sever nephropathy

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ABSTRACT

Mitochondrial diabetes (MD) is a heterogeneous disorder characterized by a chronic hyperglycemia, maternal transmission and its association with a bilateral hearing impairment. Several studies reported mutations in mitochondrial genes as potentially pathogenic for diabetes, since mitochondrial oxidative phosphorylation plays an important role in glucose-stimulated insulin secretion from beta cells. In the present report, we studied a Tunisian family with mitochondrial diabetes (MD) and deafness associated with nephropathy. The mutational analysis screening revealed the presence of a novel heteroplasmic mutation m.9276G>C in the mitochondrial *COIII* gene, detected in mtDNA extracted from leukocytes of a mother and her two daughters indicating that this mutation is maternally transmitted and suggest its implication in the observed phenotype.

Bioinformatic tools showed that m.9267G>C mutation (p.A21P) is « deleterious » and it can modify the function and the stability of the MT-COIII protein by affecting the assembly of mitochondrial COX subunits and the translocation of protons then reducing the activity of the respective OXPHOS complexes of ATP synthesis.

The nonsynonymous mutation (p.A21P) has not been reported before, it is the first mutation described in the COXIII gene which is related to insulin dependent mitochondrial diabetes and deafness and could be specific to the Tunisian population. The m.9267G>C mutation was present with a nonsynonymous inherited mitochondrial homoplasmic variation *MT-COI* m.5913 G>A (D4N) responsible of high blood pressure, a clinical feature detected in all explored patients.

1. Introduction

Mitochondrial diabetes (MD) is a group of heterogeneous disorder characterized by the presence of chronic hyperglycemia. Pathophysiological mechanisms leading to diabetes can involve an inappropriate secretion of insulin, insulin resistance, or combined defects [1]. Clinically, mitochondrial diabetes generally presents itself as a monogenetic form with a low frequency (1%) [2]. Patients with mitochondrial diabetes are often classified as diabetes types I or II [3].

Mitochondrial diabetes can be discriminated from other type of diabetes on the presence of maternal transmission in association with a bilateral hearing impairment in most of the carriers. Genetic analysis is another argument for the presence of mitochondrial diabetes [4]. Luft et al. demonstrated the implication of mtDNA mutations in oxidative phosphorylation disorders which may play an important role in glucose-stimulated insulin secretion from beta cells [5]. Indeed, recently, an increasing number of studies have

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shown that MD can be caused by point mutations [6], deletions [7,8] and duplications [9], which abolish the function of genes in the mitochondrial genome. In the large majority of cases, mitochondrial diabetes is associated with an A3243G mutation in mitochondrial *tRNA-Leu* gene; although a range of other mutations in mtDNA have also been implicated. These mutations are located in different mitochondrial genes such as the *tRNA-Glu*, the *16S rRNA*, the *12S rRNA* and the *NDI* genes [10,11].

In the present study, we detected a novel m.9276G>C heteroplasmic mutation (p.21A>P) associated with m.5913 G>A homoplasmic mutation (D4N) in cytochrome c oxidase subunit III (*MT-COIII*) and subunit I (*MT-COI*) genes, respectively, in a Tunisian family with mitochondrial diabetes and deafness associated with a sever nephropathy observed in one patient.

2. Materials and methods

2.1. Patients

This study was carried out on a Tunisian family presenting with maternally inherited diabetes and deafness (Fig. 1). The main diagnosis criterion was the maternal transmission of diabetes within generations. In fact, the diabetes should be transmitted by the mother or her relatives and not by the father. The clinical features of the members of this family are summarized in Table 1.

2.2. Controls

In addition, 100 Tunisian healthy individuals from the same ethnocultural group were tested as controls. These controls should have no personal or family history of diabetes or any other disorder. All individuals (patient and controls) provided informed consent.

2.3. Methods

2.3.1. DNA extraction

After getting informed consent from all patients, peripheral blood sample was obtained and total DNA was extracted from peripheral blood leucocytes using phenol–chloroform standard procedures [12].

2.3.2. Mitochondrial DNA sequencing

The mtDNA coding region from *ND1* to *ND6* (nucleotides 3150–14998) was amplified in 15 partially overlapping fragments [13]. Direct sequencing of PCR products was performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI PRISM/Biosystems) and the products were resolved on ABI PRISM. 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 No. NC_012920). Regions containing putative novel variations were amplified and sequenced again on both strands to exclude that they were PCR artifacts.

2.3.3. The sequence alignment and the pathogenicity prediction

The sequence alignment of the mitochondrial *COX3* gene was performed using the Clustal W program (http://www.ebi.ac.uk/Tools/msa/clustalw2). Sequences from differents species were obtained from NCBI. The assessment of the possible effect of the mtDNA change on protein function was performed using PolyPhen

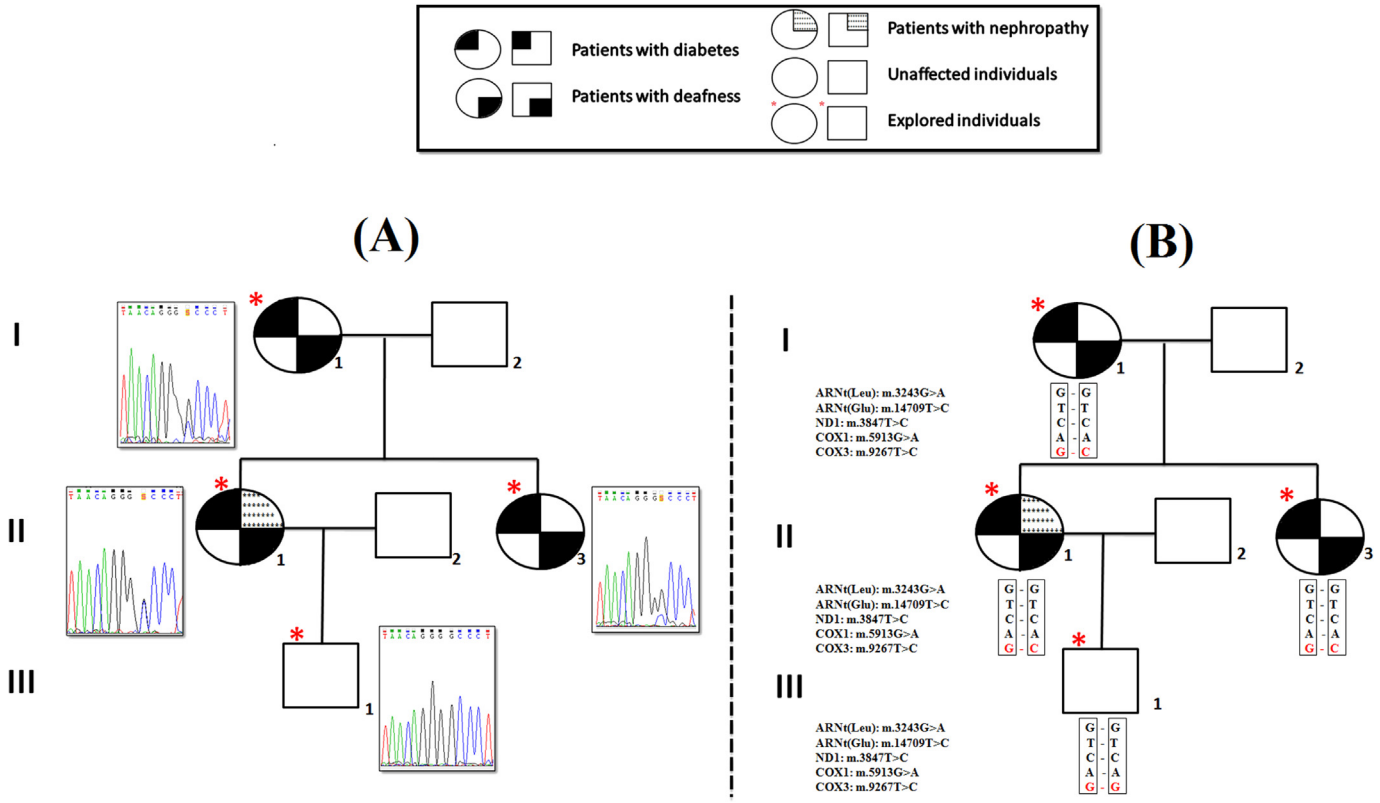

Fig. 1. (A) Pedigree of the studied Family with mitochondrial diabetes presenting the novel MT-COIII m.9267G>C mutation. Asterisks indicate the individuals from whom DNA samples were obtained and tested. Generations are indicated on the left in Roman numerals and the numbers under the individuals represent identification numbers for each generation. (B) Pedigree of the studied family showing the segregation of a characteristic haplotype and the inheritance of the m.9267G>C mutation in affected members: I.1, II.1, and II.3.

Table 1
Clinical features and mitochondrial mutations detected and associated to diabetes in the studied Tunisian family.

Subjects	Sex	Age (years)	Clinical features				m.3243A>G	m.14706T>C	m.9267G>C	m.5913G>A
			MD	Deafness	HBP	Nephropathy				
I.1	F	57	+	+	+	−	−	−	+	+
II.1	F	21	+	+	+	−	−	−	+	+
II.3	F	27	+	+	+	+	−	−	+	+
III.1	M	2	−	−	−	−	−	−	−	+

F: Female; M: male; (−): absence; (+) presence, MD: mitochondrial diabetes; HBP: high blood pressure.

program (Polymorphism Phenotyping) (http://coot.embl.de/PolyPhen/) and PROVEAN software (Protein Variation Effect Analyzer) (http://provean.jcvi.org/seq_submit.php). PolyPhen and PROVEAN structurally analyze an amino acid polymorphism and predict whether that amino acid change is likely to be deleterious to protein function [14,15].

To characterize the protein, we used ProtProgram (http://web.expasy.org/protparam/), which allows the computation of various physical and chemical parameters. The computed parameters include the instability index (II) and grand average of hydropathicity (GRAVY).

For the prediction of possible changes in hydrophobicity and changes in configuration of transmembrane domains of the COIII protein due to sequence variation, we used TopPred II online prediction software (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

To predict the 2D structure and the effect of the mutation, we used the PROTTER software V1.0, an open source tool for visualization of proteoforms and interactive integration of annotated and predicted sequence features together with experimental proteomic evidence (http://wlab.ethz.ch/protter/).

2.3.4. Generation of a 3D model of MT-COIII

To investigate the eventual effect of the nonsynonymous variation changing Alanine residue to a Proline at position p.21 (p.21A>P) of MT-COIII protein, we modeled and compared the two variants, 21A and 21P.

We used D-JIGSAW (version 2.0) (http://bmm.cancerresearchuk.org/~3djigsaw/). This server builds three-dimensional models for proteins based on homologue of known structure. The SWISS PDB VIEWER software (V4.1) was used to display and compare normal and mutated models. The quality of the models was evaluated using ProSA-web [16]. Once the two models superimposed, the RMS calculated between backbone atoms was high (2.20 Å). For the two models, the Ramachandran plot showed that more than 90%, of atoms were in favored areas.

3. Results

In the present study, we reported a Tunisian family presenting maternally inherited diabetes type II, deafness and sever nephropathy observed in the little daughter (II.1) (Fig. 1A) (Table 1).

We started the genetic study by a mutational screening of the mitochondrial genes known to be associated to diabetes. In fact, we screened the *tRNA$^{Leu(UUR)}$* and the *tRNA^{Glu}* genes, but no reported mutations were found, especially the m.3243A>G and the m.14709T>C mutations.

However, the direct sequencing of the fragment containing the entire *MT-CO* gene and parts of its flanking *MT-ND2* and *MT-ND6* genes showed the presence of a novel heteroplasmic m.9267G>C

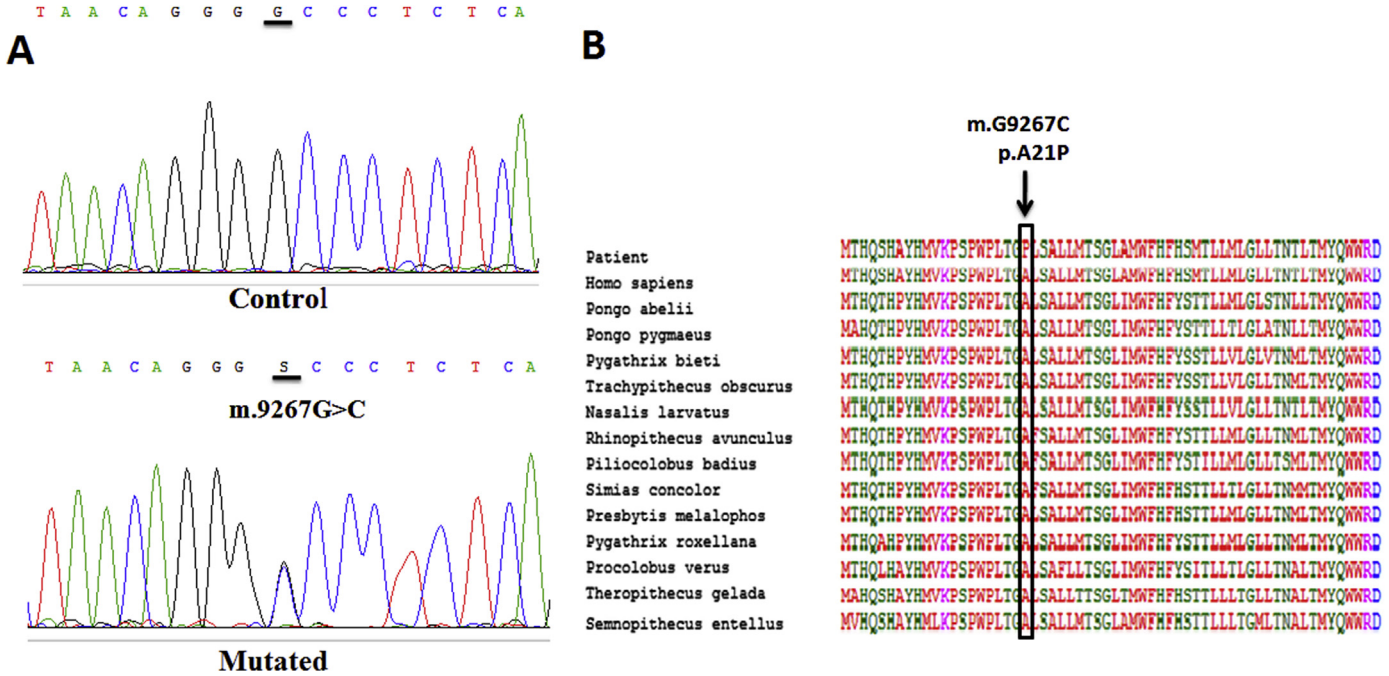

Fig. 2. (A) Sequencing chromatogram showing the heteroplasmic m.9267G>C transversion in the *MT-CO3* gene in the patient compared with a control. (B) Sequence alignment of the mitochondrial COIII protein in different species performed by the Clustal W program showing the conservation of the Alanine residue at position 21 throughout species.

transversion in the mitochondrial *COIII* gene in the DNA extracted from blood leukocytes of three studied patients (I.1, II.1 and II.3) (Figs. 1 and 2A). This substitution was absent in 100 healthy controls from Tunisian population and unaffected son (III.1) (Table 2).

This variation leads to an Alanine to Proline replacement at position 21 (p.A21P) which is a conserved residue (Fig. 2B). PolyPhen-2 analysis predicted that this variant is probably damaging with scores of 1.000 and 0.997 respectively on HumDiv and HumVar models (Fig. 3A). In addition, PROVEAN software predicted the changement of A21P is deleterious with a score of −3.592 (neutral changement >−2.5) (Fig. 3B).

In other hand, prediction of hydrophoby with Toppred II program determined that COIII is a highly hydrophobic protein, the Kyte–Doolittle algorithm demonstrated a large imbalance in the hydrophoby of the p.21A>P mutant polypeptide. The affected residue is located at the beginning of the first α-transmembrane helix of cytochrome c oxidase subunit 3 (COIII) and the substitution of Alanine (a neutral amino acid with hydropthy index 1.8) in position 21 by Proline (neutral amino acid with hydropthy index −1.6) reduced the transmembrane helix hydrophobicity (1.89–1.80) (Fig. 3C) which decreased the GRAVY (Grand average of hydropathicity) from 0.377 to 0.364.

In fact, modeling protein secondary structure with PROTTER software showed that wt MT-COIII contains seven hydrophobic domains in transmembrane region. But the substitution of the Alanine residue with Proline at position 21 caused an alteration in the structure of the protein transmembrane region and reduced the number of domains to six (first intramembrane helical domain began with F35 instead of beginning with P15). Moreover, the m.9267G>C mutation moved the first transmembrane domain to extracellular space (Fig. 4A, B). On account of the alteration protein secondary structure, instability index increased from 22.96 to 23.29 which make the protein less stable.

The comparison of normal and mutated 3D model of MT-COIII demonstrated the absence of four hydrogen bunds between P17–G20, P17–P21, L18–P21 and L18–L22 in the mutated MT-COIII protein. In fact, the absence of amine functional group in the side chain allows to A21 more chance than P21 residue to share a hydrogen bund with P17 and L18 neighbor residues (Fig. 4C, D).

Table 2
Punctual mitochondrial variations and its effects detected in the studied familly members.

Patients	Locus	Nucleotide change	Position	Amino acid change	Polyphen prediction	PROVEAN prediction	Phenotypic association
I.1	Mt-ND1	T-C	3847	Syn	–	–	Parkinson disease [17] Congenital cataract [18]
		A-G	4227	Syn	–	–	–
	Mt-ND2	T-C	5333	Syn	–	–	LHON [19]
	Mt-COI	G-A	5913	D-N	Begnin	Neutral	Blood pressure [20]
	Mt-ND4	C-T	11,761	Syn	–	–	–
	Mt-COIII	**G-C**	**9267**	**A-P**	**Probably damaging**	**Deletrious**	–
	ND5	C-T	12,705	Syn	–	–	LHON and dystonia [21] MELAS and Parkinson disease [22] Hearing loss [13]
		A-G	12,850	I–V	Pobably damaging	Neutral	–
II.1	Mt-ND1	G-A	3438	Syn	–	–	–
		T-C	3847	Syn	–	–	Parkinson disease [17] Congenital cataract [18]
		C-T	3963	Syn	–	–	–
	Mt-ND2	C-T	4805	Syn	–	–	–
	Mt-OLR	G-A	5746	Non coding	–	–	–
	Mt-COI	G-A	5913	D-N	Begnin	Neutral	Blood pressure [20]
	Mt-ND4	A-G	10,819	Syn	–	–	–
	Mt-COIII	**G-C**	**9267**	**A-P**	**Probably damaging**	**Deletrious**	–
II.3	Mt-ND1	T-C	3847	Syn	–	–	Parkinson disease [17] Congenital cataract [18]
	Mt-NC7	G-A	8292	Non-coding	–	–	
	Mt-COI	G-A	5913	D-N	Begnin	Neutral	Blood pressure [20]
	Mt-COIII	**G-C**	**9267**	**A-P**	**Probably damaging**	**Deletrious**	–
	Mt-ND5	A-G	12,950	N-S	Begnin	Neutral	–
		C-T	13,188	Syn	–	–	Parkinson disease [17]
		A-G	13,576	I–V	Possibly damaging	Neutral	–
		C-A	13,695	Syn	–	–	–
III.1	Mt-ND1	T-C	3847	Syn	–	–	Parkinson disease [17] Congenital cataract [18]
	Mt-ND2	A-C	5120	Syn	–	–	–
	Mt-COI	G-A	5913	D-N	Begnin	Neutral	Blood pressure [20]
		C-T	7028	Syn	–	–	–
		A-G	7325	Syn	–	–	–
		G-A	7362	E-K	Begnin	Neutral	–
		A-G	7363	E-G	Begnin	Neutral	–
	Mt-ND4	A-G	10,819	Syn	–	–	–
		T-C	10,873	Syn	–	–	MELAS and Parkinson disease [22]
		T-C	11,017	Syn	–	–	MELAS nd Parkinson disease [22]
		A-G	11,314	Syn	–	–	–
		C-T	11,761	Syn	–	–	–
	Mt-ND5	C-T	13,188	Syn	–	–	–
		C-T	13,695	Syn	–	–	–
	Mt-ND6	T-C	14,212	Syn	–	–	–

LHON: Leber hereditary optic neuropathy.
MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes.
Novel variations are highlighted and written in bold.

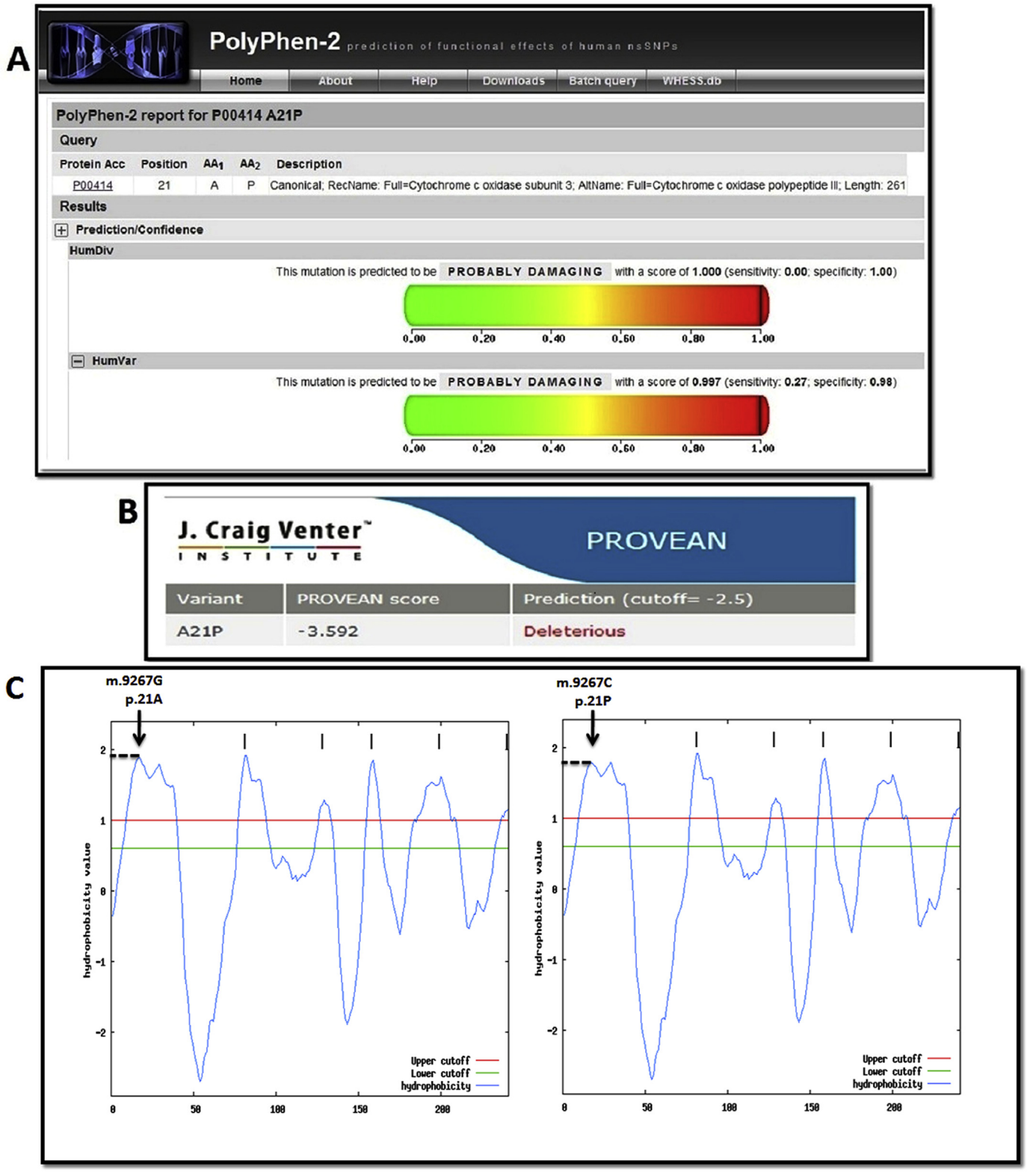

Fig. 3. (A) Results of the PolyPhen analysis predicting the possible impact of the A21P substitution on three-dimensional MT-COIII protein structure. (B) PROVEAN prediction of A21P variation on protein's function. (C) A hydropathy plot for the COIII subunit. The hydrophobicity of the wild-type COIII subunit (on the left) compared to the mutant form (on the right), mutated site shown by arrow.

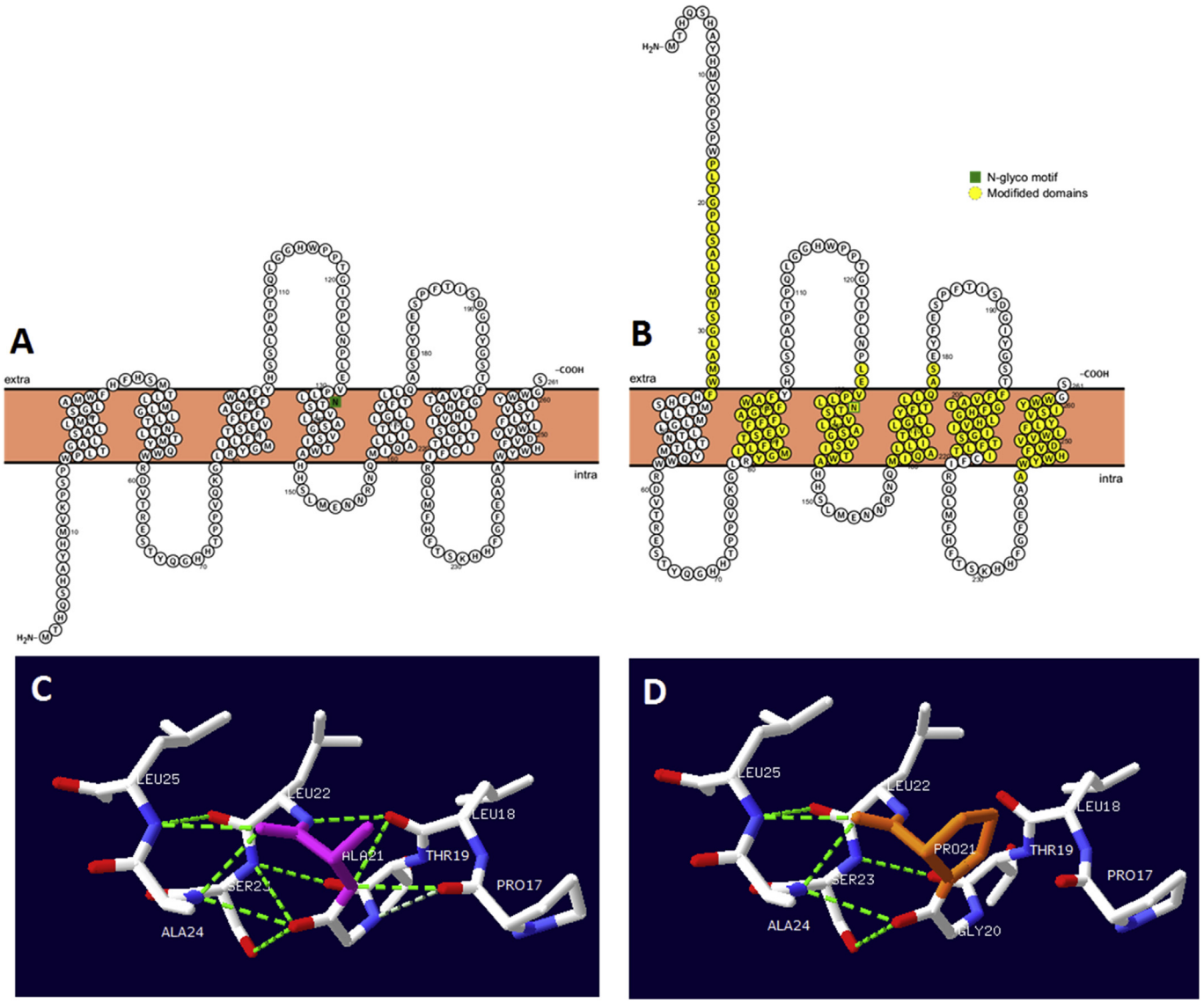

Fig. 4. Predicted transmembrane structures of human wild-type MT-COIII protein (A) and in presence of the novel m. 9267G>C mutation (B) by the Protter program. This substitution substantially reduced the number and the hydrophobicity of the intramembrane helical domain, but also influenced both contiguous matrix and intermembrane space coils. Model generation by molecular modeling by homology of MT-COIII (C): Model of 17–25 region of the wild MT-COIII protein (21A): Hydrogen bound between, P17, L18, A24 and L25 residues. (D) Mutated MT-COIII protein (21P): Hydrogen bound between A24 and L25 residues.

4. Discussion

Since the clinical investigation showed that the studied family presents mitochondrial diabetes and deafness, we carried out a screening of the mitochondrial genome which identified the presence of a novel variation and reported polymorphisms.

In fact, the screening of the *MT-CO* gene showed the presence of a novel m.9267G>C (p.A21P) heteroplasmic substitution in a highly conserved domain of the mitochondrial *COIII* gene which was absent in 100 Tunisian healthy individuals and in unaffected son (III.1). The p.A21P was not reported in the mtDB Human Mitochondrial Genome Database (http://www.mtdb.igp.uu.se/).

PolyPhen-2 analysis predicted that this substitution is "probably damaging" and PROVEAN software showed that p.A21P is « deleterious » and it can modify MT-COIII subunit function. The affected cytochrome c oxidase subunit III is 1 of 3 mitochondrial DNA encoded subunits (MT-COI, MT-COII, and MT-COIII) of the respiratory complex IV. In fact, this complex is the terminal enzyme in the respiratory chain, located in the inner membrane of mitochondria. 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 gradient is used elsewhere, for instance in the synthesis of ATP [23].

The p.A21P mutation decreased the transmembrane helix hydrophobicity (Toppred prediction), reduced the number of domains to six and moved the N terminal and the first transmembrane domain to extracellular space (PROTTER prediction). This alteration in COXIII protein proprieties could change its function and activity then reducing the activity of the whole fourth oxidative phosphorylation complex.

In addition, the highly conservation of the amino acid at the position 21 suggests that its replacement within the α-helix could change the hydrophobic part of the complex IV and consequently the tertiary structure of the COIII subunit. Indeed, generation of a 3D model of MT-COIII revealed that p.A21P substitution, caused by this mutation, may affect the function and the stability of MT-COIII

protein, as Proline's side chain α-N lost 4 hydrogen bonds with P17, L18, G20 and L22, also, Proline's cyclic structure could break the α-helix. This deviation in the protein structure could modify the protein assembly of the 3 mitochondrial subunits of COX, since COIII is the last COX subunit to be incorporated into the membrane arm and its lost is associated with instability of the membrane arm [24]. Otherwise, COXIII is an active participant in proton translocation in COX [25,26]. It could regulate the conformation of COX, and removal of COXIII would result in COX with altered conformational properties and with a decrease in transmembrane proton translocation's mechanism through COX [25]. Also, the physiological importance of the *CO3* has been shown by studies reporting that complex IV is tightly regulated by *CO3* gene expression [27,28] and the COIII subunit is essential for the activity of the fourth complex (COX) [29]. The COXIII region has been highly conserved during evolution, suggesting that it is likely to have functional importance [30]. Within this same region, a point mutation at position 9270, resulting in an amino acid change (L22F), has been described in a patient with cardimyopathy, indicating that defects in this region may associate with disease [31].

In keeping with previously described mtDNA mutations associated with disease, there were three features that suggested that C>T transition mutation at position 9267 was likely to be pathogenic. First, it was heteroplasmic, a feature generally observed in mitochondrial disease associated with mtDNA mutations. Second, it did not occur in 100 healthy control individuals and in the unaffected patient (III.1). Finally, it was situated in a highly conserved domain.

In other hand, the direct sequencing revealed 26 known substitutions (Table 2), which were previously reported in the Human Mitochondrial Database (www.mitomap.org). Among these variations, 6 substitutions were responsible for an amino acids change in several mitochondrial subunits: m.5913G>A (p.D4N), m.7362G>A (p.E487K), m.7363A>G (p.E487G), 12850A>G (p.I172V), 12950A>G (p.N205S) and m.13576A>G (p.I414V). Except the first substitution, all the others were reported as polymorphisms without negative effects on mitochondrial function. Both 3847 T>C in the *MT-ND1* subunit of Complex I and 5913G>A (p.D4N) in the *MT-COI* of Complex IV are inherited variations that were been described in the literature in patients with Parkinson disease and Congenital cataract (m.3847 T>C) and blood pressure (m.5913G>A). These two variations, presented in all family members, are frequent substitutions and may affect the functional properties of the respective OXPHOS complexes of ATP synthesis. Thus, the presence of m.5913G>A associated with the novel m.9267G>C mutation may affect the interaction of the COXI protein with the COXIII which could alter the assembly of the mitochondrial subunits of COX and eventually the proton translocation in this complex [25]. Moreover, this association could modify the expression and the generation of functional enzyme [32] then the activity of cytochrome c oxidase complex [29].

In fact, 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 [33]. According to the clinical observations, we suggest that the novel m.9267G>C mutation has the major impact on the resulting phenotype (mitochondrial diabetes and deafness which is reported in all diabetic's family members). Thus, the m.9267G>C mutation (p.A21P), found in the cytochrome c oxidase subunit III, could be also a cause of the severe chronic renal impairment detected in mitochondrial diabetes patient (II.1): The presence of nephropathy in patient (II.1) can be explained either by the raised rate of heteroplasmy compared to other patients (I.1 and II.3), either by the presence of other nuclear genes responsible of diabetic nephropathy such as the APOE gene.

In addition, 5913G>A mutation in the *MT-COI* of Complex IV is a common mutation in all family members suffering from high blood pressure, diabetes and hearing loss, which is consistent with the results found by Liu and his collaborators [19]. But the presence of 3847 T>C mutation in the *MT-ND1* subunit of Complex I in all patients could be in relation with inherited mitochondrial diabetes since *MT-ND1* is a candidate gene of mitochondrial diabetes type II [34], despite its presence in one unaffected patient.

Our study reported a Tunisian family with maternal history of diabetes and deafness, in whom we detected a novel variation m.9267G>C missense mutation in the mitochondrial *COIII* gene (p.A21P). The m.9267G>C mutation was maternally transmitted and presented in 3 affected patients (I.1, II.1 and II.3) and it could be responsible of the severe nephropathy presented in patient (II.1). In other hand, this mutation could lead to a disruption of the secondary and the 3D structure of cytochrome c oxydase protein. As well, this mutation may affect the functional properties and reduce the activity of the respective OXPHOS complex of ATP synthesis (assembly of mitochondrial COX subunits and translocation of protons).

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

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