Biochemical and Biophysical Research Communications 404 (2011) 504–510

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A novel m.3395A > G missense mutation in the mitochondrial ND1 gene associated with the new tRNA^{Ile} m.4316A > G mutation in a patient with hypertrophic cardiomyopathy and profound hearing loss

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ARTICLE INFO

Article history:
Received 25 November 2010
Available online 6 December 2010

Keywords:
m.3395A > G
m.4316A > G
ND1 gene
tRNA^{Ile} gene
Cardiomyopathy
Hearing loss

ABSTRACT

Mitochondria are essential for early cardiac development and impaired regulation of mitochondrial function was implicated in congenital heart diseases. We described a newborn girl with hypertrophic cardiomyopathy and profound hearing loss. The mtDNA mutational analysis revealed the presence of known polymorphisms associated to cardiomyopathy and/or hearing loss, and 2 novel heteroplasmic mutations: m.3395A > G (Y30C) occurring in a highly conserved aminoacid of the ND1 gene and the m.4316A > G located in the residue A54 of the tRNA^{Ile} gene. These 2 novel variations were absent in 150 controls. All these variants may act synergistically and exert a cumulative negative effect on heart function to generate the cardiomyopathy.

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1. Introduction

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise in young and adult patients at any age, as a result of dysfunction of the mitochondrial respiratory chain [1]. Many pathogenic point mutations have been described in association with different neurological disorders and with respiratory chain deficiency. About 60% of the point mutations affect mitochondrial tRNAs, 35% affect polypeptide subunits of the respiratory chain, and 5% affect mitochondrial ribosomal RNAs [2]. Some mitochondrial disorders only affect a single organ. The most frequently and severely affected organs are those that depend on high-rate aerobic metabolism: brain, skeletal and cardiac muscle, sensory organs, and kidney [3].

Indeed, mitochondrial dysfunction frequently affects the heart and may cause both hypertrophic and dilated cardiomyopathy. The cardiomyopathy is usually a part of a multisystem involvement and may rarely be isolated. In fact, it can be associated with others disorders, such as hearing loss [4], or diabetes mellitus [5–7]. The state may be stable for many years, but rapid deterioration may occur and often lead to congestive heart failure [8].

Mitochondrial cardiomyopathy has been associated with several point mutations of mtDNA in both protein encoded genes and mitochondrial tRNA genes, as well as with recessive mutations in the nuclear-encoded genes (like the nuclear gene coding for the protein NDUFS2, part of complex I, etc.) [9]. Cardiomyopathies due to mtDNA mutations can be sporadic or inherited as maternal traits and follow the rules of mitochondrial genetics.

Abnormal oxidative phosphorylation in childhood may lead to a hypertrophic or dilated cardiomyopathy [10]. When the cardiomyopathy is hypertrophic it may eventually evolve into a dilated form during the course of the disease, a form more frequently reported in adults suffering from this disease [11].

In this study, we reported a Tunisian patient with clinical features of biventricular hypertrophic cardiomyopathy associated with congenital deafness. The mutational analysis revealed 19 reported polymorphisms and 2 novel variations: the m.3395A > G (Y30C, ND1) transition and the m.4316A > G substitution in the mitochondrial tRNA^{Ile} gene.

2. Patient and methods

In this report, we studied a Tunisian family with a patient (II.1) presenting clinical features of cardiomyopathy associated to hearing loss.

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doi:10.1016/j.bbrc.2010.12.012

2.1. Patient

The patient was a 1-year-old girl, born by cesarian section after an uneventful pregnancy to healthy parents. She had an older sister of 5 years-old who had a cardiac arrhythmia in her childhood and another healthy sister of 20 months. Since the first days of live, the studied patient showed a hypertrophic cardiomyopathy. Her blood pressure was 60 mm Hg, with heart rate of 88 bpm and she showed a buccal cyanosis at the age of 2 days. Cardiology consultation showed the presence of mitral murmur and the abdominal investigation showed the absence of hepatomegaly and splenomegaly. The pulmonary, the urogenital and the dermatological examinations were normal.

Laboratory investigations at the age of 10 days revealed raised lactate blood level: 3.24 mmol/l (normal values < 2 mmol/l) and high Lactate dehydrogenase (LDH) rate: 1064 UI/l (normal values: 30–200 UI/l).

An auditory evoked potential (AEP) assessment showed a profound and bilateral cochlear perception disorder and the eye fundus revealed the absence of retinitis pigmentosa.

At the age of 1 month, she suffered from choking and cyanosis during feedings. The echocardiography revealed a biventricular hypertrophy predominating in the right ventricule that is dilated without obstruction with a systolic pulmonary artery pressure (sPAP) of 30 mm Hg. The renal examination and the eye fundus were normal but the auditory evoked potential (AEP) investigation still showed a profound and bilateral cochlear perception disorder.

At the age of 2 months, the echocardiography revealed a moderation of the hypertrophy of the right ventricule but a dramatic regression compared to the neonatal echography with PFO shunt G–D exclusive. In addition, the auditory evoked potential (AEP) examination detected a bilateral sensorineural hearing loss. At the age of 8 months, the auditory brainstem response test revealed the absence of any response at 150 dB confirming a profound and bilateral sensorineural hearing loss.

Besides, a recent clinical examination of her mother and her two sisters had been performed and no disorders were noticed.

In addition, 150 Tunisian healthy individuals from the same ethnocultural group were tested as controls.

2.2. Methods

2.2.1. DNA extraction

After getting informed consent from all the participating family members, total DNA was extracted from peripheral blood using phenol–chloroform standard procedures [12].

2.2.2. PCR amplification and sequencing of the mitochondrial genome

The whole mitochondrial genome was amplified using 24 overlapping pairs of primers [13]. After PCR amplification, each PCR product was purified and sequenced, and then, the resultant sequences were compared with the update Cambridge sequence (GenBank Accession Number: NC_012920).

2.2.3. RFLP analysis

The novel m.3395A > G mutation was confirmed by PCR-RFLP analysis with the *AccI* endonuclease enzyme (Fermentas) and the m.4316A > G mutation in the $tRNA^{Ile}$ gene was also confirmed by PCR-RFLP with the *DdeI* endonuclease enzyme (Fermentas).

2.2.4. Quantification

For the quantification of the heteroplasmic rates of the m.3395A > G and the m.4316A > G mutations, we analyzed the digestion resulting fragments with a UVIDOC-008-XD analyzer.

2.2.5. The sequence alignment and the pathogenicity prediction

The sequence alignment of the mitochondrial NADH deshydrogenase 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 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 [14,15]. The PolyPhen scores of >2.0 indicate the polymorphism is probably damaging to protein function. Scores of 1.5–2.0 are possibly damaging, and scores of <1.5 are likely benign.

To check if the studied protein contains any phosphorylation motif described in the literature (Tyrosine binding motifs and Tyrosine kinase/phosphatase motifs), we used the PhosphoMotif Finder (http://www.hprd.org/FAQ/PhosphoMotif_finder) of the Human Protein Reference Database (http://www.hprd.org/index_html).

For the sequence alignment of mitochondrial $tRNA^{Ile}$, 14 primates sequences from GenBank were used in the interspecific analysis. Then, a conservation index (CI) was calculated by comparing the human nucleotide variants with the other 13 primates. The CI was then defined as the percentage of species from the list of 14 different primate species that have the wild-type nucleotide at the corresponding position [16].

The sequence alignment of the mitochondrial $tRNA^{Ile}$ gene was performed using the Clustal W program. Sequences from the species were obtained from NCBI and the Mammalian Mitochondrial tRNA genes database (http://mamit-trna.u-strasbg.fr/).

2.2.6. Mitochondrial haplogroup analysis

After sequencing the entire mitochondrial DNA, we performed mitochondrial haplogrouping analysis based on the classifications detailed previously [17,18].

3. Results

In this study, we report a Tunisian patient presenting clinical features of mitochondrial cardiomyopathy associated with hearing loss. The mutational analysis of mitochondrial genes associated to cardiomyopathy revealed the absence of any known or new mutation. Thus, we performed a whole mitochondrial genome screening in the DNA extracted from peripheral blood of this patient. The automating sequencing revealed the presence of 19 reported polymorphisms (Table 1). In addition, we detected 2 novel variations in the studied patient: the m.3395A > G transition in the NADH-dehydrogenase subunit 1 (ND1) gene (Fig. 1A) and the m.4316A > G substitution in the mitochondrial $tRNA^{Ile}$ gene (Fig. 2A).

The m.3395A > G transition creates a *AccI* restriction site which is absent in the wild-type DNA. Therefore, we performed a PCR-RFLP analysis in all the patient's family members followed by a quantification of the digested fragments profile with the UVIDOC-008-XD analyzer to quantify the heteroplasmy rates. The results showed the presence of the m.3395A > G mutation in the ND1 gene with a high heteroplasmic load in the proband (II.1: 98%), but also in her mother (I.1: 58%) and her 2 sisters (II.2: 68% and II3: 68.5%) but not in her father (I.2: 0%) (Fig. 1B). This variant was also absent in 150 healthy individuals from the Tunisian population.

This A–G transition converted the Tyrosine (Y) at the position 30 to Cysteine (C) in a highly conserved aminoacid in the polypeptide (Fig. 1C). In addition, PolyPhen analysis predicted that this variant is probably damaging and showed that the hydropathy index changed from +2.286 to −0.875 (score = 3.157) and the number of

Table 1
Punctual mitochondrial variations detected in the studied patient. New variants were highlighted and written in bold.

Locus	Nucleotide change	Position	Aminoacid change	Previously described	Phenotypic association
MT-D-Loop (MT-HV2)	A > G	73	Non coding	Yes	Alzheimer disease; Human thyroid pathology; Prostate cancer
MT-D-Loop (MT-HV2)	T > C	146	Non coding	Yes	LHON; Mitochondrial encephalomyopathy; Muscle disease; *Hearing loss*; Parkinson's disease; auditory neuropathy; Friedreich's ataxia; Ovarian cancer; Esophageal squamous cell carcinoma
MT-D-Loop (MT-HV2)	T > C	152	Non coding	Yes	LHON; encephalomyopathy; Parkinson's disease; *Hearing loss*; Friedreich's ataxia; Mitochondrial encephalomyopathy; Maternally inherited diabetes and deafness; Nasopharyngeal carcinoma; Ovarian cancer; Alzheimer's disease
MT-D-Loop (MT-HV2)	A > G	189	Non coding	Yes	LHON; Parkinson's disease; *Hearing loss*; **Hypertrophic cardiomyopathy**
MT-D-Loop (MT-HV2)	T > C	195	Non coding	Yes	LHON; Parkinson's disease; *Hearing loss*; Sensorineural deafness; Friedreich's ataxia; Nasopharyngeal carcinoma; Ovarian cancer
MT-D-Loop (MT-HV2)	A > G	263	Non coding	Yes	LHON; MELAS; Parkinson's disease; **Infantile cardiomyopathy**; Klinefelter's syndrome; auditory neuropathy; *Hearing loss*; Diabetes and deafness; Friedreich's ataxia; **Hypertrophic cardiomyopathy**; Encephalomyopathy; Nasopharyngeal carcinoma; Ovarian cancer; MELAS
MT-D-Loop (MT-HV2)	insCC	309	Non coding	Yes	LHON; Parkinson's disease; *Hearing loss*
MT-D-Loop (MT-HV2)	insC	315	Non coding	Yes	LHON; Parkinson's disease; **Infantile cardiomyopathy**; Alzheimer's disease; *Hearing loss*; Ovarian cancer
MT-RNR1	A > G	750	Consensus	Yes	LHON; MELAS; **Hypertrophic cardiomyopathy**; Parkinson's disease; Mitochondrial encephalomyopathy; Type 2 diabetes mellitus; **Infantile cardiomyopathy**; *Hearing loss*; Chronic progressive external ophthalmoplegia; Maternally inherited hypertension; **Left ventricular noncompaction**
MT-RNR1	G > A	769	Non coding	Yes	LHON; *Hearing loss*; **Left ventricular noncompaction**
MT-RNR1	A > G	1018	Non coding	Yes	*Hearing loss*; **Left ventricular noncompaction**
MT-ND1	**A > G**	**3395**	**Y–C**	**NO**	
MT-ND1	A > G	4104	Syn	Yes	Prostate cancer; Parkinson disease
MT-TI	**A > G**	**4316**	**Non-coding**	**NO**	
MT-ND2	A > G	4769	Syn	Yes	LHON; MELAS; **Hypertrophic cardiomyopathy**; Parkinson disease; Mitochondrial encephalomyopathy; **Infantile cardiomyopathy**; *Hearing loss*; Leigh syndrome
MT-CO2	G > A	8206	Syn	Yes	**Mitochondrial cardiomyopathy**
MT-ATP6	A > G	8701	T–A	Yes	LHON; **Hypertrophic cardiomyopathy;** Parkinson disease; MELAS; Mitochondrial encephalomyopathy; **Infantile cardiomyopathy**; *Hearing loss*; Alzheimer's disease
MT-ATP6	A > G	8860	T–A	Yes	LHON; MELAS; *Hearing loss*; **Idiopathic cardiomyopathy**; Parkinson disease; Mitochondrial encephalomyopathy; **Infantile cardiomyopathy**; Type 2 diabetes mellitus; Leigh syndrome
MT-CO3	A > G	9221	Syn	Yes	LHON; Parkinson disease; Mitochondrial encephalomyopathy; *Hearing loss*; Maternally inherited diabetes and deafness; MELAS; Alzheimer's disease
MT-CO3	T > C	9540	Syn	Yes	
MT-ND4	G > A	11719	Syn	Yes	LHON; MELAS; **Cardiomyopathy**; Parkinson disease; mitochondrial encephalomyopathy; **Infantile cardiomyopathy**; *Hearing loss*; Leigh syndrome; Maternally inherited hypertension

LHON: Leber hereditary optic neuropathy.
MELAS: mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes.

structures of the 3D protein decreased from 10 to 0 (Fig. 1D). Besides, the m.3395A > G mutation is located in an extramembrane loop linking the helix A and the helix B (Fig. 1E).

The PhosphoMotif Finder analysis of the mitochondrial ND1 protein showed that the Tyr30 residue is implicated in "LGYMQL" (28–33 position) and "YMQL" (30–33) motifs. Consequently, for the mutated protein, both numbers of Tyrosine binding motifs and Tyrosine kinase/phosphatase motifs decreased, respectively from 10 to 8 and from 27 to 25.

On the other hand, the m.4316A > G transition in the mitochondrial $tRNA^{Ile}$ gene creates a novel *DdeI* restriction site. Thus, we performed a PCR-RFLP analysis with this corresponding enzyme and the digestion resulting fragments were analysed with a UVI-DOC-008-XD analyser to quantify the heteroplasmy rates. The quantification results showed the presence of the m.4316A > G substitution in heteroplasmic forms in the proband (II.1: 97%), in the mother (I.1: 82%) and her 2 sisters (II.2: 42% and II3: 46%). However, this variation was absent in the father (I.2: 0%) (Fig. 2B) and in 150 healthy individuals.

The m.4316A > G is located in the residue A54 in the T-loop of the mitochondrial $tRNA^{Ile}$ (Fig. 2C) and it affects an adenine which is highly conserved in 14 primates (Fig. 2D) and other species (Fig. 2E) defining a conservation index (CI) of 100% (14/14) [16]. This substitution was also assigned a score of pathogenicity of '14' in accordance to the scoring criteria applied to mitochondrial tRNA mutations reported by Scaglia and Wong. This score classify this mutation in the category of "possible pathogenicity" according to this same report [19].

In addition, the sequencing of the whole mitochondrial genome showed the presence of 19 known mitochondrial polymorphisms which allowed us to classify the studied patient under the haplogroup L1 (Table 1).

4. Discussion

We described a 1-year-old Tunisian patient with hypertrophic cardiomyopathy and a profound and bilateral sensorineural hearing loss. The sequencing of the whole mtDNA showed 2 novel het-

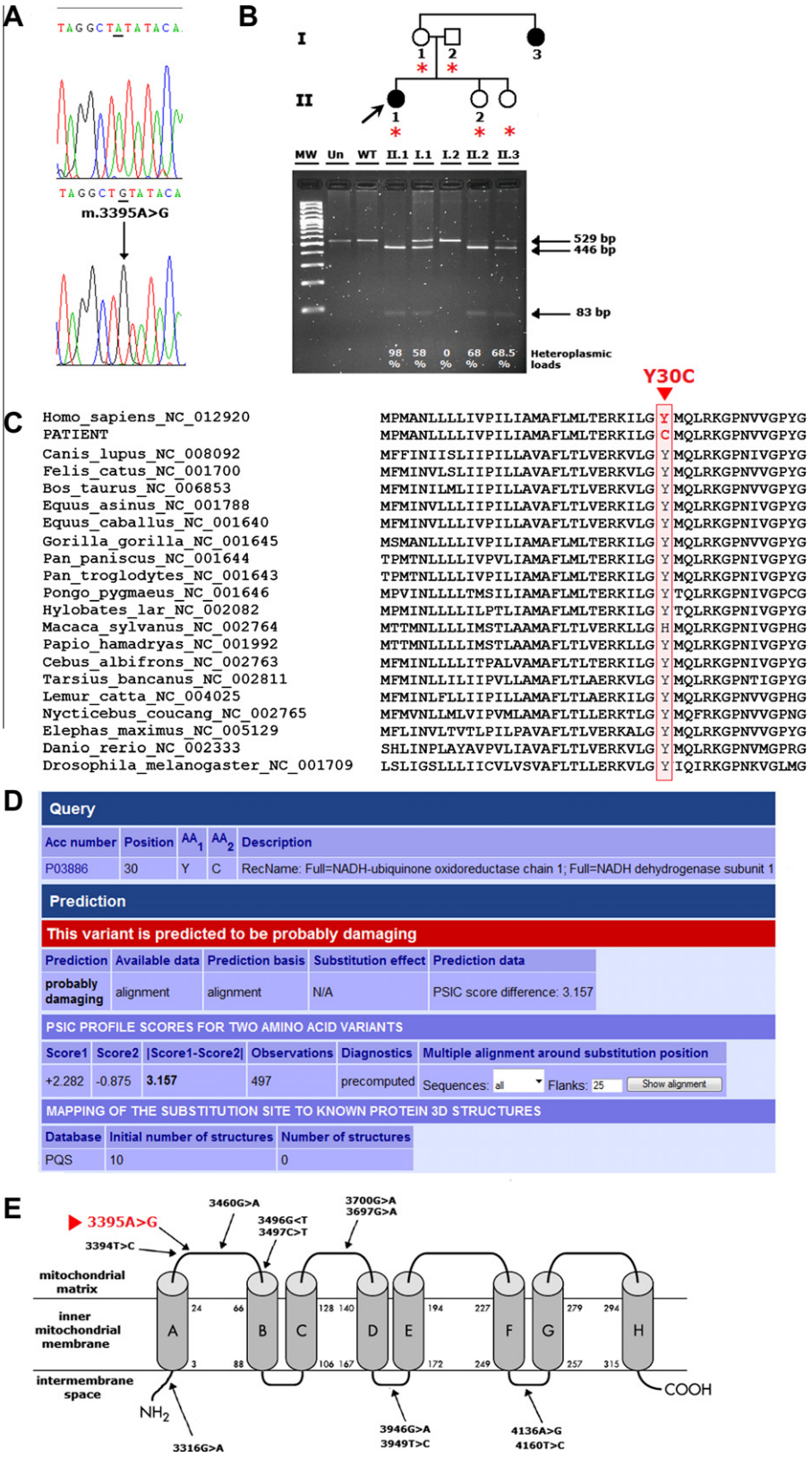

Fig. 1. (A) Sequence chromatograms showing the presence of the m.3395A > G mutation in the mitochondrial ND1 gene in the studied patient (on the bottom) and its absence in a control (on the top). (B) PCR-RFLP analysis with *AccI* restriction enzyme in DNA extracted from blood leucocytes in the members of the Tunisian family with the m.3395A > G mutation: a 529 bp PCR fragment is digested with *AccI*. The digestion in a mutated DNA shows 2 fragments of 446 and 83 bp, whereas the digestion of a wild-type DNA shows an undigested fragment corresponding to the PCR product of 939 bp. MW: DNA Ladder 100 bp; Un: Undigested PCR product; WT: digestion in wild type. (C) Alignment of the ND1 protein in different species showing the conservation of the aminoacid 30; the mutated amino acid is framed. (D) Results of the PolyPhen analysis predicting the possible impact of the Y30C substitution on 3D ND1 structure. (E) Molecular structure of ND1 and location of m.3395A > G mutation in the extramembrane of the protein.

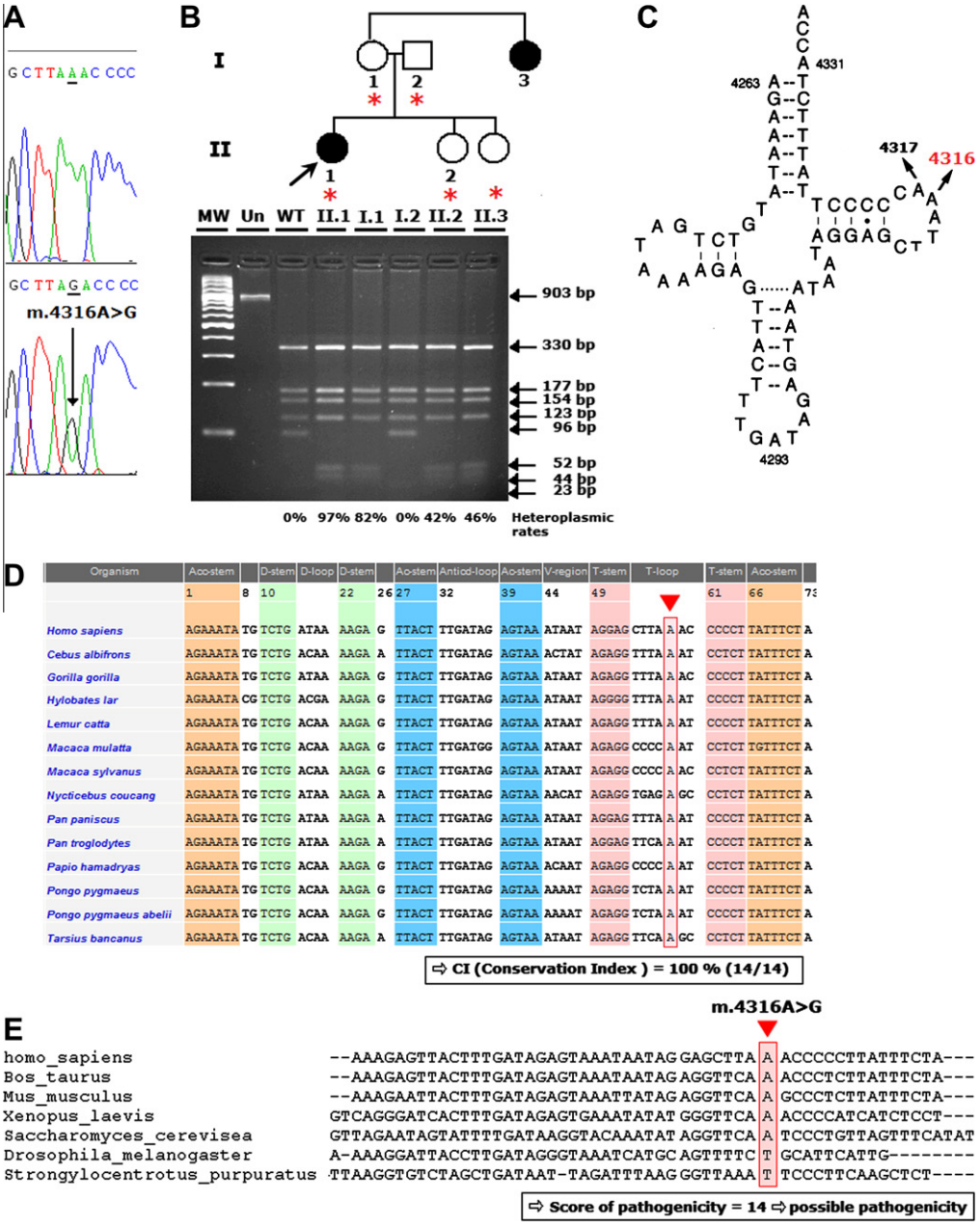

Fig. 2. (A) Sequence chromatograms showing the presence of the m.4316A > G mutation in the mitochondrial $tRNA^{Ile}$ gene in the studied patient (on the bottom) and its absence in a control (on the top). (B) PCR-RFLP analysis with *DdeI* restriction enzyme in DNA extracted in the members of the patient's family with the m.4316A > G mutation: a 903 bp PCR fragment is digested with *DdeI*. The digestion in a wild-type DNA shows 6 fragments of 330, 177, 154, 123, 96 and 23 bp, whereas the mutated DNA is cleaved in 7 fragments of 330, 177, 154, 123, 52, 44 and 23 pb in length. MW: DNA Ladder 100 bp; Un: Undigested PCR product. WT: digestion in wild type. (C) Schematic secondary structure representation of the wild type mitochondrial $tRNA^{Ile}$, showing the position of the m.4316A > G. (D) Alignment of the $tRNA^{Ile}$ gene in 14 primates showing the conservation of the adenine at nucleotide 4316. (E) Alignment of the $tRNA^{Ile}$ gene in different others species showing the conservation of the nucleotide 4316.

eroplasmic variations: the m.3395A > G transition in the ND1 gene (Fig. 1A) and the m.4316A > G variation in the $tRNA^{Ile}$ gene (Fig. 2A).

We also detected known nucleotide variations (Table 1). Some of these polymorphisms were reported to be associated with hypertrophic cardiomyopathy, infantile cardiomyopathy or other heart disorders (www.mitomap.org). Indeed, it has been demonstrated that a polymorphism in the mitochondrial D-loop can be associated with increased risk of developing cardiomyopathy [20].

The new m.3395A > G mutation (Y30C) detected in the mitochondrial ND1 was found in heteroplasmic form, with much higher level in patient (98%) than in unaffected relatives (Fig. 1B) and It was absent in large number of controls.

ND1 is one of the most important and conserved complex I subunits [21] which is responsible for electron transfer from NADH to ubiquinone. Situated in the hydrophobic arm of the L shaped complex I protein, it forms eight transmembrane spanning domains that are postulated to be involved in ubiquinone binding and proton pumping [22] processes known to be disrupted by mutations. Thus, complex I deficiency is a major cause of mitochondrial disorder and clinical presentations include a wide spectrum of phenotypes: fatal infantile lactic acidosis, cardiomyopathy, leucoencephalopathy, pure myopathy and hepatopathy [23].

More than 20 pathogenic mutations in the MT-ND1 gene were reported in patients with LHON and MELAS. Moreover, the m.3310C > T mutation, which decreases significantly the mitochon-

drial complex I activity, was described as a pathogenic mutation in a patient presenting with diabetes mellitus and hypertrophic cardiomyopathy [5].

Thus, the novel mitochondrial ND1 m.3395A > G mutation (Y30C) described in our reported patient with hypertrophic cardiomyopathy and hearing loss seems to be pathogenic. Firstly, it affected a highly conserved Tyrosine of the subunit 1 of the complex I (Tyr30) which indicates the functional importance of this residue (Fig. 1C) located in the loop between helix A and helix B. Secondly, it was detected in high heteroplasmic form in patient and not in 150 healthy individuals. Thirdly, PolyPhen analysis predicted that this mutation is probably damaging with a score of 3.157 and a number of structures of the 3D protein which decreased from 10 to 0 (Fig. 1D). And finally, the PhosphoMotif Finder analysis of the mitochondrial ND1 protein showed that the Tyr30 residue is implicated in "LGYMQL" (28–33 position) and "YMQL" (30–33) motifs. Thus, the Y30C substitution lead to a decrease of the number of Tyrosine binding motifs and Tyrosine kinase/phosphatase motifs for the mutated protein which confirm the importance of this residue in phosphorylation. Indeed, Tyrosine residue is known to be an eventual site of phosphorylation as well as Serine and threonine residues.

Further support for the pathogenic relevance of m.3395T > C comes from another mutation changing the same amino acid (Tyr30). In fact, the m.3394A > G which substitutes the Tyrosine to Histidine has been described in patients with NIDDM (non-insulin dependent diabetes mellitus) and LHON (Leber hereditary optic neuropathy) [24,25].

On the other hand, we detected another undescribed mitochondrial variation (m.4316A > G) in the studied patient with hypertrophic cardiomyopathy and profound hearing loss. It was an A–G transition in the A54 residue of the T-loop of the mitochondrial $tRNA^{Ile}$ gene (Fig. 2C). This substitution was detected with a high heteroplasmic rate in the patient (98%) and with lower rates in her mother (82%) and sisters (42% and 46%) (Fig. 2B) but it was totally absent in 150 healthy individuals. This transition affects a conserved adenine of the $tRNA^{Ile}$ gene with a conservation index of 100% and a score of pathogenicity of '14' classifying this mutation under the category of "possible pathogenicity" [19].

The mitochondrial $tRNA^{Ile}$ gene is the third most commonly mutated among the mitochondrial tRNA genes. The m.4316A > G mutation is, to our knowledge, the sixth mutation in the mitochondrial $tRNA^{Ile}$ gene reported in patients with cardiomyopathies after the m.4269A > G, m.4295A > G, m.4300A > G, m.4317A > G, m.4320C > T mutations [26–30].

Two studies revealed that the cardiomyopathy associated mutation m.4317A > G, which is just next the m.4316A > G mutation described in our report induces an aberrant secondary structure in the T-stem due to the instability introduced by the native CA pair [19]. This aberrant structure may decrease aminoacylation and it seems that for this class of mutations in this particular tRNA, a weak structure may amplify the effect of the mutation.

In the literature, several mtDNA mutations were found to be associated with cardiomyopathies in newborns and adults. These mutations were often in heteroplasmic form: m.3243A > G, m.3260A > G and m.3303C > T [31–34], m.12297T > A [35], m.4269A > G and m.4317A > G [26], m.8296A > G [36] and the m.3310C > T and m.3337G > A [37,5,6]. Recently, a novel m.8528T > C mutation in the overlapping region of ATP6 and ATP8 was identified from a patient with infantile cardiomyopathy presenting LVNC (Left ventricular noncompaction) [38].

Our studied patient presented 2 novel mutations which may act synergistically or exert a cumulative negative effect on heart function and then, their presence could predispose carriers to cardiomyopathy. In addition, the polymorphisms found in this patient, and which are described to be associated to hypertrophic cardiomyopathy and/or hearing loss (Table 1) may also be responsible for the disorder especially for the polymorphisms located in important regions of the mitochondrial D-loop.

The association or the co-segregation of 2 or 3 mutations was described in previous studies. Indeed, several reports demonstrated that some mitochondrial mutations in the ND1, ND4 or 12S rRNA genes are not individually sufficient to generate the clinical phenotype, and additional tRNA mutations were implicated to influence the phenotypic manifestation. Indeed, the $tRNA^{Glu}$ m.14693A > G or $tRNA^{Met}$ m.4435A > G, respectively, were implicated to influence the phenotypic manifestation of the primary LHON-associated mutations like ND1 m.3460G > A or ND4 m.11778G > A in Chinese families [39–41]. Moreover, the mitochondrial mutations m.5628T > C in $tRNA^{Ala}$ or G7444A in $tRNA^{Ser(UCN)}$ may have a modifying role in the phenotypic manifestation of the 12S rRNA m.1494C > T mutation in hearing loss [42,43]. The exact pathogenic role of these secondary mutations remains misunderstood.

Our report described a Tunisian newborn girl presented with hypertrophic cardiomyopathy and profound and bilateral hearing loss. The mutational analysis of the mtDNA revealed the presence of known polymorphisms which are associated to cardiomyopathy and/or hearing loss, as well as 2 novel heteroplasmic mutations which may act synergistically and exert a cumulative negative effect on heart function.

Acknowledgments

We thank the patient and her family for their cooperation in the present study. This work was supported by The Ministry of the Higher Education and the Scientific Research in Tunisia.

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