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Two novel mutations in *COII* and *tRNA^{His}* mitochondrial genes in asthenozoospermic infertiles men

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ABSTRACT

In this study we performed a systematic sequence analysis of 7 mitochondrial genes (cytochrome oxidase I, cytochrome oxidase II, cytochrome oxidase III, adenosine triphosphate synthase6, ATP synthase8, cytochrome b and *tRNA^{His}*) in 64 infertile men suffering from asthenospermia ($n = 31$) in comparison to normospermic infertile men ($n = 33$) from Tunisian population. A total of 92 nucleotide substitutions in sperm mitochondrial DNA were found; 88 of them were previously identified and reported in the human mitochondrial DNA database (www.mitomap.org) and 4 were novel. We also detected in 4 asthenospermic patients a double novels mutations, the first was found in *COXII* gene (m.8021 G/A) that was absent in normospermic infertile men. This mutation substituting the Isoleucine at position 146 to Valine in a conserved amino acid in the transmembrane functional domain of the protein. And the second was detected in the *tRNA^{His}* gene (m.12187C>A) this mutation was found in homoplasmic state and was absent in normospermic patients. It was conserved throughout evolution and affects a wobble adenine in the T-loop region at the 54 codon of mitochondrial tRNA^{His} .

1. Introduction

Approximately, 30–40% men in reproductive age have qualitative or quantitative defect in the sperm production. In about 50% of these cases, infertility can be attributed to low sperm motility (asthenozoospermia) or/and low sperm count (oligospermia) [1]. Mutation and depletion of mitochondrial DNA (mtDNA) are associated with poor motility and diminished fertility of human sperm [2,3]. Mitochondrial DNA is linked to electron transport system and is thus vulnerable to free radical damage and some of its components mutate 100 times more rapidly than nuclear DNA (DNAn) [4].

Sperms require greater amount of energy for their survival and proper functioning, so sperm mitochondrion are uniquely placed in the mild piece to provide energy for sperm motility [5]. About 70–80 mitochondria are present in a single mammalian sperm [6,7]. Mitochondrion is the only organelle in the sperm which has its own genome: mtDNA. Human mtDNA is a 16569 bp double stranded circular DNA molecule coding for 2 rRNAs, 22 tRNAs and 13 polypeptides [8]. The mitochondrial genome consists of no introns but only exons, every point mutation or deletion has the capacity to affect the mitochondria function of cellular respiration support. Mitochondrial DNA also lacks the protection of histones or DNA-binding proteins and is believed to have only a very basic repair mechanism.

Mitochondrial DNA mutations identified in several studies have been associated with poor semen quality. Indeed, absence or abnormal mitochondrion has been reported in asthenozoospeermic men [9,10].

Because there is no study investigating the role of mtDNA mutations in male infertility in the Tunisian population, we performed a systematic sequence analysis of 7 somatic mitochondrial genes involved in oxidative phosphorylation [cytochrome oxidase I (*COI*), cytochrome oxidase II (*COII*), cytochrome oxidase III (*COIII*), adenosine triphosphate synthase6 (*ATPase6*), ATP synthase8 (*ATPase8*) and cytochrome b (*Cytb*) and tRNA^{His}] in 64 Tunisian infertile patients. The aim of this study was to determine the frequency of mtDNA mutations and their eventual effect on sperm motility in Tunisian infertile patients.

2. Materials and methods

2.1. Patients

We included 64 male partners from consecutively enrolled couples who had their first infertility consultation in the Reproductive

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Biology laboratory at the faculty of medicine of Sfax (Tunisia). Patients were aged between 23 and 57 years and not related. They were picked up in an andrological evaluation. Semen analysis was performed according to WHO recommendations [WHO]. At least one sperm sample per individual, was collected after 3–5 days abstinence and analyzed after liquefaction at 37 °C during 30′. The spermiogram included the evaluation of sperm concentration and the percentage of motile spermatozoa [11]. Sperm motility analysis was performed at room temperature, and each spermatozoon was graded as "a," "b," "c," or "d," according to whether it showed rapid progressive motility, slow or sluggish progressive motility, non progressive motility, or no motility at all.

Of the 64 patients, sperm count revealed normospermia (concentration of spermatozoa $>20 \times 10^6$/mL and total progressive motility >50%) in 33 patients and asthenospermia (total progressive motility <50%) in 31 patients.

2.2. Extraction of total DNA

DNA from all experimental samples was isolated using a QIAamp DNA Mini Kit (Qiagen; Cat. No. 51304). Briefly, the sperm pellet was resuspended in sterile water and mixed with lysis solution containing 100 mg/ml proteinase K and 40 mmol/L dithiothreitol. Lysis was performed at 55 °C for 2–3 h with gentle agitation. Magnetic glass particles were added to the lysates to bind the DNA. Bound DNA was then washed and eluted from the particles.

2.3. Mutational analysis of mitochondrial genes

2.3.1. Polymerase chain reaction (PCR)

PCR amplification of the mitochondrial genes *COI*, *COII*, *COIII*, *ATPase6*, *ATPase8*, and *Cytb* and the flanking regions was performed using the primer sets as described in Table 1. PCR was performed in a thermal cycler [Gene-Amp PCR System 9700 (Applied Biosystems)] in a final volume of 50 µL using 200 ng DNA, 8 pmol of each primer, 2 mM MgCl2, 500 µM dNTP, 1xPCR buffer and 2 U Taq DNA polymerase (Promega). PCR conditions were as following: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation (94 °C, 1 min), annealing (56.5 °C, 1 min), extension (72 °C, 1 min) and a final extension at 72 °C for 5 min.

For the analysis of $tRNA^{His}$ gene, DNA fragment spanning the entire of $tRNA^{His}$ gene was amplified by PCR using oligodeoxynucleotides corresponding to the mtDNA at positions (11929 – 11948) (mt-18F 5′ TATCACTCTCCTACTTACAG 3′) and (12774–12793) (mt-18R 5′ AGAAGGATATAATTCCTACG 3′). PCR reactions were performed as previously reported [12].

2.3.2. Sequencing

After PCR amplification, each product was purified using NucleoSpin (MACHEREY–NAGEL) and sequenced on both strands. The regions containing putative novel polymorphisms were amplified and sequenced again on both strands to exclude that they were PCR artifacts. Sequencing reaction was performed in an ABI PRISM 3100-Avant automated DNA sequencer using the BigDye Terminator Cycle Sequencing reaction kit v1.1. The resultant sequences were compared with the updated Cambridge sequence (GenBank accession number, NC_012920).

2.3.3. The sequence alignment and the pathogenicity prediction

The blast homology searches were performed using the programs available at the National Center for Biotechnology Information Web site compared with the wild-type sequence.

When mutation was detected, the pathogenicity prediction was assessed by performing sequence alignment using the ClustalW program (http://bioinfo.hku.hk/services/analyseq/cgi bin/clustalw_in.pl).

2.3.4. Quantification of the m.8021A>G mutation heteroplasmy loads

Quantification of the mutant mtDNA with the mitochondrial m.8021A>G mutation was carried out using PCR–restriction fragment length polymorphism (RFLP) analysis. After an amplification of a 960 base pair (bp) fragment of the mitochondrial *COII* gene, PCR product was digested with 10 U of the MspI restriction endonuclease (Amersham), separated through 2% agarose gel, and visualized with ethidium bromide in UV. The m.8021A>G mutation creates a novel MspI restriction site that was absent in the wild type. Thus, the digestion in a mutated DNA shows 4

Table 1
Primers used for the amplification of mitochondrial genes *COI*, *COII*, *COIII*, *ATPase8*, *ATPase6* and *Cytb*.

Gene	Primer	Sequence	Sise (bp)	PCR product size (bp)
COI	mt-9F	5′ GAGGCCTAACCCCTGTCTTT 3′	20	827
	mt-9 R	5′ ATTCCGAAGCCTGGTAGGAT 3′	20	
	mt-10F	5′ CTCTTCGTCTGATCCGTCCT 3′	20	855
	mt-10R	5′AGCGAAGGCTTCTCAAATCA 3′	20	
COII	mt-11F	5′ACGAGTACACCGACTACGGC 3′	20	986
	mt-11R	5′ TGGGTGGTTGGTGTAAATGA 3′	20	
	mt-12F	5′ACGAGTACACCGACTACGGC 3′	20	909
	mt-12R	5′ TGGGTGGTTGGTGTAAATGA 3′	20	
COIII	mt-14F	5′CCCACCAATCACATGCCTAhT 3′	20	948
	mt-14R	5′TGTAGCCGTTGAGTTGTGGT 3′	20	
ATPase8 and ATPase6	mt-13F	5′ TTTCCCCCTCTATTGATCCC 3′	20	816
	mt-13R	5′ GTGGCCTTGGTATGTGCTTT 3′	20	
Cytb	mt-22F	5′ TGAAACTTCGGCTCACTCCT 3′	20	1162
	mt-22R	5′ AGCTTTGGGTGCTAATGGTG 3′	20	

Table 2
Number of polymorphisms SNPs in the asthenozoospermic and normozoospermic infertile patients.

Groupes of patients	COI	COII	COIII	ATPase6	ATPase8	Cytb	Total
Asthenospermics (n = 31)	25	9	14	6	3	15	72
Normospermics (n = 33)	0	3	0	0	2	5	10
Asthenospermics + Normospermics	0	3	0	2	1	4	10
Total	25	15	14	8	6	24	92

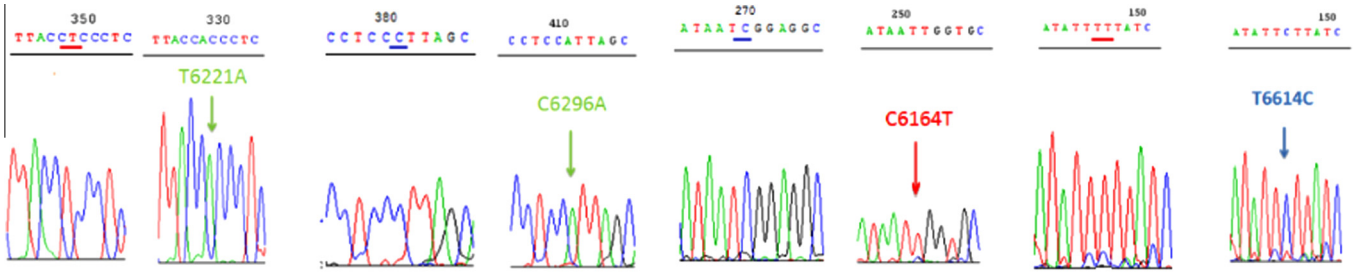


Fig. 1. Sequence chromatograms showing the 4 novel polymorphisms (T6221A, C6296A, C6164T and T6614C) detected in mitochondrial COI gene in Tunisian population. Nucleotide variants are underlined.

fragments of 670, 158, 94 and 38 bp, whereas the digestion of a wild-type DNA shows 3 fragments of 670, 252 and 38 bp. This digestion was also performed in a positive fragment (harboring an MspI restriction site) to be sure that digestion was completed and not partial. The resulting fragments were then analyzed with a UVIDOC-008-XD analyzer to quantify the heteroplasmy rate of the mutant mtDNA.

2.3.5. Secondary structure prediction and sequence alignment

Secondary structures of the wild type and mutated human mitochondrial $tRNA^{His}$ were generated using the RNAfold software from the Vienna RNA package [13]. RNAfold predicted the RNA secondary structure based on minimum energy requirements and pair probabilities. The sequence alignement of mitochondrial $tRNA^{His}$ gene was performed using ClustalW program (http://align.genome.jp/sit-bin/clustalw). Sequences from different species were obtained from NCBI and the Mammalian Mitochondrial tRNA Genes database: (http://mamit-trna.u-strasbg.fr/).

3. Results

Mitochondrial DNA sequencing of the 7 studied genes (*COI*, *COII*, *COIII*, *ATPase6*, *ATPase8*, *Cytb* and $tRNA^{His}$) with their flanking regions in all the 64 patients revealed a total of 92 nucleotide substitutions in sperm mtDNA as shown in Table 2. Of the 92 nucleotide changes, 88 were previously identified and reported in the human mitochondrial DNA database (www.mitomap.org) and 4 were novel. These 4 nucleotides changes were detected in *COI* gene (T6221A, C6296A, C6164T and T6614C) and all of them are synonymes (Fig. 1). We submitted these novel polymorphisms on the human mitochondrial genome database: www.mitomap.org with the following and respective references: (#20140520001, #20140520002, #20140520003, #20140520004) and they can be accessed at: http://www.mitomap.org/cgi-bin/ mitomap/tbl15gen.pl.

Seventy-two of the 92 nucleotide substitutions, were detected in asthenospermic patients, 10 substitutions in normospermic patients and finally 10 substitutions in the two groups of patients (asthenospermic and normospermic) (Table 2). Of the 92 nucleotide changes, 25 substitutions created missense mutations (4 in *COI*, 2 in *COII*, 4 in *COIII*, 5 in *ATPase6*, 3 in *ATPase8* and 7 in *Cytb*) while the rest were silent mutations (Table 3).

Among the 31 asthenozoospermic men an A>G transition at nucleotide 8860 was found in 20 patients and another transition A>G at nucleotide 8701 was detected in 16 cases.

The greatest number of substitutions was seen in *COI* gene ($n = 25$) and in the *Cytb* ($n = 24$). All the substitutions in *COI* ($n = 25$) and *COIII* ($n = 14$) were found only in asthenospermic men (Table 2).

We also detected a novel missense mitochondrial mutation (m.8021A>G) in *COII* gene (Fig. 2A) in 4 asthenospermic patients

Table 3
The mtDNA variations detected in the mitochondrial *COI*, *COII*, *COIII*, *ATPase6*, *ATPase8* and *Cytb* genes.

Locus	Nucleotide change	Position	Aminoacid change	Reported in mitomap
MT-COI	T>C	5999	Syn	Yes
	G>A	6026	Syn	Yes
	A>G	6047	Syn	Yes
	C>T	6146	Syn	Novel
	G>A	6150	V-I	Yes
	T>C	6221	Syn	Yes
	T>C	6221	Syn	Novel
	T>C	6253	M-T	Yes
	G>A	6267	A-T	Yes
	C>A	6296	Syn	Novel
	A>G	6575	Syn	Yes
	T>C	6614	Syn	Novel
	T>G	6620	Syn	Yes
	T>C	6671	Syn	Yes
	T>C	6776	Syn	Yes
	T>C	6827	Syn	Yes
	C>T	6848	Syn	Yes
	A>G	6989	Syn	Yes
	C>T	7028	Syn	Yes
	A>G	7055	Syn	Yes
	A>G	7146	T-A	Yes
	C>A	7196	Syn	Yes
	C>T	7256	Syn	Yes
	T>C	7389	Syn	Yes
	G>A	7521	Syn	Yes
MT-COII	A>C	7597	Syn	Yes
	G>A	7598	A-T	Yes
	T>C	7609	Syn	Yes
	G>A	7642	Syn	Yes
	G>A	7664	A-T	Yes
	A>G	7768	Syn	Yes
	A>G	7771	Syn	Yes
	G>A	7789	Syn	Yes
	G>A	7805	Syn	Yes
	C>T	7858	Syn	Yes
	C>T	7867	Syn	Yes
	C>T	8137	Syn	Yes
	G>A	8206	Syn	Yes
	A>G	8248	Syn	Yes
MT-COIII	A>G	9347	Syn	Yes
	T>C	9540	Syn	Yes
	G>A	9755	Syn	Yes
	C>T	9818	Syn	Yes
	G>A	9932	Syn	Yes
	G>A	9477	V-I	Yes
	A>G	9494	Syn	Yes
	C>T	9301	A-V	Yes
	C>T	9302	Syn	Yes
	A>G	9336	M-V	Yes
	A>G	9390	T-A	Yes
	A>G	9425	Syn	Yes
	T>C	9656	Syn	Yes
	T>C	9770	Syn	Yes
MT-ATP6	A>G	8860	T-A	Yes

Table 3 (*continued*)

Locus	Nucleotide change	Position	Aminoacid change	Reported in mitomap
	A>G	8701	T-A	Yes
	C>T	8655	Syn	Yes
	T>C	8868	Syn	Yes
	G>A	8697	Syn	Yes
	C>T	8684	T-I	Yes
	T>C	8618	I-T	Yes
	A>G	8531	N-S	Yes
MT-ATP8	G>A	8392	Syn	Yes
	C>T	8468	Syn	Yes
	T>C	8383	Syn	Yes
	A>G	8435	T-A	Yes
	A>G	8531	T-A	Yes
	T>C	8448	M-T	Yes
MT-Cytb	G>A	15320	Syn	Yes
	T>C	15670	Syn	Yes
	C>T	15265	Syn	Yes
	G>A	15346	Syn	Yes
	A>G	15712	Syn	Yes
	T>C	15784	Syn	Yes
	A>G	15326	Syn	Yes
	C>T	14766	Syn	Yes
	G>A	15301	Syn	Yes
	C>T	14766	Syn	Yes
	T>C	15115	Syn	Yes
	A>G	15607	Syn	Yes
	T>C	15697	Syn	Yes
	G>A	14905	Syn	Yes
	C>G	15247	Syn	Yes
	G>A	15043	Syn	Yes
	T>C	14783	Syn	Yes
	A>G	15218	T-A	Yes
	G>A	15257	D-G	Yes
	A>G	15203	I-V	Yes
	G>A	14861	A-T	Yes
	G>A	15077	E-K	Yes
	C>A	15452	L-I	Yes
	G>A	15734	A-T	Yes

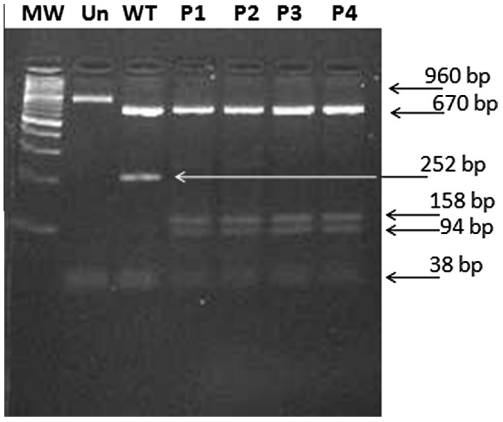


Fig. 3. PCR-restriction fragment length polymorphism (RFLP) analysis of asthenozoospermic patient with the m.8021A>G mutation: a 960-bp PCR fragment was digested with the restriction endonuclease MspI. The digestion in mutated DNA shows 4 fragments (670, 158, 94 and 38 bp) whereas digestion of wild-type DNA shows 3 fragments (670, 252 and 38 bp). MW, 100-bp DNA Ladder; Un, undigested PCR product; WT, wild-type DNA digestion.

(12.9%). This mutation was absent in normospermic patients, suggesting that it could be associated to asthenospermia.

This A-to-G transition found converted the Isoleucine at position 146 to Valine in a conserved amino acid in the transmembrane functional domain of the protein (Fig. 2B). It may affect the electron transfer from reduced cytochrome c to molecular oxygen.

Because, low heteroplasmic loads of mitochondrial mutations cannot be observed by simple sequencing and should be detected by radioactive polymerase chain reaction or by polymerase chain reaction-restriction fragment length polymorphism followed by a quantification of the heteroplasmic rates of the mutant mtDNA. Polymerase chain reaction-restriction fragment length polymorphism analysis was therefore performed, using the MspI restriction endonuclease because the m.8021A>G mitochondrial mutation

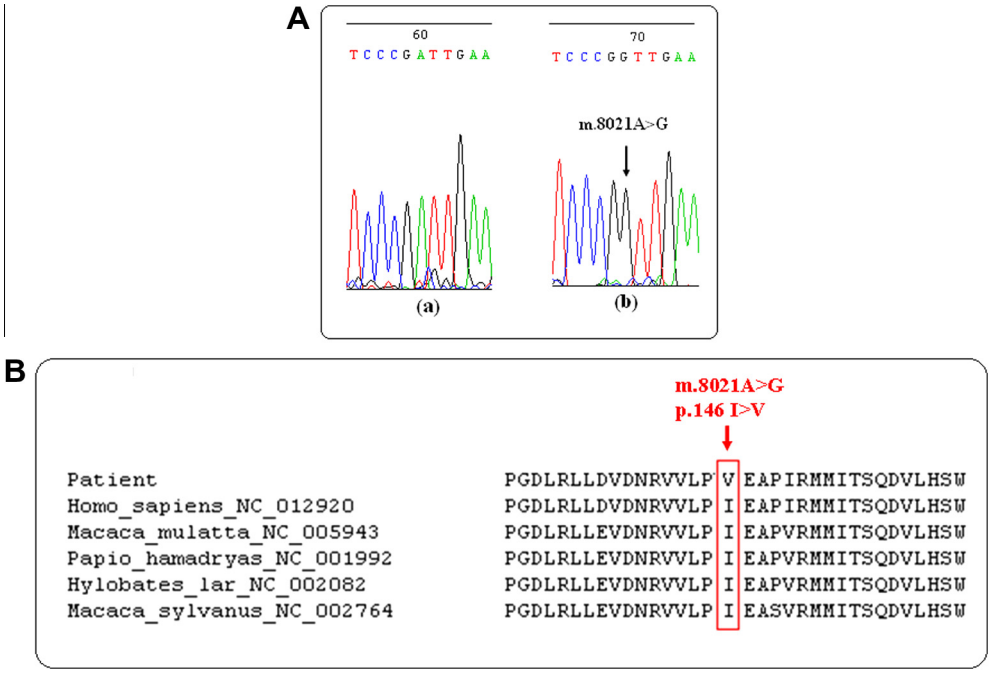
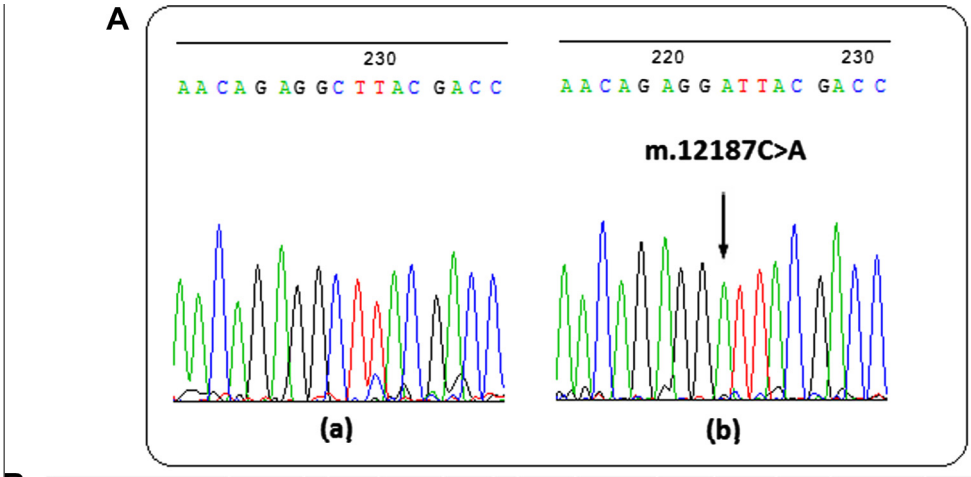


Fig. 2. (A) Sequence chromatograms showing the presence of the m.8021A>G mutation in the mitochondrial *COII* gene (b) and its absence in a control (a). (B) Alignment of the COII protein in different species showing the conservation of the amino acid 146; the mutated amino acid is framed. COII, subunit II of cytochrome c oxidase.



B

Organism	Aco-stem		D-stem	D-loop	D-stem		Ao-stem	Anticd-loop	Ao-stem	V-region	T-stem	T-loop	T-stem	Aco-stem
	1	8	10		22	26	27	32	39	44	49		61	66
Homo sapiens	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	C TTACGA	CCCCT	TATTTAC
Colobus guereza	GTAAATA	TA	ATTT	AACTA	AAAT	A	CTAGA	TTGTGAA	CCTAG	ACAT	AGAAG	C TTACCG	CTTCT	TATTTAC
Gorilla gorilla	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	TAAC	AGAGG	C TCACAA	CCCCT	TATTTAC
Hylobates lar	GTAAACA	TA	GTTT	AATCA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CAAT	AGAGG	C TCGAAA	CCTCT	TGCTTAC
Lemur catta	GTAAATA	TA	GTTT	AAAA	AAAC	A	CTAGA	TTGTGAA	TCCAG	AAAT	AGAAG	C TCAAAC	CTTCT	TATTTAC
Macaca mulatta	GTAGATA	TA	GTTT	AACTA	AAAC	A	TTAGA	TTGTGAA	TCTAA	CCAC	AGAGA	C TTACCA	CCTCT	TATTTAC
Macaca sylvanus	GTAAATA	TA	GTTT	AATTA	AAAC	A	TTAGA	CTGTGAA	TCTAA	CTAT	AGAAG	C TTACCA	CTTCT	TATTTAC
Pan paniscus	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	C TCACGA	CCCCT	TATTTAC
Pan troglodytes	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTGA	CAAC	AGAGG	C TCACGA	CCCCT	TATTTAC
Papio hamadryas	GTAGACA	TA	GTTT	AATCA	AAAC	G	TTAGA	CTGTGAA	TCTAA	CTAT	AGAAG	C CCACCA	CTTCT	TGTTTAC
Pongo pygmaeus	GTAAATA	TA	GTTT	AACCA	AAAC	A	TTAGA	TTGTGAA	TCTAA	TAAT	AGGGC	C CCACAA	CCCCT	TATTTAC
Pongo pygmaeus abelii	GTAAATA	TA	GTTT	AACCA	AAAC	A	TCAGA	TTGTGAA	TCTAA	TAAT	AGGGC	C CACAA	CCCCT	TATTTAC
Tarsius bancanus	GTAAATA	TA	GTTT	AAACA	AAAC	A	TTAGA	TTGTGAG	TCTAA	CAAT	AGAAG	C CAAAAA	TTTCT	TATTTAC
Trachypithecus obscurus	GTAAATA	TA	ATTT	AACTA	AAAT	A	TTAGA	TTGTGAA	TCTAA	ACAT	AGAAA	C TCACCC	CTTCT	TATTTAC

Fig. 4. (A) Sequence chromatograms showing the presence of the m. 12187C>A mutation in the $tRNA^{His}$ gene (b) and its absence in a control (a). (B) Sequence alignment of the $tRNA^{His}$ gene in different species. The cytosine at position 12187 is conserved throughout evolution.

create a novel MspI restriction site. Digestion results indicated that m.8021A>G mutation was present in a heteroplasmic state (Fig. 3). Quantification using the UVIDOC-008-XD analyzer indicated that patients had high levels of mutant mtDNA (98%) (Fig. 3).

Results of the screening of mitochondrial *tRNA^{His}* gene, revealed a novel mutation (m.12187C>A) in homoplasmic state in the same 4 asthenospermic patients who had the m.8021A>G mutation in *COII* gene (Fig. 4A); this mutation was absent in normospermic patients. It was conserved throughout evolution (Fig. 4B) and affects a wobble adenine in the T-loop region at the 54 codon of mitochondrial $tRNA^{His}$, and thus, it could not disturb the secondary structure of this tRNA.

4. Discussion

The effect of mtDNA mutations on male infertility has been studied more extensively in recent years, few studies have shown that mutations in the mitochondrial genome are associated with poor semen parameters, such as sperm maturation [14], sperm motility [15,16], and other related disorders [17]. It was also proposed that if sperm dysfunction is the main, or the only phenotypic consequence of a mtDNA mutation, reduced sperm motility or asthenospermia is one of the major causes of male infertility just next to reduced count or oligospermia [15,18–25].

In the present study, we performed a systematic sequence analysis of 7 mitochondrial genes (*COI*, *COII*, *COIII*, *ATPase6*, *ATPase8, Cytb* and *tRNA^{His}*) and their flanking regions in 64 Tunisian patients (31 asthenospermic and 33 normospermic). We did not find any reported pathogenic mtDNA mutation, but the analysis showed several known mitochondrial polymorphisms ($n = 88$) which were reported in various mitochondrial disorders and 4 novel polymorphisms. Certain substitutions ($n = 25$) were missense mutations (4 in *COI*, 2 in *COII*, 4 in *COIII*, 5 in *ATPase6*, 3 in *ATPase8* and 7 in *Cytb*) while the rest were silent mutations.

In a similar study by Thangaraj et al. in 2003, the authors screened 4 mitochondrial genes (*COI*, *COII*, *ATPase6* and *ATPase8*) and revealed 36 substitutions in an oligoasthenoteratozoospermic man who also had varicocele of the left testis (8 in *COI*, 13 in *COII*, 5 in *ATPase8*, and 10 in *ATPase6*). The fourth of these substitutions ($n = 9$) were missense mutations (3 in *COI*, 3 in *COII*, 1 in *ATPase8*, and 2 in *ATPase6*) [2].

Among the 92 nucleotide substitutions, 72 were detected exclusively in asthenospermic patients, suggesting that they are responsible for reduced sperm motility.

The several polymorphisms found in *COI*, *COII*, *COIII*, *Cytb*, *ATPase6* and 8 plays an important role and disrupt ATP production and thus the partial and complete spermatogenesis arrest and impaired sperm motility. Since sperm require a substantial amount of energy to swim fast enough to reach the oviduct during

fertilization, the appropriate bioenergetic function of mitochondrion is crucial for male infertility [5].

Our study revealed also that 4 asthenospermic patients harbored a novel heteroplasmic missense mitochondrial mutation (m.8021A>G) in *COII* gene, converting the Isoleucine at position 146 to Valine in the trans membrane domain of the polypeptide. None of the 33 normospermic patients had this mutation, suggesting that this change may affect the electron transfer from reduced cytochrome c to molecular oxygen and compromises sperm motility leading to asthenospermia.

Previously, Thargaraj et al. (2003) detected a novel 2-bp deletion (nucleotides 8195 and 8196) in *COII* gene in oligoasthenoteratozoospermic Indian men, which might have given rise to a truncated protein and the authors suggested that the diminished sperm motility would be due to this 2-nucleotide deletion in *COII* gene [2].

More recently, Selvi Rani et al. reported a novel missense mutation (m.11994C>T) in ND4 gene in oligoasthenozoospermic Indian patients ($n = 34$) that was absent in fertile men ($n = 150$) [26]. In contrast, Pereira et al. reported the absence of this m.11994C>T mutation in their oligoasthenozoospermic infertile Portuguese patients ($n = 43$), suggesting that this ND4 mutation may be population specific [27]. In Kumar et al. (2009) study, a different transition (m.9098T>C) was detected only in infertile cases [28].

In this study, our results showed also the presence of an homoplasmic mutation (m.12187C>A) in mitochondrial $tRNA^{His}$ gene, in the same asthenospermic patients who had m.8021A>G mutation in *COII* gene. In 2002, Spiropoulos et al., correlated high levels of mutant mtDNA with low sperm motility in men who had inherited the A3243G mtDNA mutation in *tRNA* leucine from their mother [25].

To elucidate the effect of mtDNA changes, functional studies using the cybrid cells (a cell culture model used for the study of mitochondrial disorders), should be taken to evaluate the eventual change of complex II activity and of protein synthesis, and the impact of novel mutation on respiratory chain function.

In conclusion, the screening of 7 mitochondrial genes revealed that some Tunisian infertile men carry 4 new mitochondrial polymorphisms in the *COI* gene and that 12.9% of asthenospermic patients present with a double undescribed mutations (m.8021A>G) in the *COII* gene and m.12187C>A in the $tRNA^{His}$ gene) that was absent in normospermic patients and in controls, suggesting that this mutations could be associated to asthenospermia and male infertility.

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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