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Original Article

Association of Pathogenic Missense and Nonsense Mutations in Mitochondrial COII Gene with Familial Adenomatous Polyposis (FAP)

Zahra Shaker Ardakani[1], Mohammad Mehdi Heidari[1*], Mehri Khatami[1], Morteza Bitaraf Sani[2]

1. Department of Biology, Faculty of Science, Yazd University, Yazd, Iran.

2. Animal Science Research Department, Yazd Agricultural and Natural Resources Research and Education Center, Agricultural Research, Education & Extension Organization (AREEO), Yazd, Iran.

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Nuclear genetic mutations have been extensively investigated in solid tumors. However, the role of the mitochondrial genome remains uncertain. Since the metabolism of solid tumors is associated with aerobic glycolysis and high lactate production, tumors may have mitochondrial dysfunctions. Familial adenomatous polyposis (FAP) is a rare form of colorectal cancer and an autosomal dominant inherited condition that is characterized by the progress of numerous adenomatous polyps in the rectum and colon. The present study aimed at understanding the nature and effect of mitochondrial cytochrome c oxidase subunit 2 (*COII*) gene mutations in FAP tumorigenesis. Fifty-six (26 familial and 30 sporadic) FAP patients and 60 normal controls were enrolled in this study. *COII* point mutations were evaluated by PCR and direct sequencing methods, and a total of 7 mtDNA mutations were detected (3 missense, 1 nonsense, and 3 synonymous variations). Novel non-synonymous *COII* gene mutations were mostly in heteroplasmic state. These mutations change amino acid residues in the N-terminal and C-terminal regions of COXII. Bioinformatics analysis and three-dimensional structural modeling predicted that these missense and nonsense mutations have functional importance, and mainly affected on cytochrome c oxidase (complex IV). Also, FAP patients carried a meaningfully higher prevalence of mutations in the *COII* gene in comparison with healthy controls (P <0.001). Analysis of cancer-associated mtDNA mutation could be an invaluable tool for molecular assessment of FAP so that these findings can be helpful for the development of potential new biomarkers in the diagnosis of cancer for future clinical assessments.

Key words: Familial adenomatous polyposis, mitochondrial genes, *COII*, point mutations

Mitochondria are the key organelles involved in the process of oxidative phosphorylation. Human mitochondrial genome (mtDNA) is made up of 16,569 nucleotide pairs, and is more

*Corresponding author: Department of Biology, Faculty of Science, Yazd University, Yazd, Iran.
E-mail: heidarimm@yazd.ac.ir

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susceptible to mutagen-induced damage than nuclear DNA due to the lack of introns and protective histones. In addition, the globular, coiled structure of mtDNA may be a binding platform for environmental chemical carcinogens. Also, mtDNA is particularly exposed to the high concentration of reactive oxygen species (ROS) (1). Pathogenic mtDNA mutations can severely affect mitochondrial respiration and increase the production of endogenous ROS which are important factors in the inactivation of tumor suppressor genes such as TP53, which is involved in apoptosis and neoplastic transformation, cell proliferation, and metastasis of cancer cells (2). After much effort in analyzing the role of the nuclear genome in the progress of neoplasms, researchers have examined the mitochondrial genome in cancer cells for somatic mutations accumulated in cancer cells. Such mutations have been reported in various types of human cancers including prostate, breast, esophageal, pancreatic, and colon (3-7). MtDNA mutations effect encoding protein genes of the respiratory chain complexes leading to an impairment flow of protons and electrons through the membrane, which subsequently increases its potential role in the development of neoplastic cells (8).

Mutations in the complex IV genes including *COI*, *COII*, and *COIII* have been observed in different hematological disorders such as sideroblastic anemia (9) or myelodysplastic syndrome (10, 11), cardiac disorders such as coronary artery disease (12), and solid tumors such as colon neoplasms (13). In the mitochondrial respiratory chain, cytochrome c oxidase (COX) is the terminal electron acceptor. This enzyme complex comprises 13 subunits, in which three of them (subunits I–III) are encoded by the mitochondrial genome, and form the core of the enzyme that catalyzes the transfer of electrons from reduced cytochrome c to O_2 to form H_2O (14). However, the role of the mtDNA mutations in cancer continues to be the subject of conflict and controversial discussion in the literature (15, 16). Global profiling and mitochondrial genome analysis have not been performed in patients with FAP, to date, especially.

Familial adenomatous polyposis (FAP) is an autosomal dominant disease characterized by *APC* gene mutations, and typically detected by the presence of hundreds to thousands of colorectal adenomas with different sizes as well as several extracolonic manifestations during childhood and adolescence (17, 18). Almost all such patients are at risk of developing colorectal cancer (CRC) if they are not recognized and treated at early stages. It is estimated that FAP affects approximately 1 in 15 000 births (19). Clinically, FAP affects both sexes equally and in the majority of patients, polyps begin to develop as small intramucosal nodules in the rectosigmoid segment (distal colon), during childhood. Patients with FAP can also have an increased risk of tumors and malignancy in extra-intestinal sites, including skin, thyroid, pancreas, adrenal gland, brain, bone, retina, liver, and gastric fundic gland (20). However, 20–35% of cases are ‘‘de novo’’ without clinical or genetic evidence of FAP in their parents (21). Due to the increased risk of malignancy, screening protocols have been suggested for patients with FAP. The results of our previous research in FAP patients also confirmed the role of the pathogenesis of mitochondrial *COI* gene mutations in familial and sporadic patients (22). Therefore, in this article, we analyzed the mtDNA of patients and healthy cases in an attempt to find sequence variants of mtDNA putatively associated with the occurrence of FAP.

Materials and methods

Patients and healthy controls

Fifty six patients with FAP symptoms were enrolled in this study. Clinical and demographic characteristics of participants are described in Table 1. All patients with a confirmed diagnosis of classic

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Table 1. Clinical and demographic characteristics of participants.

Clinical characteristics	Number=56	Range	Frequency/ Mean
Gender	-	-	-
Male	23	9 with a family history	41.08%
Female	33	17 with a family history	58.92%
FAP with family history	26	From 9 different family	46.43%
FAP without family history (sporadic)	30	Based on clinical diagnosis of classical FAP	53.57%
Age in familial cases	26	0.7 to 20 years	12.5 years
Age in sporadic cases	30	1.5 to 20 years	15 years
Median follow-up in familial cases	3	1 to 10 years	6.16
Median follow-up in sporadic cases	5	2.5 to 20 years	11.8

Table 2. Primer pairs used to amplify the mitochondrial *COII* gene.

Pairs	Position	Length (bp)	Forward primer (5′-3′)	Reverse primer(5′-3′)
1	7377-8470	1093	CTGGAGTGACTATATGGATG	TAGGTGGTAGTTTGTGTTTA
2	7900-8558	658	ACCAATGGTACTGAACCTACG	AATGAATGAAGCGAACAGAT

FAP presented to Imam Khomeini Hospital (Tehran) between 2016 and 2018 and with a mean age of 13.5 years (range 0.7 to 20 years, median 15 years) were enrolled. These patients were from nine different Iranian families (17 females and 9 males) and 30 sporadic and unrelated patients (16 females and 14 males). Blood specimens were collected after informed consent was obtained following protocols approved by the Ethics Committee of Yazd University, and all procedures were in accordance with the Helsinki Declaration of 1975, as revised in 2008 and ethical standard. FAP was diagnosed with clinical findings according to the characterization of more than 100 adenomatous polyps in colon and rectum and a dominant inheritance in family history. Additionally, an age and sex-matched (2-19 years) healthy normal group of 60 unrelated healthy cases from the same geographic area and without a familial history of any type of cancer was also enrolled (31 male and 29 female). In both the patient and control groups, gender distribution was about identical, with 54 (46.55%) males and 62 (53.45%) females.

DNA extraction, amplification, and sequence analysis

DNA was extracted from 5 ml peripheral blood samples using the Qiagen DNA kit (Qiagen Co., Tehran, Iran), and was resuspended in TE buffer (10 mM Tris and 1 mM EDTA in pH 7.5).

To directly sequence the *COII* gene of mtDNA, two specific primer pairs were designed using the primer design software (Primer Premier 5.0; Premier Biosoft Inc., Canada) (Table 2). The *COII* gene at mtDNA map position 7,586 to 8,269 was amplified by polymerase chain reaction (PCR) to produce two completely overlapping fragments. The PCR mixture in a final volume of 25 µL consisted of 70 ng of total DNA, 2 pmol of each forward and reverse primers, 0.5 mM of each dNTPs, 1.5 mM $MgCl_2$, 0.4 units Taq DNA polymerase, 5 µL of 10×PCR buffer. Amplification was performed in a DNA thermal cycler (Perkin-Elmer, Foster City, CA) with one denaturation cycle at 95 °C for 5 min, followed by 35 cycles of

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30 s at 95 °C; 40 s at annealing temperature (55 °C for the first primer pair and 62 °C for the second primer pair), 30 s at 72 °C, and finally, one cycle of 72 °C for 5 min. The amplified DNA was electrophoresed in 1% agarose gel and stained with ethidium bromide to evaluate the size and purity of the PCR products. PCR products were directly sequenced and sequencing results were evaluated by the Geneious program and subsequently compared to the published sequences in MITOMAP (a human mitochondrial genome database), Blast2 program (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2. htlm) and MitoAnalyzer (http://www.cstl.nist.gov/ biotech/ strbase/mitoanalyzer.html). Fisher's exact test version X2 was used for statistical analysis. A P-value <0.05 was considered statistically significant.

***In silico* analysis**

In order to obtain preliminary evidence for polymorphisms, mutations, and alterations in the amino acid sequences, we performed the sequence alignment by using the multiple sequence alignment software; MEGA6 and the Standard Protein Blast (blastp) program available at http://www.ncbi.nlm.nih. gov/Blastp. The prediction of the functional consequences of mutations, damaging effects and pathogenicity scores of missense and nonsense mutations were performed by polymorphism phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/ pph2/), scale- invariant feature transform (SIFT) (http://sift. jcvi.org/), I-Mutant online service (http://gpcr2.biocomp. unibo.it/cgi/ predictors /I- Mutant3.0), PMUT, Provean, and MutPred software for annotation of biological function of protein and prediction of pathological mutations (https://www.bsc.es/medicahead/scientific-outputs/pmut, http://provean.jcvi.org/, and http://mutpred.mutdb.org), and ENTPRISE-X (23) (http://cssb2.biology.gatech.edu). The hydrophobicity or hydrophilicity scales in the proteins were determined using a plot created by the Expert Protein Analysis System (ExPASy) Protscale tool (http://web.expasy.org/protocol) and hydrophobicity indexes were calculated with Peptide-2 prediction tool (www.peptide-2.com). Finally, to an assessment of the 3D structures and interaction changes in the mutant and normal protein, PyMol software (https://www.pymol.org/) was used.

Results

We have studied 56 Iranian familial and sporadic cases with FAP symptoms, and have identified seven somatic mutations in the mitochondrial *COII* gene. These somatic mutations include three heteroplasmic missense mutations in 24 patients, one nonsense heteroplasmic mutation in 10 patients and three synonymous homoplasmic and heteroplasmic mutations in 4 patients. The results of the screening of *COII* somatic mutations are presented in Table 3. Direct-sequence analysis of *COII* gene revealed four novel and unpublished mutations which were absent in the mitochondrial databanks (http://www.mitomap.org) at nucleotide positions A7602C, A7877G, C7892G, and A7878G of mtDNA, resulting in three missense substitutions and one nonsense mutation at amino acid residues Q6P, K98E, Q103E, and K98Ter of the polypeptide, respectively. Interestingly, all of these novel mutations appeared as overlapping peaks at the same position, thus were detected as heteroplasmic in the sequence chromatogram (Fig. 1 and 2). The same analysis was performed for the parents' blood of the FAP patients; their sequencing results for two heteroplasmic mutations (A7877G and A7878G) were positive, but were negative for other observed mutations. These missense and nonsense mutations were not detected in 60 healthy controls from the Iranian population, and in the blood samples of the investigated family members. Three synonymous mutations (G7600A, T7645C, and G7897A) have been previously reported in Mitochondrial Database as variations and

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Table 3. *COII* gene variations and their distribution in FAP patients and healthy controls.

Gene variation	Amino acid change	Hetero /Homo	Distribution of mutations in patients	Distribution of mutations in controls	Novel/ Reported*
7600 G>A	p.Ala5Ala (silent)	Homo	3 Patients from 2 Family and 5 sporadic cases (14.28%)	2 girls and 1 boy (5%)	Reported in mitochondrial encephalomyopathy
7602 A>C	p.Gln6Pro (missense)	Hetero	2 Patients from 2 Family and 1 sporadic case (5.35%)	-	Novel
7645 T>C	p.Leu20Leu (silent)	Homo	3 Patients from 1 Family and 5 sporadic cases (14.28%)	-	Reported in thyroid tumors
7877 A>G	p.Lys98Glu (missense)	Hetero	11 Patients from 6 Family and 10 sporadic cases (37.5%)	-	Novel
7878 A>G	p.Lys98Ter (nonsense)	Hetero	10 Patients from 6 Family and 5 sporadic cases (26.78%)	-	Novel
7892 C>G	p.Gln103Glu (missense)	Hetero	4 Patients from 4 Family and 2 sporadic cases (10.71%)	-	Novel
7897 G>A	p.Trp104Trp (silent)	Hetero	1 Patient from 1 Family and 2 sporadic cases (5.35%)	-	Reported in Leber's hereditary optic neuropathy

Hetero/Homo: heteroplasmy or homoplasmy.*According to Mitomap (http://www.mitomap.org) and Ensembl (a genome browser: https://ensembl.org/index.html).

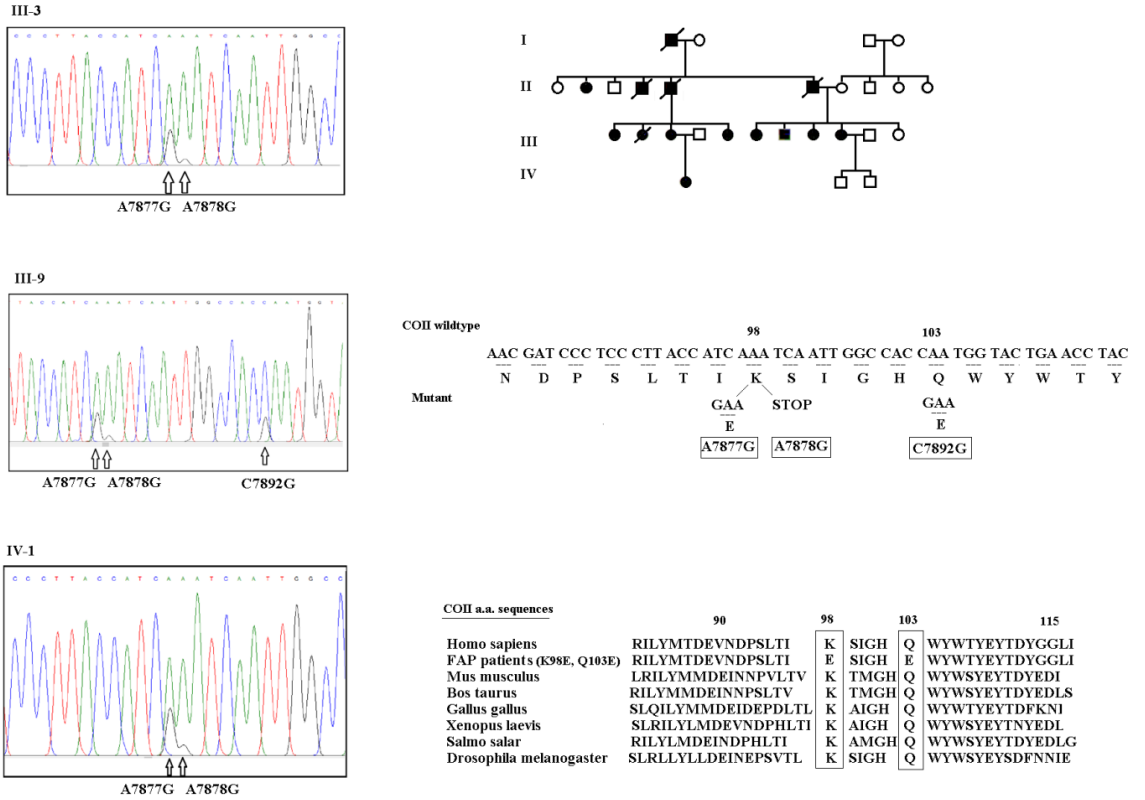

Fig. 1. Sequence chromatogram and nucleotide positions of three *COII* gene mutations in three members from a family with FAP. Arrows indicate the position of mutations. Evolutionary conservation in COII amino acid (aa) positions are also shown.

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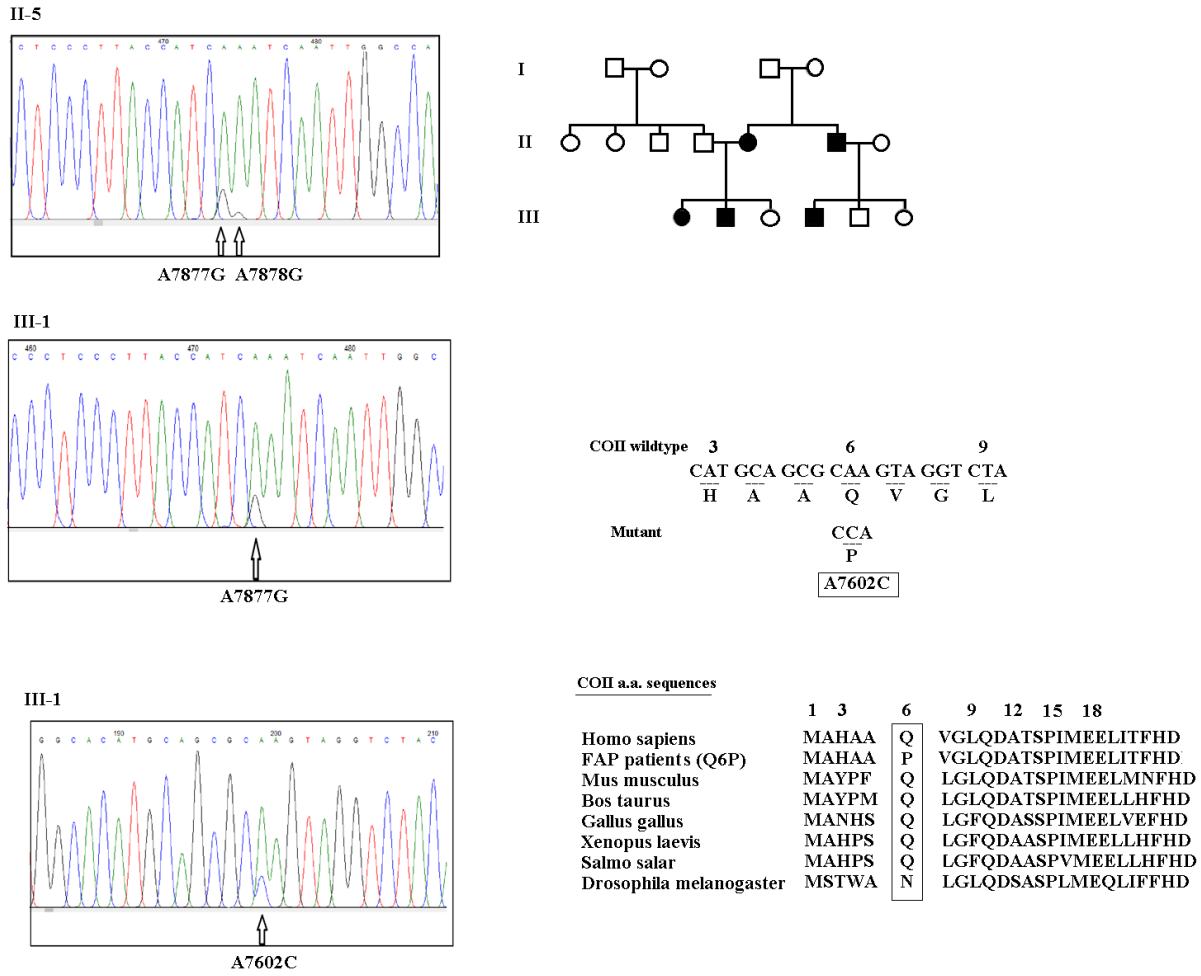

Fig. 2. Sequence chromatogram and nucleotide position of A7602C mutation in *COII* gene in a family with FAP. Arrows indicate the position of mutations. Evolutionary conservation in COII amino acid (aa) positions are also shown.

associated with several diseases that have been listed in Mitomap and Genome browser Ensemble databases (Table 3).

For the evaluation of these mutation effects, we focused on non-synonymous mutations in the *COII* gene. A total of 4 different non-synonymous mutations, including 3 missense and 1 nonsense mutations were analyzed by various bioinformatics tools. *In silico* analysis determined that the three novel missense mutations at positions 6, 98 and 103 in the *COII* gene were deleterious variations and probably damaging in protein structure and function (Table 4). The analysis of the mitochondrial polypeptide sequences from different species showed that the amino acid residues in these positions of the MT-COII protein were located in evolutionarily stable domains (Figures 1 and 2). Also, FAP patients carried a meaningfully higher prevalence of missense and nonsense mutations of the *COII* gene than healthy controls ($P < 0.001$) (Table 5). Poly Phen-2 analysis predicted that these missense mutations are probably damaging with scores of 0.999, 1.000 and 0.970, respectively. The secondary structural modeling of protein identified that MT-COII is mostly a hydrophobic protein and has only two hydrophilic domains. However, m.A7602 transversion (Q6P) substitutedpolar and neutral glutamine amino acid (with hydropathy index -3.5) in position 6 of the transmembrane domain of COII subunit to a hydrophilic proline (with hydropathy index -1.6). Also, m.A7877G transition (K98E) changed a basic and hydrophilic

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Table 4. Potential pathogenic *COII* gene variations identified in protein-coding region.

Mutation	SIFT/Score	Polyphen-2/ Score	PMut/Score (%)	I-Mutant/DDG	Provean/ Score
p.Gln6Pro	Affect protein function/ 0.00	Probably amaging/0.999	Pathogen/0.56 (81%)	Decrease Stability/ -0.53	Deleterious/ -5.68
p.K98E	Affect protein function/ 0.00	Probably amaging/1.000	Pathogen/0.51 (79%)	Decrease Stability/ -1.06	Deleterious/ -3.98
p.Q103E	Affect protein function/ 0.00	Probably amaging/0.970	Pathogen/0.51 (79%)	Increase Stability/ 0.59	Deleterious/ -2.99

SIFT/Score: threshold for intolerance is 0.05: in other words, score <0.05 is predicted to be deleterious; Polyphen-2/Score: sensitivity: 0.00 and specificity: 1.00; PMut: prediction of pathogenicity percentage of mutation; I-Mutant/DDG: free energy change value (DDG): DDG <0: decreased stability and DDG> 0: increased stability; the Provean scores are averaged within and across alignment scores. If the Provean score is equal to or below a predefined threshold, the protein variant is predicted to have a "deleterious" effect.

Table 5. Association of *COII* gene mutations in controls and patients with FAP.

	FAP Patients (n=56)	Control cases (n=60)	P value
Nonsense mutations (%)	10 (17.85)	0/ 0%	< 0.001
Missense mutations (%)	24 (42.85)	0/ 0%	0.000
Synonymous mutations (%)	4 (7.14)	3/ 5%	0.710
P value for total mutations	< 0.001		

P-value < 0.05 was considered statistically significant.

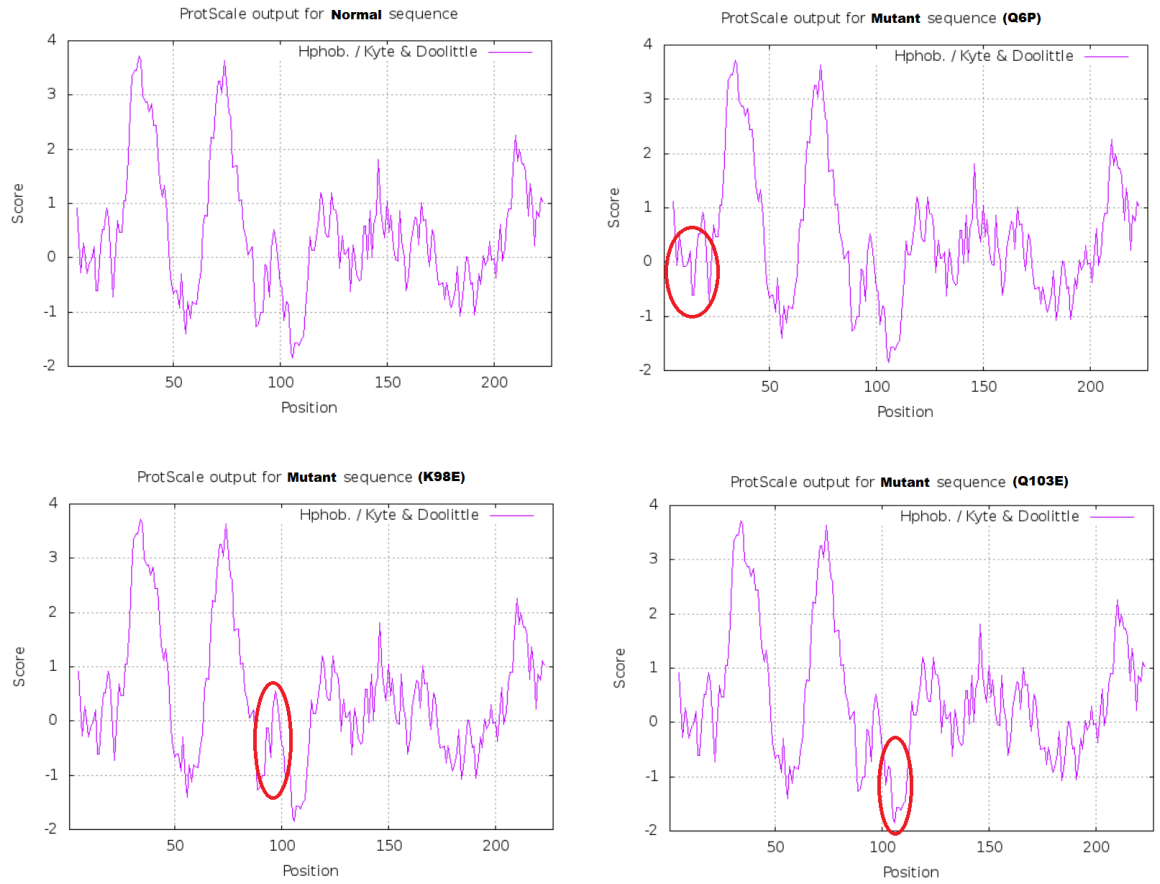

Fig. 3. Hydropathy plots for the COII subunit. The hydrophobicity of the wild-type COII subunit is compared to the mutant forms for Q6P, K98E and Q103E mutations. Mutated sites are shown by circle.

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lysine amino acid (with hydropathy index -3.9) to an acidic and hydrophilic glutamic acid (with hydropathy index -3.5) in position 98 of the COII protein. Another missense mutation at position 103 of the protein (m.C7892G) exchanged a polar and neutral glutamine amino acid (with hydropathy index -3.5) with an acidic and hydrophilic glutamic acid (with hydropathy index -3.5). Both K98E and Q103E occurred in the C-terminal domain of the protein. A hydropathy plot of these mutant polypeptides generated with the Kyte–Doolittle algorithm (ProtScale program) presented slight alterations caused by the Q6P and K98E mutations in the hydrophobicity of beta-sheet domains of protein and could be the cause of the slight alterations in the structure of the MT-COII protein membrane (Figure 3). In addition, all three missense mutations (Q6P, K98E and Q103E) change evolutionarily highly conserved amino acids and had MutPred scores > 0.5 (a predicted score for identification of pathogenic or benign amino acid substitutions in human), so probably altering protein structure and activity. Furthermore, another single nucleotide variation (A7878G, K98ter) causes premature termination of the translation of protein. According to pathogenicity-based studies using a bioinformatics tool (ENTPRISE-X), this nonsense mutation was deleterious and had a pathogenicity score higher than 0.5.

Discussion

In the present study, we reported a *COII* mitochondrial gene screening of 56 familial and sporadic patients with FAP. Our results showed the presence of four novel heteroplasmic (three missense and one nonsense) mutations in the mitochondrial *COII* gene in a total of 47 familial and sporadic FAP patients. Mutations in *COII* have been reported and associated with a variety of disease phenotypes, such as colon neoplasms (7), MELAS (24), sideroblastic anemia (25), Alpers-Huttenlocher-like disease (26), myelodysplastic syndrome (10), myopathy (27), severe lactic acidosis (28), myalgia and myoglobinuria (29), and non-syndromic mitochondrial encephalomyopathy (30).

The mutational analysis of the *COII* gene showed the presence of some of the mutations, especially, the A7877G (K98E) and the A7878G (K98ter) in other family members. In fact, these two mutations which occur in the same amino acid position, show maternally inheritance but were not very rare in Iranian patients (37.5% and 26.78%, respectively).

Also, the molecular study of the mitochondrial *COII* gene of these patients revealed three previously reported substitutions (p.Ala5Ala in mitochondrial encephalomyopathy, p.Leu20Leu in thyroid tumors, and p.Trp104Trp in Leber's hereditary optic neuropathy) and four novel heteroplasmic variations m.7602 A>C (p.Gln6Pro), m.7877 A>G (p.Lys98Glu), m.7878 A>G (p.Lys98Ter) and m.7892 C>G (p.Gln103Glu). These non-synonymous variations were absent in the mitochondrial databanks, and in the normal control population. The bioinformatics analysis predicted that they have probably damaging effects with high pathogenicity scores. The Q6P and K98E mutations in mitochondrial inter-membrane and C terminal region caused hydrophobicity scale alterations and that probably could affect the structure of MT-COII protein. Also, The m.7892 C>G mutation (p.Gln103Glu) is located in the C-terminal region and *in silico* analysis suggested it as a deleterious and pathogenic mutation. In the wild type, MT-COII is one of the most important subunits in complex IV, the terminal enzymatic complex of the mitochondrial respiratory chain that catalyzes the electron transfer from cytochrome c through its binuclear copper A (CuA) center to the bimetallic core of the catalytic MT-COI (31).

Additionally, the mutation of a single adenine nucleotide at position 7878 of the mitochondrial genome to guanine results in introducing a stop

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codon (AAA codon is exchanged to AGA: stop codon) at position 98 of MT-COII. This nonsense mutation results in the deletion of 56.83% of protein, and another important point about this mutation is its occurrence in familial patients. Since the C-terminal hydrophilic domain of protein is mostly an important inte-rmembrane region, and contains the binuclear CuA center (32), it is likely that the truncated MT-COII does not have sufficient function to the assembly of complex IV and forming stabilizing bands with the redox group, copper A, or the other complex IV subunits, (MT-COI, MT-COIII, COX4I1, COX5B, COX6A, COX6B, COX6C, COX7A, and COX7C). These associations among mitochondrial respiratory complexes have been demonstrated to be vital for mitochondrial structural assembly, stability, and function (33). Results of the immunoblot analyses and immunohistochemical assays performed in a 14-year-old boy with a proximal myopathy and lactic acidosis indicated that *COII* mutations arrest the assembly of COX holoenzyme with 13-subunits, due to the impairment of assembly or stability of the COX holoenzyme and ultimately, the electron-transfer function of protein in complex IV (34).

Also, analysis of the 3D structure revealed that Q6P and K98E missense mutations described here are able to affect the 3D structure (Figure 4). The structural modeling of these two new mutations could confirm that mutations in the transmembrane and C-terminal regions probably damage the protein conformation, and could disrupt its function. In addition, generation of a 3D model of Q6P mutation in *COII* demonstrated the presence of one hydrogen bond between P6 (mutant) with W449 (3.4 Aº), instead of two hydrogen bonds sharing between Q6 (wild-type) with S433 (2.8 Aº), and W449 (3.4 Aº) neighbor residues in COI protein. Furthermore, in normal state, four interactions have been proposed between residues K98 (wild-type) with C65 (2.4 Aº), E109 (3.1 and 3 Aº) and T111 (2.7 Aº), via a complex network of hydrogen bonds, while in mutant state, E98 only has two hydrogen bonds with E108 (3.1 Aº) and T107 (2.7 Aº) adjacent amino acids in COII protein.

So, these missense mutations may affect the spatial conformation and the shape of these significant regions regarding electron transport. Therefore, such mutations are likely to affect the protein structure, and could probably decrease the activity of oxidative phosphorylation complex IV and cause mitochondrial dysfunction.

Because four heteroplasmic mutations (3 missense and 1 nonsense mutations) reported here for the first time, were also observed in other affected family members of patients, and also, they all occur in highly evolutionarily conserved amino acids, and in that most bioinformatics results and structural and functional prediction software predicted them as pathogenic variations, and finally, these mutations were not identified in ethnically matched control subjects, we suggest the implication of these variations in the progress of FAP in Iranian population.

In conclusion, we reported 26 patients from nine different families and 30 sporadic patients from Iranian population with FAP and presenting 7 point mutations in COII: “A7602C, A7877G, A7878G, and C7892G” in heteroplasmic state and “G7600A, T7645C and G7897A” in homoplasmic state. Interestingly, all of the novel missense and nonsense mutations were present in a heteroplasmic state. We provided bioinformatics evidence that these novel mutations affect the assembly or stability of the COX holoenzyme. These findings suggest that structural and functional modifications in the mitochondrial genome, especially the *COII* gene participate in tumor development of FAP. However, it should be noted that the importance and main role of these *COII* gene mutations in the pathogenesis of FAP cannot be fully understood by

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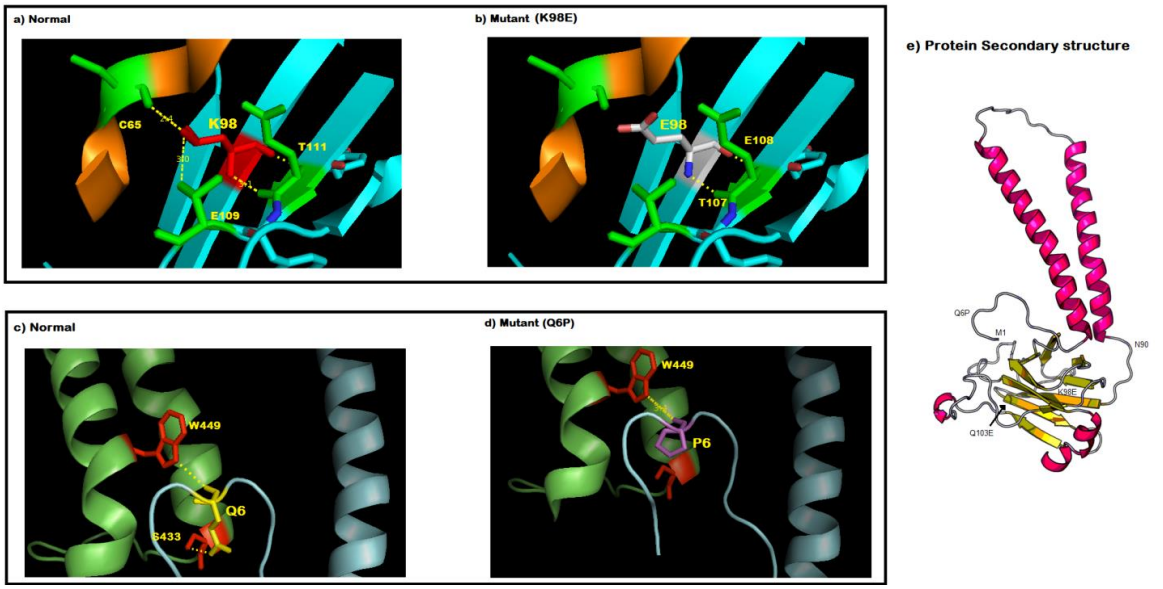

Fig. 4. Molecular 3D modeling of COII protein. a-b) 3D Model of region of the wild COII protein (K98) and mutant protein (E98). Hydrogen bounds between F219, P215 and L216 residues (a) and mutated COII protein (219C) as well as hydrogen bounds between C65, T111, and E109 residues in normal and E108 and T107 residues in mutant protein are shown. c-d) Comparison between two normal and mutated models of COII for Q6P mutation: hydrogen bounds between W449 and S433 residues in normal and W449 residue in mutant protein are shown. e) Secondary structure model of COII protein and positions of three missense mutations.

sequencing analysis alone and further functional analyses should be performed in the laboratory.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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