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Original article

Novel Missense Mitochondrial *ND4L* Gene Mutations in Friedreich's Ataxia

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Abstract

Objective(s)
The mitochondrial defects in Friedreich's ataxia have been reported in many researches. Mitochondrial DNA is one of the candidates for defects in mitochondrion, and complex I is the first and one of the largest catalytic complexes of oxidative phosphorylation (OXPHOS) system.
Materials and Methods
We searched the mitochondrial *ND4L* gene for mutations by TTGE and sequencing on 30 FRDA patients and 35 healthy controls.
Results
We found 3 missense mutations [m.10506A>G (T13A), m.10530G>A (V21M), and m.10653G>A (A62T)] in four patients whose m.10530G>A and m.10653G>A were not reported previously. In two patients, heteroplasmic m.10530G>A mutation was detected. They showed a very early ataxia syndrome. Our results showed that the number of mutations in FRDA patients was higher than that in the control cases (P= 0.0287).
Conclusion
Although this disease is due to nuclear gene mutation, the presence of these mutations might be responsible for further mitochondrial defects and the increase of the gravity of the disease. Thus, it should be considered in patients with this disorder.

Keywords: Friedreich's ataxia (FRDA), mtDNA, Mutation, *ND4L* gene

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Introduction

Friedreich's ataxia (FRDA) is the most common inherited ataxia. In whites, it affects 1 in 50,000 people (1). Clinically, FRDA is characterized by progressive spinocerebellar ataxia, diabetes mellitus, and hypertrophic cardiomyophathy (2). Friedreich's ataxia is caused by a GAA-trinucleotide expansion in the frataxin gene located on chromosome locus 9q13, resulting in a reduced expression of frataxin, a small mitochondrial protein (3, 4). Owing to the mitochondrial localization of frataxin, the neurological and cardiological degenerations observed in FRDA are thought to be the result of a mitochondrial defect (5).

NADH ubiquinone reductase (complex I) is the first and one of the largest catalytic complexes of oxidative phosphorylation (OXPHOS) system. Defects of this complex either alone or in combination with other complexes are probably the most frequently observed defect in the respiratory chain (RC). This predominance of complex I dysfunction suggests that its structure or function is relatively easily perturbed (6). Dysfunction of the mitochondrial RC is seen in patients with neurological diseases including alzheimer disease (AD) (7), parkinson disease (PD) (8), Multiple sclerosis (MS) (9, 10), and Friedreich's ataxia (FRDA) (11). We suggested previously that our patients had a biochemical defect in complex I activity and ATP production (12) and several polymorphisms in mitochondrial *ND* genes (13). These findings prompted us to revisit our cases of Friedreich's ataxia. Although heart tissue was no longer the case, we investigated the mitochondrial *ND4L* gene in blood, and its mutations was assumed to be of special interest with regard to FRDA.

Patients and methods

Patients

We studied thirty Iranian patients (14 females and 16 males) from unrelated families with a diagnosis of FRDA regarding their clinical aspects. We basically adopted the clinical criteria of Harding and Geffroy *et al* (1, 14). We also chose 35 healthy controls (16 females and 19 males) matched for age, sex, and ethnicity. All of the patients and the control group were informed of the aims of the study and gave their informed consents for the genetic analysis.

Molecular analysis

DNA was isolated from the peripheral blood samples using a DNA extraction kit (DNAfast Kit-Genfanavaran, Tehran, Iran). For *ND4L* gene amplification, PCR was carried out using DNA thermal cyclers (eppendorf, master cyclers, 5330) and ONP93 (5'-TCTGGCCTATGAGTGACTAC-3') and ONP13 (5'-TTCACTGGATAAGTGGCGTT-3') primers in 25 µl of a solution containing 2.5 mM of $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 100 ng total DNA and 1 U Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). The PCR reaction was performed for 30 cycles composed of the following steps: 95 ºC for 50˝, 60.5 ºC for 50˝, and 72 ºC for 35˝. For TTGE assay, PCR products (660 bp) were denatured at 95 °C for 30 seconds and slowly cooled to 45 °C for a period of 45 min at a rate of 1.1 °C/min. TTGE was performed according to the manufacturer's instructions (Dcode Universal Mutation Detection Systems, BioRad, Hercules, CA). A volume of 10 µl of the denatured and reannealed PCR products was loaded on the gel. Electrophoresis was carried out at 140 V for 5-6 hr at a constant temperature increment of 1-2 °C/hr. The temperature range was determined by computer simulation (Win Melt software; Bio-Rad Laboratories).

Analysis of secondary structure and pathogenicity prediction for amino acid changes

The results of DNA sequence analysis were compared with those of the published Cambridge sequence (15). Each sequence variation was then checked against the Mitomap database (16). Those not recorded in the database were categorized as novel mtDNA variations. The conservation of the novel polymorphism bases was assayed using

DNASTAR software (MEGALIGN program). This software was used to determine the DNA sequence alignment of the *ND* genes in humans and in other species (chimpanzee, bovine, horse, rat, gallus, and drosophila).

The predicted amino acids sequence was analyzed using the FASTA program information system which is available at URL: http://fasta.bioch.virginia.edu/fasta_www2/fasta_list2.shtml. The secondary protein structure was analyzed by theoretical prediction using the algorithm of Garnier-Robson (17). A hydropathy plot of the amino acids sequence was calculated by the method of Kyte and Doolittle (18).

The prediction of the pathogenicity of amino acids changes was accomplished by SIFT database at http://www.blocks.fhcrc.org.

Statistical analysis

Fisher's exact probability test was used, and the values of $P< 0.05$ were regarded as statistically significant. The statistical analysis was performed using the GraphPad Prism software.

Results

The $(GAA)_n$ repeats of FRDA patients were observed in both alleles, ranging from 245 to 991 GAA repeats. TTGE analyses for the mitochondrial *ND4L* gene were carried out on a total of 30 patients and 35 healthy controls. DNA fragments showing abnormal banding patterns on TTGE analysis were sequenced to identify the exact mutations (Figure 1). The analyzed mtDNA sequences were compared with those of the published sequence (MITOMAP 2009).

We found three different types of point mutations in four of our patients and did not find any of these mutations in the healthy controls. The first mutation was m.10506A>G transition (homoplasmic state), changing a Thr codon (ACC) to Ala codon (GCC) at amino acid position 13 (designated T13A) in patient 1. It was previously reported in one LHON patient (19) and one PEO patient (20). SIFT predicted that the substitution at position 13 from T to A could be tolerated with a score of 0.10 and median sequence conservation of 3.64.

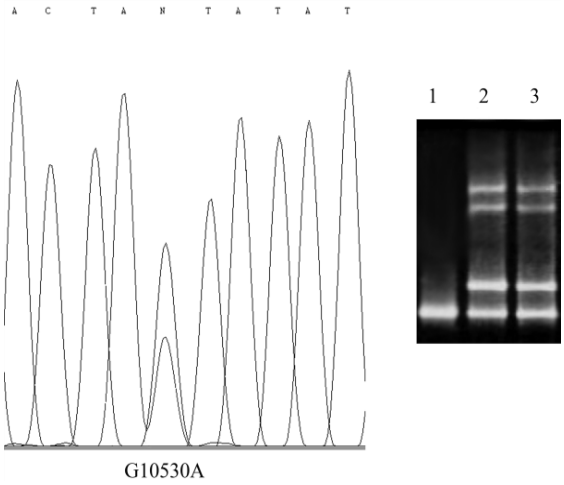

Figure 1. The detection of heteroplasmic G10653A mutation by TTGE and sequencing. Lane 1 and 2, the heteroplasmic multi-band patterns belonging to FRDA patients. Lane 3, wild type.

The second mutation was m.10530G>A transition, replacing a Val codon (GTA) with a Met (ATA) in a heteroplasmic state in patients 2 and 3 (V21M). We found this mutation in patients 2 and 3 with about 60% heteroplasmic level, but the degree of heteroplasmic in their mothers was about 25%. They showed a very early ataxia syndrome, and other signs included diabetes mellitus and cardiomyopathy. The GAA expansion size in these patients was smaller than the mean GAA repeats in FRDA patients (651±220) (Table 1). The calculated chemical distance (D) between Thr and Ala was relatively large (D= 58) (21) and these mutations changed the hydropathy profile at a hydrophobic region (Figure 2). SIFT predicted that this protein function would be affected with a score of 0.00 for V21M, and the median sequence conservation was 3.64.

The third mutation was m.10653G>A transition (homoplasmic state), changing Ala codon (GCC) to Thr codon (ACC) at A62T in patient 4. The substitution at position 62 from A to T was predicted by SIFT to affect protein function with a score of 0.00. The results of amino acids alignment revealed that the mutations T13A, V21M and A62T were high, moderate, and low conserved, respectively.

Table 1. The summary of the clinical and genetic analysis of FRDA patients.

Patient	Age of onset	GAA repeats	Mutation	Heteroplasmic/ Homoplasmic	Novel/Reported	Activity of complex I (nmol. Min^{-1}. mg $protein^{-1}$)*
1	15	498	m.10506A>G	Homoplasmic	LHON, PEO	84.18
2	12	256	m.10530G>A	Heteroplasmic	Novel	67.44
3	14	384	m.10530G>A	Heteroplasmic		78.53
4	21	247	m.10653G>A	Homoplasmic	Novel	81.45

Mean value for the control subjects (n= 35) were 119.42±13.48 (mean±SD) (12).

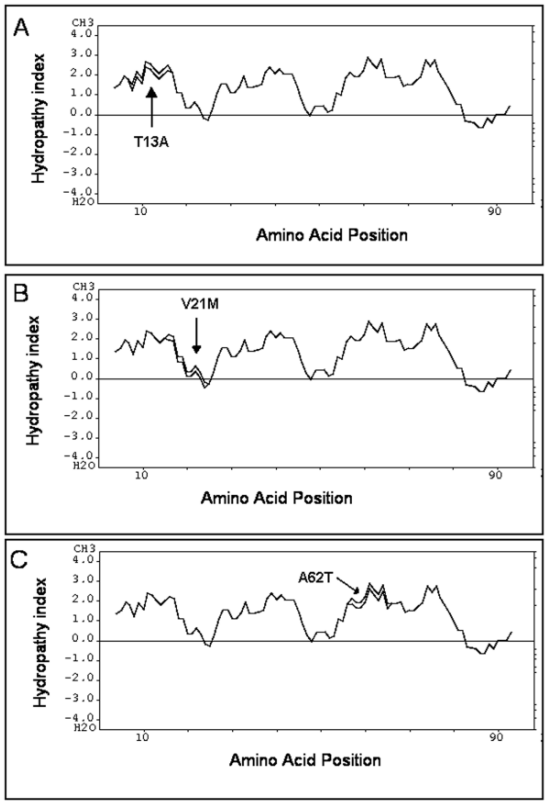

Figure 2. The hydropathy plot of the amino acids sequence of ND4L protein. The positive values indicate the hydrophobic regions, whereas the negative values indicate the hydrophilic regions. The arrows indicate the changes in hydrophobicity caused by the replacement of amino acids in the hydrophobic region. A) 10506, B) 10530 and C) 10653.

Discussion

Friedreich's ataxia is an inherited disease that causes a progressive damage to the nervous system resulting in symptoms ranging from gait disturbance and speech problems to heart diseases. Rotig *et al*, (1997) suggested that the frataxin gene plays a role in the regulation of mitochondrial iron content (22). They found a combined deficiency of a Krebs cycle enzyme, aconitase, and 3 mitochondrial respiratory chain complexes in the endomyocardial biopsy samples from patients with FRDA. Wilson and Roof (1997) suggested that mitochondrial dysfunction contributes to FRDA pathophysiology (23). Lodi *et al* (1999) reported *in vivo* evidence of impaired mitochondrial respiration in the skeletal muscles of FRDA patients (24).

Therefore, pathogenic mtDNA mutations may be recognized as a secondary case and a predominant risk factor. ND4L protein is coded by mtDNA genome. It has 98 amino acids and is a transmembrane protein. The secondary structure prediction by the algorithm of Garnier-Robson showed that this protein has a 79.3% alpha helix structure. We consider the m.10530 G>A mutation pathogenic for a number of reasons. First, this mutation alters the residues in the sequence of the protein, which is moderately conserved during the evolution and is located in a structurally/functionally important region. If the site is conserved across species, this implies that it is functionally important, and a mutation at this site is likely to be deleterious. Second, the mutation is consistent with the biochemical defect because the patients had complex I deficiency in their lymphocyte cells (Table 2) (12). Third, this mutation is never reported as a neutral polymorphism and is not detected in normal individuals from different ethnic backgrounds. Fourth, the mutation is heteroplasmic in the lymphocyte cells analyzed (the degree of heteroplasmy was about 60%), and heteroplasmic is a common feature of pathogenic mtDNA mutation. For

Table 2. The results of the secondary structure prediction of three mutations on ND4L protein.

ND4L	AA change	Alpha helix (%)	Extended strand (%)	Turn (%)	Random coil (%)
Normal	-	79.3	26.8	11.0	2.4
A10506G	Thr to Ala	81.7	24.4	11.0	2.4
G10530A	Val to Met	87.8	22.0	8.5	1.2
G10653A	Ala to Thr	70.7	34.1	11.0	3.7

heteroplasmic mutations this means that affected individuals have a high percentage of mutated mtDNA, while unaffected individuals have a lower percentage. Fifth, the chemical distance of a valine amino acid from a methionine amino acid (V21M) is relatively large (D= 21). The more chemical distance shows, the more amino acids conservation. Sixth, SIFT predicted the affected protein function. SIFT is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect. Seventh, this mutation caused to increase the alpha helix percentage to 87.8% (Table 2). Eighth, the mutation changes the hydropathy profile at a hydrophobic region (Figure 2). The changes in protein secondary structure and hydropathy profile might affect on protein function and be pathogenic.

The homoplasmic m.10653G>A mutation has not been reported previously. We found this mutation in patient 4 that had a small expanded allele of 247 repeats. This patient had a severe, typical Friedreich's ataxia. Our present study indicates that these missense mutations might affect the function of the ND4L protein. Also, our result show that the number of mutations in FRDA patients is higher than that in the control cases (P= 0.0287).

Conclusion

Although, this disease is due to nuclear gene mutations, the presence of these mutations might be responsible for further mitochondrial defects and the increase of gravity of the disease. So it should be considered seriously in patients with this disorder.

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