Increased Prevalence 12308 A > G mutation in Mitochondrial tRNA$^{\text{Leu (CUN)}}$ Gene Associated with earlier Age of Onset in Friedreich Ataxia

Abstract
Friedreich ataxia (FRDA) is an inherited recessive disorder. Mitochondrial DNA is a candidate modifying factor for FRDA. The purpose of this study was to investigate the relationship between the tRNA$^{\text{Leu (CUN)}}$ 12308 A> G mutation and age of onset in Friedreich ataxia.

Materials & Methods
The 12308 A> G substitution in mitochondrial tRNA$^{\text{Leu (CUN)}}$ was examined in DNA samples from 30 Friedreich ataxia patients and 48 control subjects by temporal temperature gradient gel electrophoresis (TTGE) and sequencing. Logistic regression was used to determine of cutoff age of onset.

Results
Twenty-two patients had the 12308 A> G mutation, and we found that its overall prevalence was significantly higher in 20 patients aged 17 years or younger than in 2 patients aged over 17 years (90% versus 10%). The 12308 A> G mutation lies in a region that has been highly conserved between species.

Conclusion
Our results show that the 12308 A > G mutation is associated with earlier age of onset in Friedreich ataxia. Thus, this mutation might cause the younger age of onset in FRDA.

Keywords: Friedreich ataxia; tRNA$^{\text{Leu (CUN)}}$ gene; mitochondrial DNA; Mutation; TTGE

Introduction
Friedreich Ataxia (FRDA) is a progressive neurodegenerative disease that results from a deficiency in frataxin, a protein that localizes to mitochondria. In typical FRDA patients, the gene has undergone a triplet (GAA) expansion in the first intron (1, 2). The progressive gait and limb ataxia, hypertrophic cardiomyopathy, and diabetes mellitus in FRDA patients are attributed to lowered levels of ATP that are generated in these energy-intensive tissues (3). The FRDA gene encodes a widely expressed 210-aa protein, frataxin, which is located in mitochondria and is severely reduced in FRDA patients (4). Yeast strains that carry a disruption in the Frataxin homolog (YFH1) develop a severe defect in the mitochondrial respiration chain (RC) and experience a loss in mitochondrial DNA (mtDNA) (5, 6) that is associated with elevated intramitochondrial iron (7-9). mtDNA is considered a candidate modifying factor for FRDA. Dysfunction of the mitochondrial respiratory chain is observed in patients with neurological diseases, including Alzheimer disease.
(10), Parkinson disease (11), multiple sclerosis (12), and Friedreich ataxia (13, 14). tRNA<sub>Leu</sub>(CUN) encodes for the most common amino acid in the mt-respiratory chain, implicating it in mtDNA-coded OXPHOS subunits. Van den Ouweland et al. identified a mutation in the mitochondrial tRNA<sub>Leu</sub>(CUN) gene in Chronic Progressive External Ophthalmoplegia (CPEO) and Wolfram syndrome (15).

The purpose of this study was to investigate the relationship between the tRNA<sub>Leu</sub>(CUN) 12308 A> G mutation and age of onset in Friedreich ataxia.

**Materials & Methods**

**Patients**

We studied 30 Iranian patients (14 females and 16 males) from 30 unrelated families with a diagnosis of FRDA, based on their clinical aspects, essentially adopting the clinical criteria of Harding and Geffroy et al. (16, 17). We also enrolled 48 healthy controls (23 females and 25 males) who were matched for age, sex, and ethnicity. Control subjects had no signs of FRDA on enrollment. All patients and control subjects were informed on the aims of the study and gave their informed consent for participation in the genetic analysis. Patients were referred for assessment by consultant neurologists in Iran.

**Molecular Analysis**

DNA was isolated from peripheral blood samples using a DNA extraction kit (DNAfast Kit-Genfanavaran, Tehran, Iran). The GAA repeat length was calculated per Campuzano et al. 1996 (18, 19). The tRNA<sub>Leu</sub>(CUN) gene was amplified by PCR in 25 µl using 2 primers (nt 11901-11920 (F) 5'-TGCTAGTAAACCACGTCTCC-3’ and nt 12420-12401 (R) 5'-TTTGTTAGGGTTAACGAGGG-3’). The reaction mixture comprised 2.5 mM MgCl<sub>2</sub>, 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 reactions were performed in thermal cyclers (Eppendorf, master cyclers, 5330) for 30 cycles as follows: 95°C for 50 s, 57°C for 1 min, and 72°C for 55 s. After the reactions, the PCR product (519 bp) was separated on a 1.5% agarose gels.

**Temporal temperature gradient gel electrophoresis (TTGE)**

The PCR product (519 bp) was denatured at 95°C for 30 s and cooled slowly to 45°C for 45 min at 1.1°C/min. TTGE was performed per 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 onto the gel and electrophoresed at 140 V for 5-6 h as the temperature was ramped at 1.2°C/h. The temperature range was determined by computer simulation (WinMelt software; Bio-Rad Laboratories). The DNA fragments from controls and patients were analyzed and compared. Any DNA fragments that showed differences in banding patterns between controls and patients were sequenced to identify the exact mutations (19). If a band shift or length heteroplasmy pattern was detected by TTGE, it was confirmed by direct DNA sequencing on an ABI 3700 capillary sequencer (Macrogen Seoul, South Korea). Sequences were compared with a comprehensive mitochondrial databank (20).

**Restriction enzyme analysis**

A 144-bp fragment encompassing the tRNA<sub>Leu</sub>(CUN) mutation site, located at nt 12,308, was amplified by mispairing PCR (15) (primers nt 12,190-12,209 (f) and nt 12,338-12,309 (r)). The reverse 30-mer oligonucleotide (5’- ATTACTTTATTTGGAGTTGCACCAGAATT) contains a nucleotide that differs from the Cambridge mtDNA sequence (21) (A to G substitution; underlined in the oligonucleotide sequence). This modification created an EcoRI site. The mtDNA fragment was digested with EcoRI for 1 hour at 37°C, and the fragments (119 and 25 bp) were separated on a 2.5% agarose gel. Bands were visualized by ethidium bromide staining.

**Statistical analysis**

Fisher’s exact probability test was used to examine the association between two groups and to find the relation between the 12308 A> G mutation and earlier age of onset in Friedreich ataxia. Logistic regression was used to determine the cutoff age of onset. When we fit the logistic regression to observations, we obtained a cutoff point 17. P<0.05 was regarded as statistically significant. The statistical analysis was performed using GraphPad Prism and Minitab.

**Results**

The age range of patients and controls was 8-32 and 11-34 years, respectively. The age of FRDA onset in our
patients was 13.6 ± 4.8 years (mean ± SD). TTGE analysis was performed for 30 patients and 48 healthy controls. Our 30 patients were homozygous for an expanded (GAA)n repeat in the FRDA gene. The size of the GAA expansion in patients was 634 ± 242 repeat (mean ± SD), ranging from 243 to 967 GAA repeats. All patients presented with the classical Friedreich ataxia phenotype, with absence of tendon reflex. Normal controls of the same ethnicity were also genotyped to establish the frequency of mutations. DNA fragments that showed abnormal banding patterns by TTGE analysis were sequenced to identify the exact mutations (Figure 1). The analyzed mtDNA sequences were compared with those of the published sequence (20).

Twenty-two of 30 FRDA patients had a homoplasmic 12308 A> G mutation. A subsequent analysis by mispairing PCR showed that this mutation was homoplasmic in blood from the 5 patient’s mother (healthy individual) and 3 healthy controls (Figure 2). The data showed that the homoplasmic 12308 A> G mutation in FRDA samples was more frequent than in normal controls (P = 0.000).

Discussion

The expansion of the GAA repeat in intron 1 of the FRDA gene results in a reduction in frataxin expression. Although skeletal muscle cell involvement is not a prominent clinical feature of patients with FRDA, it has been shown that frataxin is absent from or severely reduced in skeletal muscle cells in FRDA patients, although frataxin mRNA is normally expressed at intermediate levels (18, 19, 22). Houshmand et al. demonstrated that the rate of D-loop variations was higher in FRDA patients than controls (P < 0.05); mtDNA deletions were present in 76% of our patients who presented with mtDNA damage, which may be due to the accumulation of iron in mitochondria (23). We suggested previously that our patients had a biochemical defect in complex I activity and ATP production (24) and several point mutations in mitochondrial ND genes (25). In 22 patients with the 12308 A> G mutation, the overall prevalence of this mutation was significant higher in the 20 patients aged 17 years or younger than in the 2 patients aged over 17 years (90% versus 10%, represented P = 0.000) (Figure 3). Note that the cutoff point, 17 years, is the earlier age of onset for which the probability of having the 12308 A> G mutation is 0.5.

When we analyzed GAA repeats in these 22 patients, that mean ± SD of longer GAA repeats in patients aged 17 years or younger was higher than those aged 17 years (691 ± 142 versus 468 ± 188, represented P = 0.01). The tRNA-Leu(CUN) sequence and the two-dimensional (2D) structure comparison, per the Mamit-tRNA database (http://mamit-tRNA.u-strasbg.fr), showed that the 12308 A > G mutation is highly conserved between species. The 12308 A > G mutation is found in breast cancer (26), and Pulkses et al. reported an increased risk of stroke that is associated with the presence of a homoplasmic 12308 A > G variant in 48 patients (27). Iron accumulation in the mitochondria of patients with FRDA results in hypersensitivity to oxidative stress as a consequence of a Fenton reaction. (Fe2+-catalyzed production of hydroxyl radicals). As reactive oxygen species (ROS) are continuously generated by the respiratory chain, they may cause significant oxidative damage to mtDNA (such as mtDNA deletions and mutations) if they are not efficiently eliminated (28).

More than 60 different pathogenic mutations and more than 50 polymorphic sites have been described in these tRNA genes (see also http://www.gen.emory.edu/mitomap.html). The difficulties in deciding to what extent mtDNA (and nuclear DNA) analyses should be performed in patients with RC deficiencies underscore the importance of investigating a large group of patients for the presence of pathogenic mitochondrial mutations. The tRNA-Leu(CUN) mutation in MELAS and diabetes patients gives rise to severe impairments in 16S ribosomal RNA transcription termination, resulting in an imbalance between the amounts of ribosomal and other RNA transcripts (29). The effect of all other tRNA gene mutations on the transcription and translation of mitochondrial genes has not been investigated. Our results show that the 12308 A > G mutation associates with earlier age of onset in Friedreich ataxia. It is possible that the 12308 A > G mutation in mtDNA is a predisposing factor that lowers age of onset in FRDA.

Acknowledgments

This research was supported by Yazd University. We thank all patients for providing blood samples for the
scientific research as well as the Special Medical Center (Tehran, Iran), whose cooperation and support were essential to our work. The study was approved by the Yazd University Human Research Ethics Committee.

Financial Disclosures
None declared.

Funding/Support
None declared.

Fig 1. Detection and Identification of a Homoplasmic 12308 A > G Mutation in FRDA Patient by TTGE and Sequencing
Lane 1, 2 and 3 homoplasmic band shifts belong to FRDA patients. Lane 4, normal control. Sequencing result revealed this mutation.

Fig 2. Detection of tRNA_{Leu}^{CUN} Mutation by EcoRI Digestion in One Control (Lane 1), FRDA Patient and Her Mother (Lane 2 and 3), Control with this Mutation (Lane 4) and One Patient and Two Controls Without this Mutation (Lane 5, 6 and 7). Fragment sizes in base pairs are indicated.
**Fig 3.** The Graph of Probability of Having the 12308 A > G Mutation Versus the Earlier Age of Onset in FRDA

**Table 1:** The Probability of Having the 12308 A > G Mutation with the Earlier Age of Onset for Total of 30 Patients are Used in this Clinical Study. This Table Shows That the Probability of Having the 12308 A > G Mutation is Closed to One for the Small Values of the Earlier age of Onset. On the Other Hand the Probability of Having the 12308 A > G Mutation Decreased Where the Earlier Age of Onset Increased and Vice Versa.

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References


23. Houshmand M, Mahmoudi T, Panahi MS, Seyyedena Y, Saber S, Ataei M. Identification of a new human mtDNA 12308 A > G mutation in Mitochondrial tRNA<sup>Leu</sup><sup>(CUN)</sup> Gene


