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## Accepted Manuscript

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**HIGHLIGHTS**

- MtDNA G15927A or G15928A variation is said to be associated with MS.
- Using PCR-RFLP, we tested this association in an Iranian population.
- We found no association between the mtDNA variation and MS.

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## Mitochondrial DNA G15927A and G15928A variations in patients with multiple sclerosis

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**Abstract:**

**Background:** Modern genetics has offered a fresh perspective on the pathology of Multiple Sclerosis (MS). As mitochondrial DNA (mtDNA) variations are held to be potential contributors to the complex pathobiology of MS, the present study tests the claim that mtDNA G15927A or G15928A variations, or both, are associated with MS in an Iranian population.

**Materials and methods:** Following DNA extraction from blood samples of 100 subjects with relapsing-remitting MS, and 100 healthy unrelated control subjects, PCR-RFLP analyses was carried out by HpaII restriction enzyme reaction. Electrophoresis was then performed with 3% Agarose gel. As the restriction enzyme did not differentiate between two neighboring nucleotide positions (G15927A and G15928A), all PCR products with a variant allele were sequenced to determine the exact position of the variation.

**Results:** The MtDNA G15927A or G15928A variations were observed in 11 of all 100 cases of MS (11%) and in 7 of 100 healthy control subjects (7%) ( $P=0.3$ ,  $OR=1.6$ ,  $95\% CI=0.5-5.2$ ). Having sequenced all the PCR products with the variant allele (11 cases and 7 controls), the mtDNA G15927A variation was found in one of the 100 cases (1%) and 3 of 100 controls (3%) ( $P=0.3$ ,  $OR=0.3$ ,  $95\% CI=0.0-4.1$ ). Therefore, the mtDNA G15928A variation was present in 10 of the 100 cases (10%) and in 4 of 100 controls (4%) ( $P=0.09$ ,  $OR=2.6$ ,  $95\% CI=0.7-12.0$ ).

**Conclusion:** Neither mtDNA variation, G15927A or G15928A, was associated with MS in the studied Iranian population. There was a non-significant association of the G15927A and the G15928A variations separately with MS.

**Keywords:** G15927A; G15928A; mtDNA variation; mitochondrial DNA variation; Multiple Sclerosis; MS

## Introduction

Multiple sclerosis (MS) affects the brain and the spinal cord (Andalib et al., 2015b). The disease is characterized by a wide spectrum of neurological symptoms such as sensory loss (Namerow, 1968), motor (Guthrie, 1951), bladder, bowel, and sexual dysfunctions (DasGupta and Fowler, 2003), and optic neuritis (Plant, 2008), fatigue (Braley et al., 2012), pain (Archibald et al., 1994), cognitive dysfunction (Lovera and Kovner, 2012), and depression (Ghojzadeh et al., 2014). MS diagnosis is made by clinical observation and paraclinical tests (Andalib et al., 2016). Neuroimaging such as magnetic resonance imaging (Barkhof et al., 1997) is necessary for diagnosis of MS, while lumbar puncture (Gajofatto et al., 2013), evoked potential analysis (Parisi et al., 1998) and optic coherence tomography (Talebi et al., 2013) provide additional information. On the basis of the course of the disease, MS is divided into several subtypes (Roudbary et al., 2017), of which the relapsing-remitting form is the most frequent. Inflammatory processes contribute to MS as well as neurodegeneration (Andalib, Sasan et al., 2017b). The specific cause of MS remains elusive, but hypotheses of the underlying etiology abound, (Andalib et al., 2015a). More recently, increasing attention has been directed to the neurogenetics of MS. Attempts to unravel a potential genetically initiated pathogenesis of MS have led to the claim that mitochondrial gene changes may contribute to the disease, beyond the possible alterations of nuclear genes. Mitochondria are cellular engines, and many investigations have shown that an oxidizing environment affects the mitochondrial DNA (mtDNA). MtDNA encodes enzymes contributing to oxidative phosphorylation, transfer RNA (tRNA) and ribosomal RNA (rRNA), which are subject to maternal inheritance (Andalib, S et al., 2017).

MtDNA variations are thought to be associated with many neurological disorders such as Parkinson's disease (Andalib et al., 2014), Alzheimer's disease (Grazina et al., 2006), stroke (Andalib, Sasan et al., 2017a), and MS (Andalib et al., 2013a). In the only study of the association of the

mtDNA G15927A and G15928A variations with the tRNA Thr gene in MS Mayer-Wohlfart et al. (Mayr- Wohlfart et al., 1996) found a higher frequency of the variation in German MS patients. Because of the potential importance of mtDNA variations, we set out in the present study to test whether the mtDNA G15927A and G15928A variation is associated with MS in an Iranian population.

## **Materials and Methods:**

### **Study design, study size, setting and participants:**

We used a case-control design of the present study, as approved by the Ethics Committee of Tabriz University of Medical Sciences. Using sample size calculation test, the subject number was determined with a power of test of 80% with STATA software (version 12), with a case control ratio of one-to-one. In order to avoid similar risk factors, 100 relapsing-remitting MS subjects were selected according to the McDonald criteria from several therapeutic centers of Tabriz University of Medical Sciences, Tabriz, Azerbaijan province, Iran. One hundred healthy unrelated control subjects were recruited from the general population of Azerbaijan province. Exclusion criteria included a family history of neurodegenerative or inherited disorders. The confounding effect of heterogeneity of the genetic background of the participants, triggering possible false-positive or false-negative results, was controlled by selecting participants from Azerbaijan Province. A frequency matching was performed for age to restrict its possible confounding effect. The potential confounding effect of gender was controlled by individual matching in that each case was associated to one control.

### **MtDNA genotyping:**

Following blood sampling from each participant, DNA was extracted using a salting-out method. Appropriate forward and reverse primers (Table 1) were designed to amplify the target

sequence using primer3 designing tool. Polymerase Chain Reaction (PCR) was thereafter carried out with a previously applied standard protocol (Andalib et al., 2013b; Motavallian et al., 2013). Afterwards, Restriction Fragment Length Polymorphism (RFLP) analysis was carried out on PCR products using a restriction endonuclease enzyme which target sequence was influenced by the nucleotide change (Table 1). For this purpose, PCR products with restriction enzyme was incubated for a day. Using a UV transilluminator, the restriction products were assessed on 3% agarose gel electrophoresis with DNA safe stain. As the restriction enzyme was unable to differentiate between two neighboring nucleotide positions (G15927A or G15928A), all the samples with variant allele were sequenced (PerkinElmer sequencer) to identify the exact position of the variation.

### Statistical methods

Collected data were analyzed by using STATA (Version 12.0) with chi-square test and a P-value of  $\leq 0.05$  was considered statistically significant. Odds ratio (OR) with 95% confidence interval (CI) was calculated using multivariate logistic regression.

### Results:

In the initial analysis, the mtDNA G15927A or G15928A variation was found in 11 of all 100 cases (11%) and in 7 of 100 control subjects (7%). No heteroplasmy was seen for the mtDNA G15927A or G15928A variations (Figure 1). In Figure 2, we compare the sequencing results for the mtDNA G15927A and G15928A variations. The comparison of allelic frequencies between case and control groups for the mtDNA G15927A or G15928A variation is illustrated in Figure 3. The Chi-squared analysis showed a non-significant association between MS and the mtDNA G15927A or G15928A variation ( $P=0.3$ ) (Table 2).



In order to detect the exact site of variation, when PCR products showed G15927A or G15928A variation, we sequenced the 11 patients and 7 control subjects identified above. In the second round of analysis, the sequencing results showed that the mtDNA G15927A variation was present in a single of the 100 cases (1%) and in 3 of the 100 control subjects (3%). In Figure 4, we compare allelic frequencies of the mtDNA G15927A variation in the case and control subject groups. No significant association was observed between the MS diagnosis and the mtDNA G15927A variation ( $P=0.3$ ). Bivariate logistic regression analysis yielded an OR of 0.3 (Table 3).

The mtDNA G15928A variation was found in 10 of the 100 cases (10%) and in 4 of the 100 control subjects (4%). Figure 5 compares allelic frequencies of the mtDNA G15928A variation in the case and control subject groups. The chi-squared analysis revealed no significant association between the MS diagnosis and the mtDNA G15928A variation ( $P=0.09$ ), with a bivariate logistic regression analysis OR of 2.6 (Table 4).

### **Discussion:**

The G15927A and G15928A are frequently studied mtDNA variations in MS. We carried out the present study to test the hypothetical association of the variations with susceptibility to MS. The restriction enzyme HpaII detected adjacent mtDNA at np, 15927 and 15928. In the first round of analysis, the mtDNA G15927A or G15928A variation was present in 11 of a total of 100 cases (11%) and 7 of a total of 100 controls (7%), with no significant association between the MS diagnosis and the mtDNA G15927A or G15928A variation ( $P=0.323$ , OR=1.6, 95% CI=0.5-5.2).

These findings do not corroborate those of Mayer-Wohlfart et al. (Mayer- Wohlfart et al., 1996) who reported higher frequencies of G15927A and G15928A in patients with MS (14%) than in control subjects (2%) ( $P = 0.00018$ ). Furthermore, the mutations in patients and control subjects

were shown to coincide with one (3 cases) or more (16 cases) secondary LHON mutations. The authors concluded that the two substitutions may be involved in failures of vision in MS.

Here, we sequenced all PCR products with G15927A or G15928A variation (11 cases and 7 control subjects) to detect the exact nucleotide position of the variation. Consequently, in the second round of analysis, the mtDNA G15927A variation was present in one of the 100 cases of MS (1%), and in 3 of the 100 control subjects (3%), but with no significant association between the MS diagnosis and the mtDNA G15927A variation ( $P=0.3$ ,  $OR=0.3$ ,  $95\% CI=0.0-4.1$ ). The mtDNA G15928A variation was present in 10 of the 100 cases of MS (10%) and in 4 of the 100 control subjects (4%), likewise with no significant association between the MS diagnosis and the mtDNA G15928A variation ( $P=0.096$ ,  $OR=2.6$ ,  $95\% CI=0.7-12.0$ ).

The findings of the present study indicate that the mtDNA G15927A and G15928A variations have no association with the MS diagnosis in this population of Iranians, and no significant association between the MS diagnosis and the G15927A and G15928A variations separately. As the results failed to confirm those of the only previous study, replication of the current findings is required to reach a firm conclusion.

#### **Contribution:**

SA contributed to the design of the experiments and the drafting of the manuscript. MT, MF, and NM collected participants and contributed to the drafting of the manuscript. ES supervised genetic analyses and contributed to the drafting of the manuscript. HS contributed to the epidemiological design and revision of the manuscript. TM, MS, and AG contributed to the interpretation of findings and revision of the manuscript.

#### **Conflicts of interest:**

The authors declare no conflicts of interest in this study.

**Acknowledgement:**

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| SNP                      | Forward primer (5'-3')         | Reverse primer (3'-5')          | Total segment length | Restriction enzyme | Restriction sequence | Expected Length of fragments following RFLP |
|--------------------------|--------------------------------|---------------------------------|----------------------|--------------------|----------------------|---|
| G15927A<br>or<br>G15928A | GTAGC<br>ATCCG<br>TACTA<br>TAC | GTACC<br>GTACA<br>ATATT<br>CATG | 331 bp               | HpaII              | c/cgg                | 123 bp+208 bp+331 bp                        |

Table 1. Primers, restriction enzyme, and length of mtDNA fragments before and after enzymatic digestion

| Cases N (%)   | Controls N (%) | P value | OR  | 95% CI  |
|---------------|----------------|---------|-----|---------|
| 11/100 (0.11) | 7/100 (0.07)   | 0.3     | 1.6 | 0.5-5.2 |

Table 2: Statistical significance, odds ratio (OR), and 95% confidence interval (95% CI) for mtDNA G15927A or G15928A variation.

| Cases N (%)  | Controls N (%) | P value | OR  | 95% CI  |
|--------------|----------------|---------|-----|---------|
| 1/100 (0.01) | 3/100 (0.03)   | 0.3     | 0.3 | 0.0-4.1 |

Table 3: statistical significance, odds ratio (OR), and 95% confidence interval (95% CI) between case and control groups for mtDNA G15927A variation.

| Cases N (%)   | Controls N (%) | P value | OR  | 95% CI   |
|---------------|----------------|---------|-----|----------|
| 10/100 (0.10) | 4/100 (0.04)   | 0.09    | 2.6 | 0.7-12.0 |

Table 4: statistical significance, odds ratio (OR), and 95% confidence interval (95% CI) between case and control groups for mtDNA G15928A variation

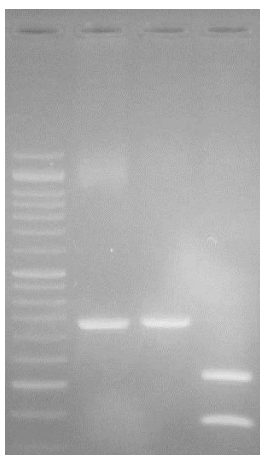


Figure 1: Electrophoresis of PCR and restriction products for mtDNA G15927A or G15928A variation (DNA ladder, Undigested PCR product, Homoplasmic 15927A or 15928A mtDNA fragment, and Homoplasmic wild type mtDNA fragments, left to right, respectively)

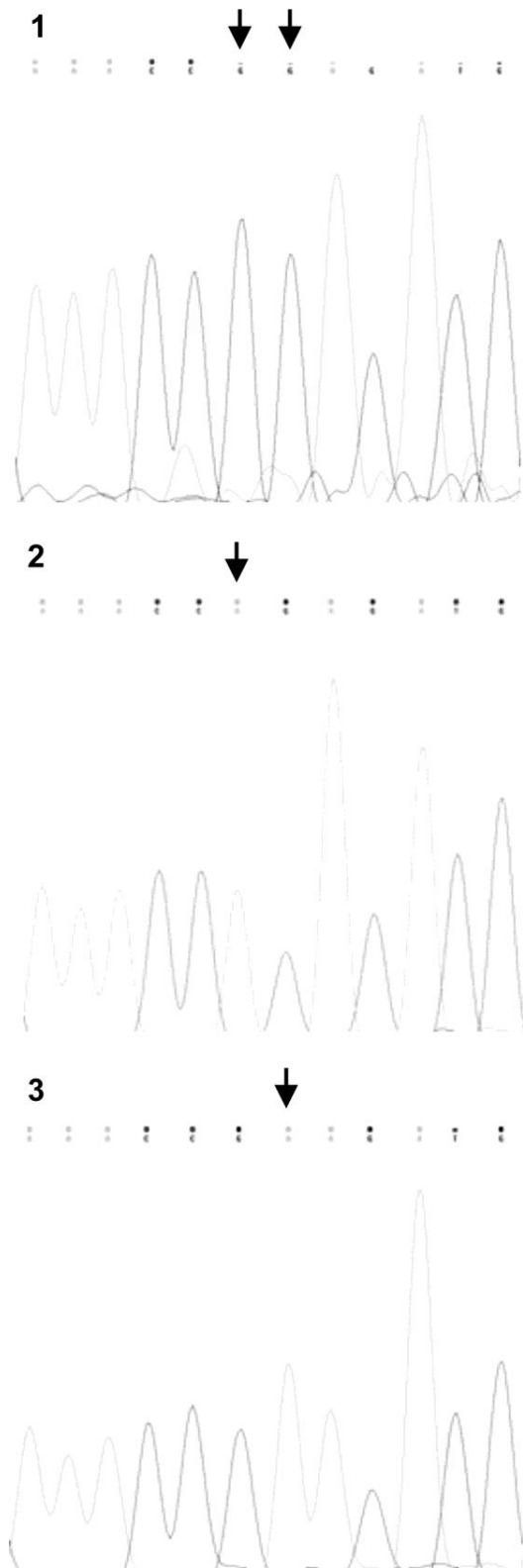


Figure 2: Sequencing results of mtDNA G15927A or G15928A variation



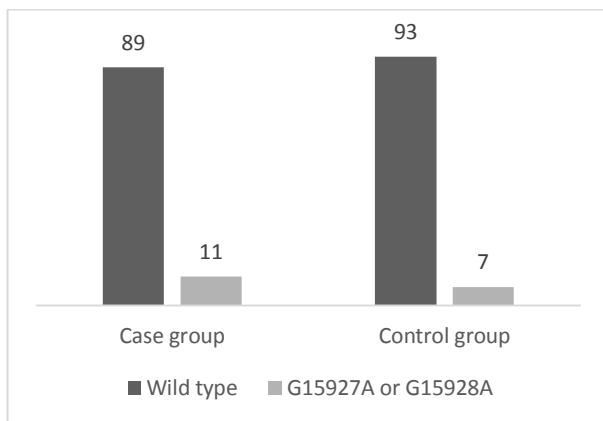


Figure 3: Allelic frequencies of mtDNA G15927A or G15928A variation in the case and control groups

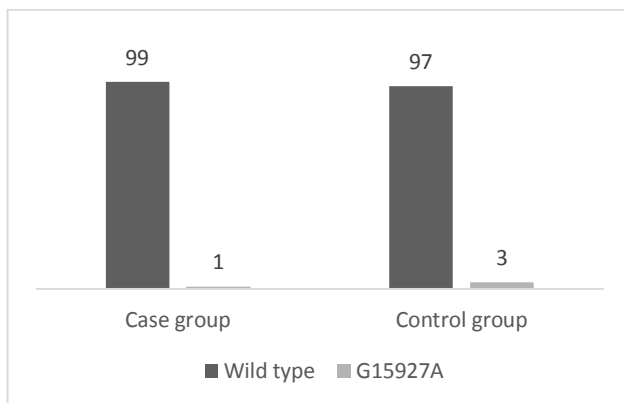


Figure 4: Allelic frequencies of mtDNA G15927A variation in case and control groups

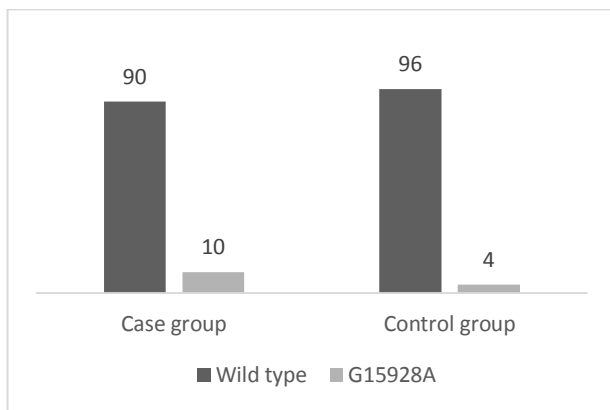


Figure 5: Allelic frequencies of mtDNA G15928A variation in case and control groups