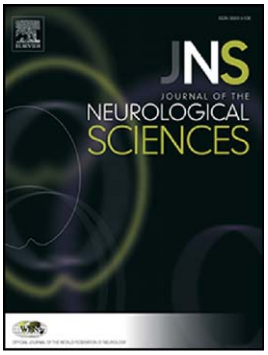

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Lack of association between mitochondrial DNA G15257A and G15812A variations and Multiple Sclerosis

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Abstract

Background: Multiple Sclerosis (MS) is a debilitating disease of the central nervous system for which no definitive therapy has yet been developed. The etiology remains uncertain, but there is evidence of genetic susceptibility to the disease, including contributions from mitochondrial DNA (mtDNA) variations to the pathogenesis of MS. G15257A and G15812A are variations of the mtDNA tRNA(Thr) gene in MS sufferers of different populations. The present study tested the hypothesis of an association of the G15257A and G15812A variations of the mtDNA tRNA(Thr) gene to the susceptibility to MS in an Iranian population.

Material and Methods: Two hundred subjects included 100 MS patients and 100 unrelated healthy controls. DNA was extracted from blood samples by means of the salting-out method. The mtDNA fragment was amplified by polymerase chain reaction (PCR). Restriction Fragment Length Polymorphism (RFLP) analysis was done by digestion of the PCR products with Acc I and Rsa I restriction endonuclease enzymes for mtDNA G15257A and G15812A variations, respectively. Afterwards, the restriction products were visualized by electrophoresis using 3% Agarose gel and safe DNA gel staining. To confirm the accuracy of genotyping procedure, sequencing of the mtDNA fragments was carried out in randomly selected samples.

Results: The mtDNA G15257A variation was found in one of the 100 patients and one of the 100 controls (P=0.637) (odds ratio [OR] =1, 95% confidence interval [95% CI] =0.0-79.2). The mtDNA G15812A variation was not found in any of the 100 patients or 100 controls (0%) (P=1) (OR =1, 95% CI =0.0-79.2).

Conclusion: The evidence from the present study is inconsistent with the hypothesis that the G15257A and G15812A variations in the mtDNA tRNA(Thr) gene are associated with susceptibility to MS in the selected populations.

Keywords: Multiple Sclerosis, MS, Mitochondrial DNA, MtDNA variation, G15257A, G15812A, tRNA(Thr) gene, Iranian population

Introduction

Multiple Sclerosis (MS) is a neurodegenerative disorder of uncertain etiology. Patients afflicted with MS experience a broad spectrum of neurological symptoms, including dysarthria [1], dysphagia [2, 3], bladder, bowel and sexual dysfunction [4], nystagmus [5], hypoesthesia [6], fatigue, tiredness, lack of energy and sleepiness [7], depression [8], fear of disease progression [9], memory impairment [10], tremor [11, 12], and spasticity [13, 14]. Traditionally, MS is held to stem from lymphocyte attacks on the oligodendrocytes that provide the myelin sheaths of nerve fibres. The consequence is damage to the pathways in the white matter of the brain and spinal cord containing myelin [15, 16]. MS has recently been characterized further as a neurodegenerative disorder [17] with axonal injury, neuronal loss, and atrophy of the central nervous system (CNS) [18] associated with deterioration of cerebral gray matter [19]. Magnetic resonance imaging (MRI), cerebrospinal fluid (CSF) analysis [20], and optic coherence tomography (OCT) [21] make important contributions to the diagnosis. The incidence of MS is rising but the etiology is still a mystery.

In terms of genetics, susceptibility to MS may arise from alterations of nuclear or mitochondrial genes, or both. Of the nuclear genes, MHC [22] and HLA [23-25] have been found to contribute to susceptibility to MS. There is also evidence that mutations of nuclear genes expressing mitochondrial proteins can be associated with MS. Some nuclear genes, including mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α), and nuclear respiratory factor 1 (NRF1), involved in the maintenance of the mtDNA content [26, 27], are held to be associated with MS. Mitochondrial dysfunction as a cause of axonal degeneration has also been postulated to contribute to the susceptibility to MS [28], and mitochondrial dysfunction may stem from mitochondrial gene alterations. Mitochondrial DNA (mtDNA) is more vulnerable to damage and mutation than nuclear DNA, due to the highly oxidizing environment of the mitochondria, with the relative exposure of mtDNA and the poor repair mechanisms [29]. The damage diminishes the ATP synthesis efficiency and augments the generation of toxic reactive waste products [29]. Notwithstanding the fact that all cells may be affected by this process neurons are particularly vulnerable, and mtDNA mutations either as cause or contributing factor may give rise to several neurological disorders [29], such as Leber's Hereditary Optic Neuropathy (LHON) [30], Alzheimer's Disease (AD) [31], Parkinson's disease

(PD) [32], and MS [33], Mitochondrial Encephalopathy with Lactic Acidosis and Stroke-like episode (MELAS) [34], Myoclonus Epilepsy with Ragged Red Fibers (MERRF) [35].

Mitochondria are cytoplasmic organelles that contribute to oxidative phosphorylation and thus to ATP production. MtDNA, which contains multiple copies of a circular structure of 16569 base pairs [36], produces mitochondrial RNA and proteins. The mitochondrial genome, which is inherited exclusively from mothers [37], has 37 intronless genes , expressing 13 subunits of the electron-transfer chain, 2 ribosomal RNAs, and 22 transfer RNAs [38]. The respiratory chain in mitochondria consists of 5 protein complexes, of which the genes of complex I are considered to be the most vulnerable part of mtDNA [39].

It is increasingly difficult to ignore the role of mitochondrial dysfunction arising from mtDNA variations or acquired mutations in the pathogenesis of MS**.** When axonal damage occurs in MS, gene products specific for the mitochondrial electron transfer chain diminish in the brain [28] along with a decreased capacity of respiratory chain complexes I and II [28]. Active MS lesions mainly exert their impact upon complex IV of the respiratory chain and is found in axons, oligodendrocyte in addition to astrocytes [40]. The acute mitochondrial injury in active lesions, in turn, gives rise to a compensatory growth in mitochondrial density and enzymatic activity in chronic established lesions [41]. In chronic active MS lesions, oxidative damage to mtDNA and impaired activity of complex I have been demonstrated [42]. Andalib et al. [33] also reviewed and highlighted the role of mtDNA gene variations in MS in multiple populations. With this in mind, the G15257A and G15812A are variations of the tRNA(Thr) mtDNA gene commonly found in various populations such as a German population [43] and an American population [44]. There is insufficient evidence of the association of mtDNA G15257A and G15812A variations with MS in Iran. Therefore, the present study tested the hypothesis that mtDNA G15257A and G15812A variations are associated with MS also in a population of Iranians.

Materials and Methods:

Study design, setting and participants:

The current study adopted a case-control design, as approved by the ethics committee of Tabriz University of Medical Sciences. Eligible patients afflicted with relapsing-remitting MS were diagnosed according to the McDonald criteria (n=100) [45], with 100 healthy and unrelated volunteers serving as controls. Exclusion criteria included a family history of neurodegenerative or inherited diseases for cases and controls. In order to avoid similar risk factors, the patients were recruited from several medical centers belonging to Tabriz University of Medical Sciences, Tabriz, Iran. To restrict the possible confounding effects of age and gender, frequency and individual matchings were carried out, respectively, with informed consent obtained in writing from each subject.

Study size:

The study size was determined using STATA software (version 12) with a test power of 80%.

MtDNA genotyping:

Blood samples were collected from eligible participants who met the selection criteria and DNA was thereafter extracted from their blood samples by means a salting-out method [46]. It is worth noting that the same DNA stock in a previous research [47] was used for the present study. Quantitation of the extracted DNA samples, showing optimal DNA extraction, was assessed by the spectrophotometry. Appropriate forward and reverse primers were utilized for specific mtDNA segments (Table 1). Using a gradient thermocycler (Peqlab, Germany), temperatures and cycling times were optimized for each DNA template target and primer pair. The polymerase chain reaction (PCR) amplification was thereafter carried out by using a thermocycler (Peqlab, Germany) according to a previously used PCR standard protocol [48, 49]. For the mtDNA G15257A and G15812A variations, Restriction fragment length polymorphism (RFLP) was identified by appropriate restriction endonuclease enzymes (Thermo Fisher Scientific Inc., USA) (Table 1) which target sequences were influenced by the nucleotide change. The digestion with restriction enzyme was followed by visualization of the restriction products by electrophoresis with 3% Agarose gel and DNA safe stain. Furthermore, accuracy of genotyping method was

confirmed by mtDNA sequencing. In order to confirm the PCR-RFLP results, several samples from each mtDNA variation were selected and purified with QIAquick Spin^{R} Purification Kit and directly sequenced by Macrogen Inc. (Soth Korea), using an automated ABI Prism 3730XL DNA sequencer (Perkin-Elmer).

Table1. Forward and revers primers and restriction endonuclease enzymes for analysis of the mtDNA G15257A and G15812A variations

Statistical methods

Data management and analysis were performed using STATA software (Version 12.0). Data were analyzed by Chi-square test and P-value <0.05 was considered statistically significant. In addition, using bivariate logistic regression analysis, odds ratio (OR) accompanied by 95% confidence interval (95% CI) was calculated.

Results

Demographic summary

In the present study, a ratio of 1 case per 1 control was applied, with 100 cases with MS and 100 healthy unrelated control subjects selected for the study. The demography of these cases and controls groups is listed in Table 2. Frequency matching was performed in 60% of cases and controls aged 20-35 years. Individual matching of gender in case and control groups resulted in ultimate recruitment of 63 females and 37 males.

Table 2: demographic summery of case and control groups

MtDNA G15257A variation findings

RFLP analysis (Figure 1) revealed that the mtDNA G15257A variation was present in 1 of 100 cases (1%) and in 1 of 100 controls (also 1%) (Figure 2). The chi-squared analysis showed no significant association between MS and the G15257A variation (P=1) (Table 3). Bivariate logistic regression analysis yielded an OR of 1 (Table 3). No heteroplasmy was found for the G15257A variation. Figure 3 illustrates sequencing confirmation for the RFLP results of the mtDNA G15257A variation.

Figure 1: Comparison of electrophoresis of PCR and restriction products for the mtDNA G15257A variation (DNA ladder, Undigested PCR product, Homoplasmic 15257A variant, and Homoplasmic normal variant, left to right, respectively)

Figure 2: Comparison of allelic frequencies between case and control groups for the mtDNA G15257A variation

Table 3: Statistical significance, odds ratio (OR), and 95**%** confidence interval (CI) between case and control groups for the mtDNA G15257A variation

Figure 3: Comparison of the sequencing results in the normal (1) and the mtDNA G15257A (2) variants

MtDNA G15812A variation findings

RFLP analysis (Figure 4) showed that the mtDNA G15812A variation was was absent from all of the 100 cases (0%) and the 100 controls (0%) (Figure 5). The chi-squared analysis showed no significant association between MS and the G15812A variation (P=1) (Table 4). Bivariate logistic regression analysis yielded an OR of 1 (Table 4), and no heteroplasmy was found for the mtDNA G15812A variation. Figure 6 shows sequencing confirmation in the RFLP results of the mtDNA G15257A variation.

Figure 4: electrophoresis of PCR and restriction products for the mtDNA G15812A variation (Homoplasmic normal variant, Homoplasmic normal variant, Undigested PCR product, and DNA ladder, left to right, respectively)

Figure 5: Comparison of allelic frequencies between case and control groups for the mtDNA G15812A variation

Table 4: statistical significance, odds ratio (OR), and 95**%** confidence interval (CI) between case and control groups for the mtDNA G15812A variation. * Note: inasmuch as exact confidence intervals are not possible to be calculated with zero count cells using STATA, one (1) was added to each count cell.

Figure 6. Normal sequencing result for the mtDNA G15812A variation

Discussion

The causes of MS remain uncertain, including the role (if any) of genetics as a contributing factor in the pathogenesis of MS. We previously assessed the role of mtDNA T4216C and A4917G variations in Iranian MS patients and found the variations are not associated with the disease ([T4216C: P=0.61, OR=1.1, 95% CI=0.5-2.4], [A4917G: P=0.637, P=0.637, 95% CI=0.4-3.5] [47]. The main issue addressed here is the association with MS of the mtDNA G15257A and G15812A variations in Iranian MS sufferers. In the present study, 1 out of the 100 MS subjects (1%) had the mtDNA G15257A variation, as well as 1 out of the 100 healthy controls (1%), and the statistical analysis revealed no association between the mtDNA G15257A variation and MS (P=1). Likewise, the mtDNA G15812A was seen in none of the MS (0%) or control (0%) subjects, and the statistical analysis revealed no association between the mtDNA G15812A variation and MS (P=1).

Recent years have seen an increasing interest in the genetics of mtDNA in MS. Association studies have had contradictory results on the roles of mtDNA G15257A and G15812A in the susceptibility to MS. The present findings agree with those of Hwang et al. [50] who found no association between MS and the LHON mutations in an Korean population. The authors analyzed 12 MS subjects for the mtDNA G15257A variation and found no MS subject with the variation. Moreover, the mtDNA G15257A variation was shown to have no pathogenetic significance in Italian MS subjects [51]. In that study, 4 out of 74 (5.4%) of the MS subjects presenting with MS with early and prominent optic nerve involvement and 5 out of 99 (5.1%) healthy controls had the mtDNA G15257A variation in a homoplasmic state.

On the other hand, however, the present results do not agree with those of Mayer-Wohlfart et al. [43] who found a possible association of mtDNA variations in MS with optic involvement. In that study in Germany, DNA was extracted from 100 Caucasian MS subjects with restarted visual evoked potentials and 100 Caucasian controls. Subsequent PCR-RFLP and sequencing analyses showed that the mtDNA G15257A variation was present in 3 MS subjects (2 male and 1 female), once alone and twice in combination with variation at np 13708, and the mtDNA G15812A variation was only seen in a female control subject, curiously in association with three other secondary LHON mutations. Also, Kalman et al. [44] investigated LHON associated mtDNA mutations in MS patients in the USA. Using sequencing and RFLP analysis in this

study, 11 out of the 53 MS subjects (20.8%) were shown to be positive for at least two (4216 and 4917 or 13,708) or three (4216, 13,708, 15,257) simultaneous secondary LHON mutations, along with 7 out of the 7 (9.5%) controls (P = 0.036). The authors concluded that the high incidence of the simultaneous secondary LHON variations in MS patients (and LHON) versus controls suggests that certain sets of the mtDNA variations are associated with, and predispose to, MS.

The main conclusion drawn from the findings of the present study is the lack of association of the mtDNA G15257A and G15812A variations with susceptibility to MS in the Iranian population. Nonetheless, because of the equivocal findings reported for this topic, further investigations into different populations are warranted.

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Conflict of interest:

Not declared.

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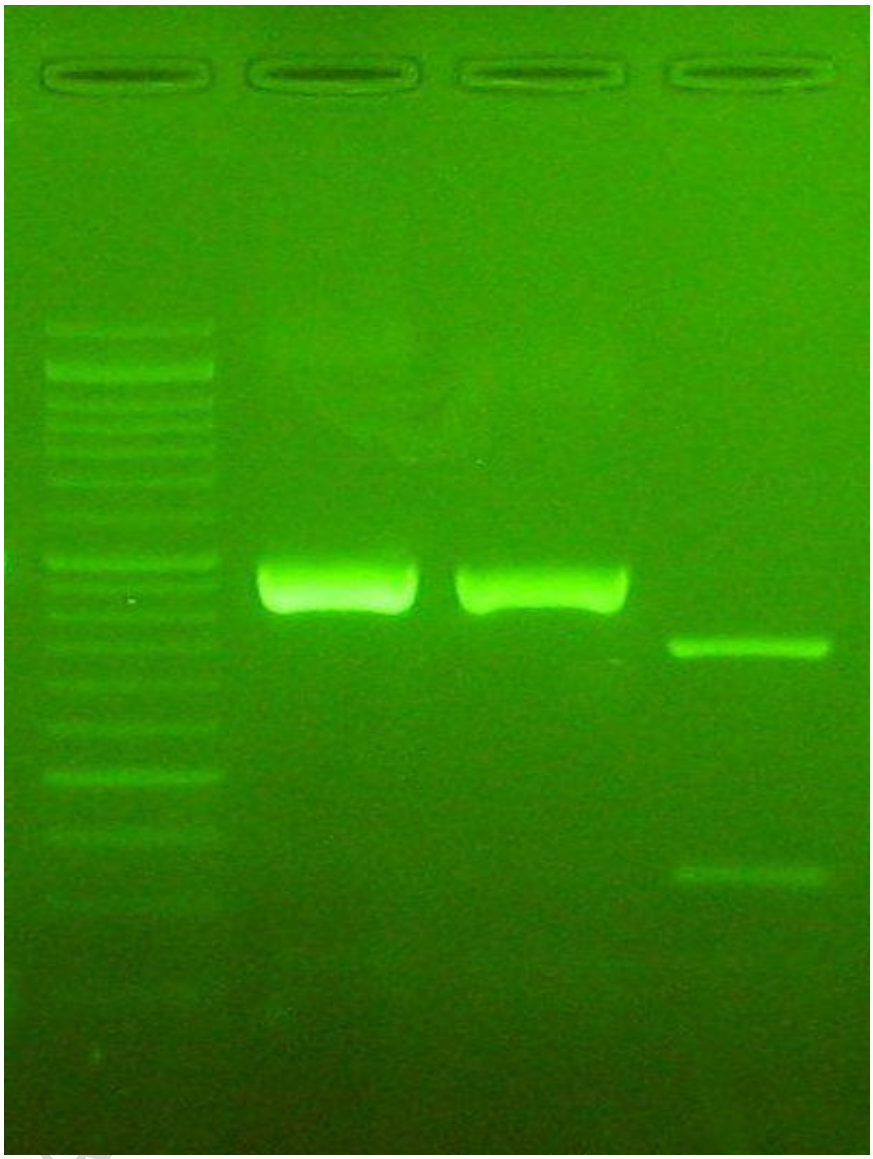

Figure 1

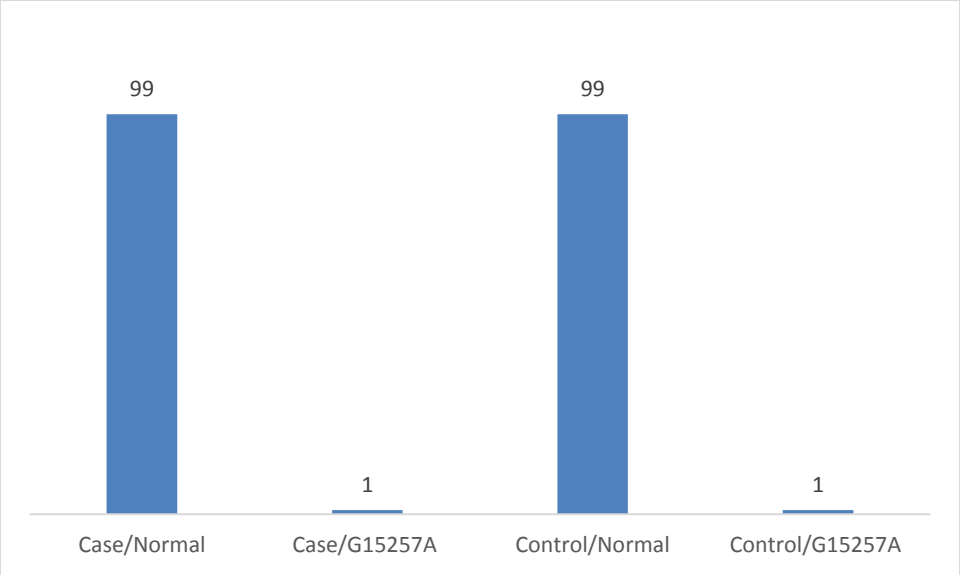

Figure 2

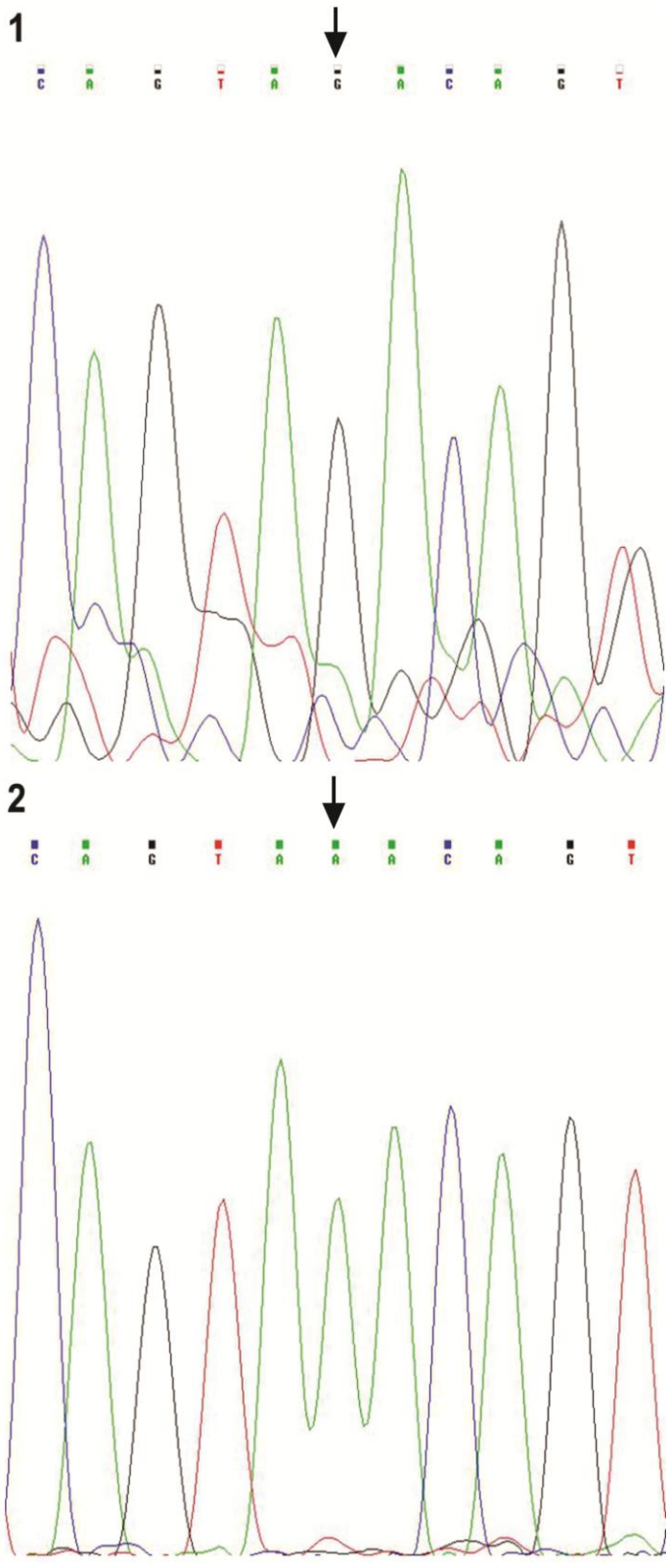

Figure 3

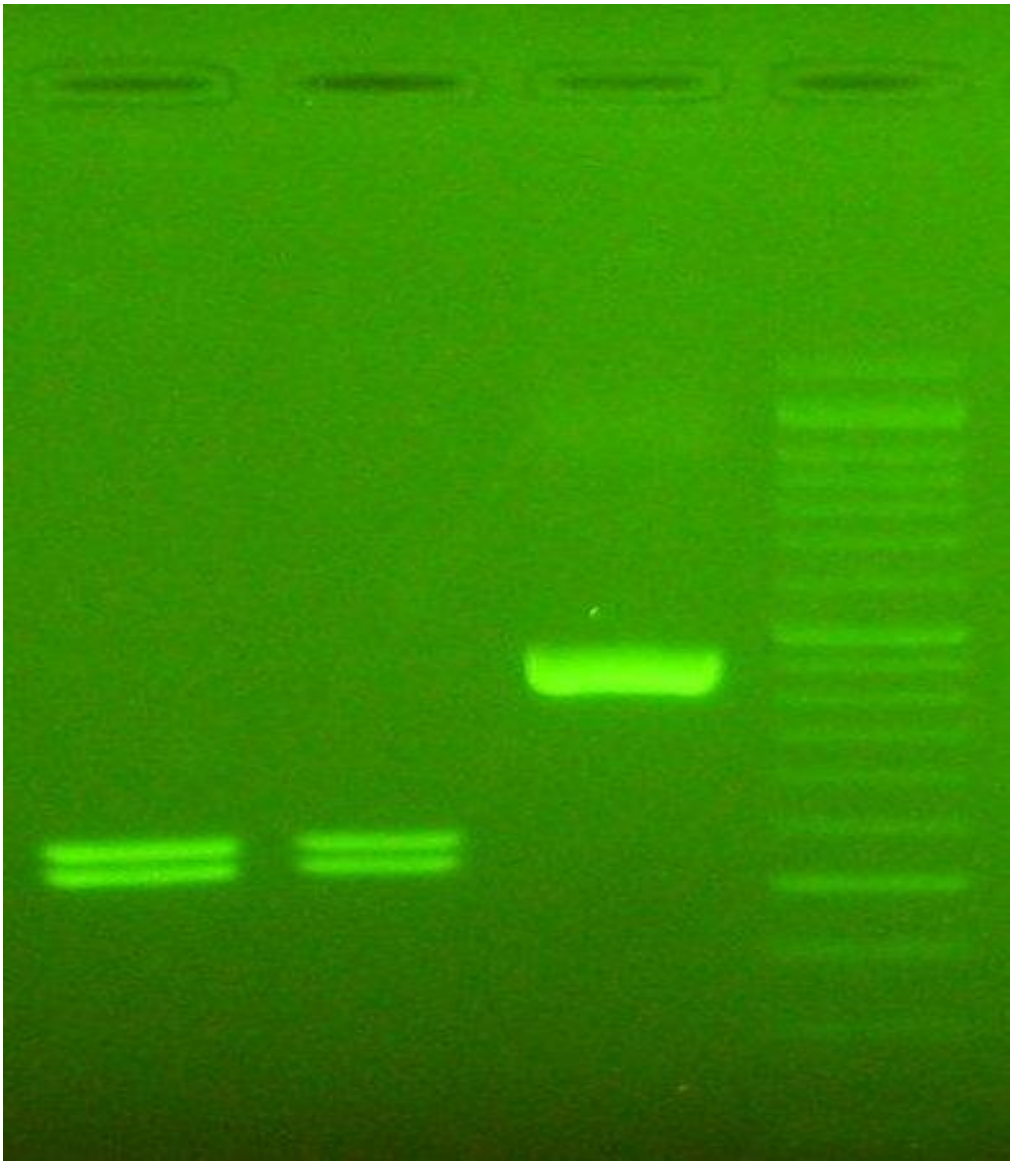

Figure 4

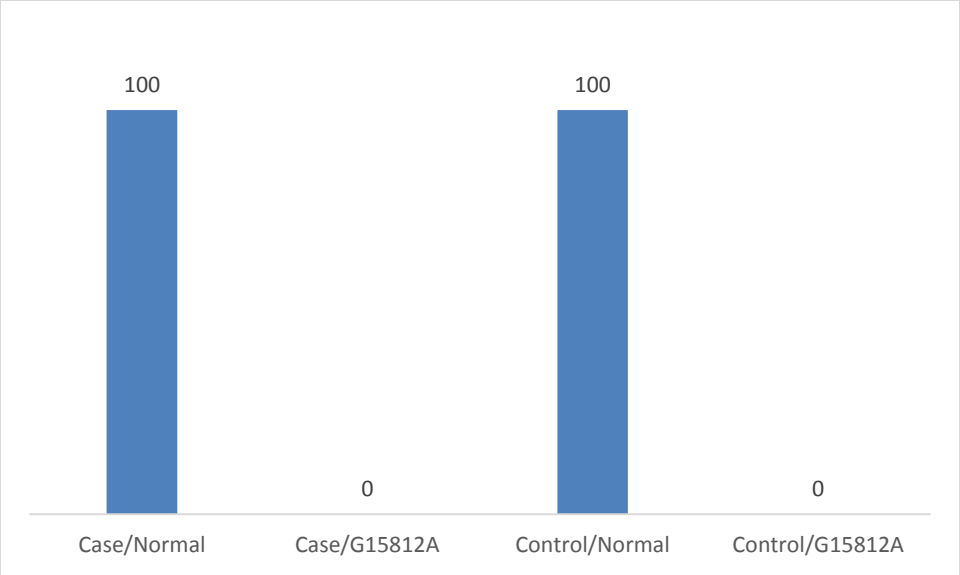

Figure 5

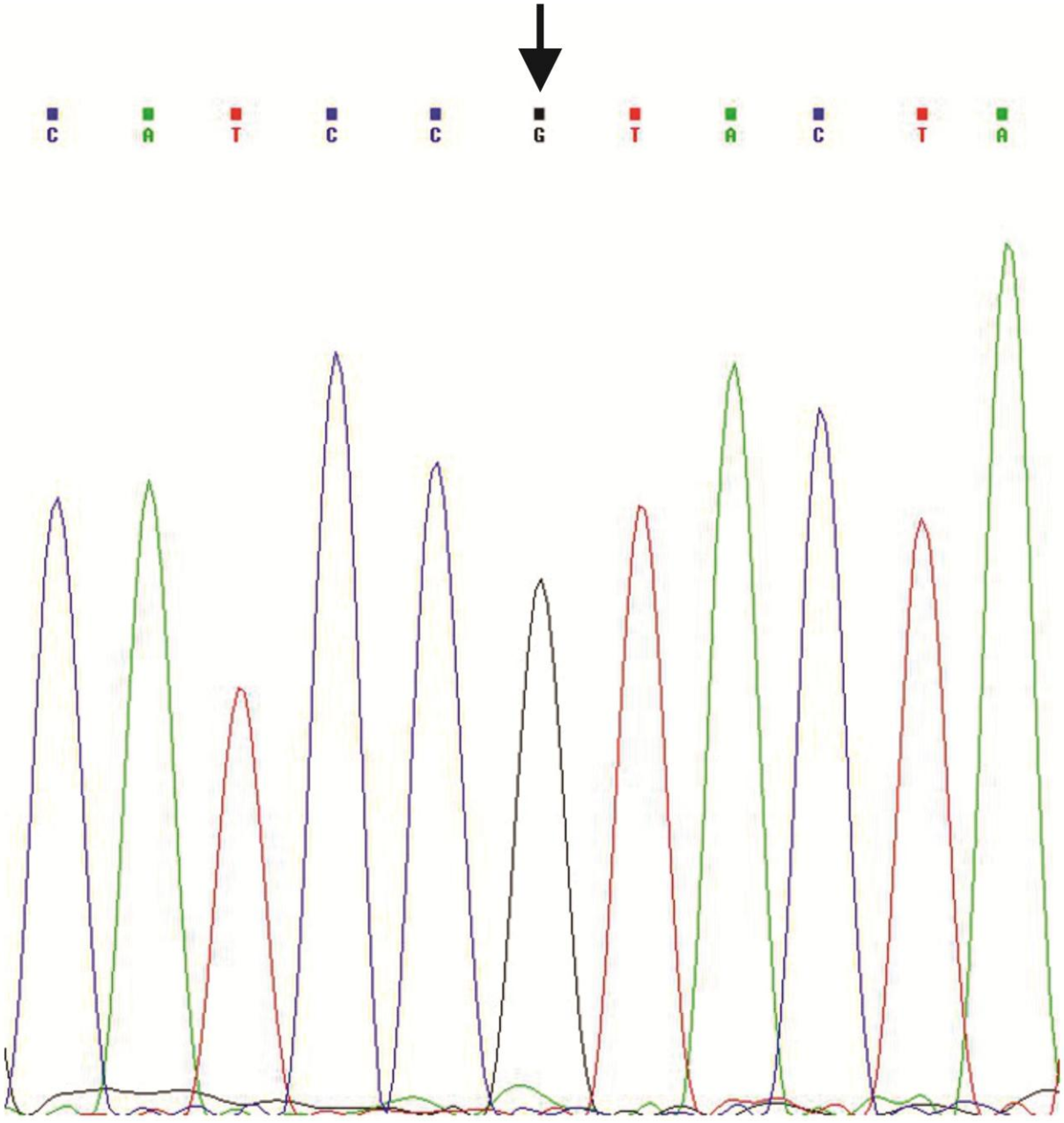

Figure 6

Table 1

MtDNA variation	Forward primer	Reverse primer	Endonuclease: Target sequence
G15257A	TCCTCCCGTGAGGCCAAATA	GGGACGGATCGGAGAATTGT	Acc I : gt/mkac
G15812A	TAACAAACTAGGAGGCGTCC	CTTGGGTGGTACCCAAATCT	Rsa I : gt/ac

Table 2

	Case group	Control group
Age (mean ± SD)	32.47±8.13 y	30.24±15.49 y
Gender	F=63(63%) M=37(37%)	F=63(63%) M=37(37%)

Table 3

Cases N (%)	Controls N (%)	P value	OR	95% CI
1/100 (0.01)	1/100 (0.01)	1	1	0.0-79.2

Table 4

Cases N (%)	Controls N (%)	P value	OR	95% CI
0/100 (0.00)	0/100 (0.00)	1	1	0.0-79.2*

Highlights

- Association of mtDNA G15257A and G15812A variations and MS was assessed.
- No association was found between mtDNA G15257A variation and MS.
- No association was found between mtDNA G15812A variation and MS.