

Four novel mutations in the mitochondrial *ND4* gene of complex I in patients with multiple sclerosis

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Abstract. Multiple sclerosis (MS) is an immune-mediated neurological, inflammatory disease of the central nervous system. Recent studies have suggested that genetic variants in mitochondrial DNA (mtDNA)-encoded complexes of respiratory chain, particularly, complex I (NADH dehydrogenase), contribute to the pathogenicity of MS among different ethnicities, and targeting mitochondrial function may represent a novel approach for MS therapy. In this study, we sequenced *ND* genes (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6*) encoding subunits of complex I in 124 subjects, 60 patients with relapsing-remitting MS and 64 healthy individuals, in order to identify potential novel mutations in these patients. We found several variants in *ND* genes in both the patients and controls, and specific variants only in patients with MS. While the majority of these variants were synonymous, 4 variants in the *ND4* gene were identified as missense mutations in patients with MS. Of these, m.11150G>A was observed in one patient, whereas m.11519A>C, m.11523A>C and m.11527C>T were observed in another patient. Functional analysis predicted the mutations, m.11519A>C, m.11523A>C and m.11150G>A, as deleterious with a direct impact on *ND4* protein stability and complex I function, whereas m.11527C>T mutation had no effect on *ND4* protein stability. However, the 3 mutations, m.11519A>C, m.11523A>C and m.11527C>T, which

were observed in the same patient, were predicted to cause a cumulative destabilizing effect on *ND4* protein, and could thus disrupt complex I function. On the whole, this study identified 4 novel mutations in the mtDNA-encoded *ND4* gene in patients with MS, which could lead to complex I dysfunction, and further confirmed the implication of mtDNA mutations in the pathogenicity of MS. The identified novel mutations in patients with MS may be ethnic-related and may prove to be significant in personalized treatment.

Introduction

Multiple sclerosis (MS) is a neurological chronic inflammatory disorder affecting the white matter of the central nervous system (CNS) that involves immune-mediated mechanisms. The disease is characterized by demyelination and axonal loss as a result of myelin sheath damage by the body's own immune system, which affects the ability of nerve cells in the brain and spinal cord to effectively communicate with each other (1,2). However, the exact immunopathogenic mechanisms responsible for the initiation and progression of the disease remain unknown. Clinically, MS presents with a range of signs and symptoms that appear as episodic or progressive neurological impairments, such as numbness and tingling, blurry vision, mobility and balance issues, muscle weakness and tightness, bladder and bowel dysfunction, decreased memory and fatigue, as well as other conditions, such as depression (3,4). These symptoms present in each of the 4 types of MS that are as follows: i) Relapsing-remitting MS (RRMS), which is the most common type and occurs in 85-90% of patients; ii) secondary progressive MS (SPMS), which occurs in 70-80% of patients with RRMS within 10-15 years; iii) primary progressive MS (PPMS), which occurs in 15% of cases; and iv) progressive relapsing MS (PRMS), the least common form which occurs in 5% of patients (5). MS affects approximately 2.5 million individuals worldwide and commonly appears in young adults with a mean age of 32 years (6). Moreover, MS usually occurs 2-3 times more frequently in females than in males (7). Higher rates of MS have been reported in Europe, Southern Canada, Northern United States, New Zealand

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and Southeast Australia (8,9). Studies from the Arab Gulf countries, including Kuwait, Saudi Arabia and United Arab Emirates have also demonstrated noticeable increases in the incidence and prevalence of MS (10,11).

Although the cause of MS remains unclear, combinations of genetic and environmental factors may contribute to the etiopathogenicity of the disease (12-14). Recently, MS has become increasingly viewed as a neurodegenerative disorder, in which mitochondrial dysfunction occurs early in the pathogenic process and plays an important role in axonal degeneration and demyelination, as well as in disease progression (15-17). Therefore, it has been suggested that targeting mitochondrial pathways along with neuroprotection and immunomodulation may provide a novel approach for the treatment of MS (16-18).

The mitochondria are the main site of energy production in the cell and are also the major source of reactive oxygen species (ROS). Mammalian mitochondria have their own genome [mitochondrial DNA (mtDNA)], a single, circular double-stranded molecule of 16,569 base pairs. mtDNA encodes 2 rRNAs and 22 tRNAs, as well as 13 polypeptides that are all subunits of complexes of the respiratory chain, located in the inner mitochondrial membrane, that drives oxidative energy metabolism. While subunits of complex II are entirely encoded by mtDNA, subunits of complexes I, III, IV and V are encoded by either mtDNA or nuclear DNA (nDNA) (19). Specifically, mtDNA encodes 7 subunits of complex I (NADH dehydrogenase), 1 subunit of complex III (ubiquinol-cytochrome *c* oxidoreductase), 3 subunits of complex IV (cytochrome *c* oxidase) and 2 subunits of complex V (20). It has been demonstrated that mtDNA has a higher mutation rate than the nuclear genome. A number of factors contribute to the increased rate of mutations in mtDNA, such as the close proximity of mtDNA to the site of ROS production, the lack of protective histones and the low efficiency of DNA repair pathways (21,22). The loss of mitochondrial genomic integrity can lead to a progressive decline in mitochondrial function (23,24) and, eventually, to a reduction in energy within the cell, which has been implicated in a number of neuroinflammatory and neurodegenerative diseases (25). The role of defects in mtDNA in MS stems from the observation that a number of patients with Leber hereditary optic neuropathy (LHON), a maternally inherited mitochondrial disease caused by mutations in the *ND1*, *ND4*, *ND4L* and *ND6* genes of complex I, develop neurological features, including inflammatory demyelinating disorders compatible with a diagnosis of MS (26,27). Studies on mtDNA in patients with MS from different ethnic backgrounds have identified variations within complex I genes to be associated with the pathogenicity of MS. A specific variant in the *ND2* gene of complex I has been reported to play a role in the susceptibility to MS in Caucasians (28). Other variants in different genes of complex I have been shown to be associated with the risk of developing MS in a Filipino population (29). These observations suggest that although the disease etiology is common between populations, genetic variants can be population-specific. Therefore, in the current study, we carried out genetic analysis of mtDNA-encoded complex I genes to identify specific mutations in Saudi patients with MS. The identification of ethnicity-related mtDNA variations

in MS may lead to the development of novel approaches for personalized treatment.

Subjects and methods

Subjects. Between October, 2016 and June, 2017, a total of 124 Saudi subjects were enrolled in this study, 60 patients with RRMS and 64 healthy control subjects. Patients diagnosed with RRMS at the Neurology Outpatient Clinic at King Khalid Hospital, King Saud University were selected for this study. The diagnosis of RRMS was performed according to the McDonald Criteria (30) with at least 2 previous relapses in different CNS regions, as confirmed by a neurological examination, medical history, clinical examination, magnetic resonance imaging (MRI) and electrophysiological analyses. The inclusion criteria for the patients were the following: Either sex, a relapsing-remitting course with at least 1 documented relapse during the previous year or 2 documented relapses during the previous 2 years, and a Kurtzke Expanded Disability Status Scale (EDSS) score of 0-5.5 inclusive. The exclusion criteria for the patients were the following: A documented history of infections, chronic diseases, such as diabetes, chronic diseases of the immune system other than MS, immunodeficiency syndrome, malignancy and pregnancy.

The healthy control subjects were recruited from King Khalid hospital Blood Bank, Kingdom of Saudi Arabia. Their medical history confirmed the absence of any neurological disorders, active infections or other medical conditions. The inclusion criteria for the controls were as follows: Either sex, and the absence of any neurological disorders, active infections or other medical conditions. Individuals with a documented history of chronic diseases, such as diabetes, immune system or inflammation disorders, immunodeficiency syndrome and malignancy were excluded from the study. Age, body mass index (BMI) and hypertension data were collected from the medical records of the patients and healthy controls. Medication, disease duration and disability status, evaluated using the Kurtzke EDSS were collected from the patients.

Ethics statement. Ethics approval was obtained from the Scientific and Ethics Committee in King Saud University, College of Medicine, Kingdom of Saudi Arabia and from the Medical Research and Ethics Committee in the College of Medicine and Medical Sciences, Arabian Gulf University, Kingdom of Bahrain. The participants were given a complete description of the study. Informed consent was obtained from all individual participants included in the study.

Extraction of genomic DNA. Venous blood samples were collected from the participants into ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was extracted from peripheral blood samples using the QIAMP DSP DNA kit (Qiagen) as previously described (31). In brief, 20 μ l protease were mixed with 200 μ l EDTA-blood followed by the addition of lysis buffer (200 μ l). The mixture was incubated at 56°C for 10 min, and then centrifuged at 20,000 x g for 1 min at 4°C. Absolute ethanol (200 μ l) was then added to the mixture followed by centrifugation at 6,000 x g for 1 min at room temperature. Washing steps were carried out using 500 μ l washing buffer followed by centrifugation at 6,000 x g for

Table I. Primers of mtDNA-encoded complex I genes.

Target genes	Forward sequence (5'-3')	Reverse sequence (5'-3')	Annealing temperature (°C)	PCR product (bp)
ND1	CTCAACTTAGTATTATACCC	GAGCTTAGCGCTGTGATGAG	59	1,249
ND2	GTCATCTACTCTACCTAC T	GGCGGGAGAAGTAGATTGAA	52	689
ND3	CACTATCTGCTTCATCCGCC	GAGCGATATACTAGTATTCC	54	1065
ND4	GCGCAGTCATTCTCATAATC	TTTGTTAGGGTTAACGAGGG	54	729
ND4L	TCTGGCCTATGAGTGACTAC	ACTGTGAGTGCGTTTCGTTTCGTA GTTTGG	54	1,415
ND5	TTTTGGTGCAACTCCAAA	GGTTGACCTGTTAGGGTGAG	50	1,369
ND6	CTCCAAAGACCACATCATCGAAAC	TTCATCATGCGGAGATGTTGG ATGGGGTGG	52	1,334

1 min and then at 20,000 x g for 3 min (at room temperature). To elute the genomic DNA, elution buffer (200 µl) was added, incubated at room temperature for 1 min and then centrifuged at 6,000 x g for 1 min (at room temperature). The concentration of DNA was determined using a NanoDrop ND-1000 ultraviolet-visible light spectrophotometer (Thermo Fisher Scientific, Inc.). All DNA samples were stored at -20°C until further analysis.

Polymerase chain reaction (PCR) and DNA sequencing. mtDNA-encoded ND1, ND2, ND3, ND4, ND4L, ND5 and ND6 genes of complex I were amplified by PCR. The sequences of the primers of the 7 subunits (Table I) were as previously described by Zonouzi *et al* (29). Each PCR reaction contained 50 ng of DNA, 0.4 µl of each primer, 0.2 µl Super Taq polymerase (Invitrogen; Thermo Fisher Scientific, Inc.), 0.8 µl MgCl₂, 0.4 µl dNTPs master mix (10 mM of each), and 2.5 µl 10X PCR buffer to a final volume of 25 µl. PCR was performed using an automated thermal cycler (Perkin-Elmer 2400) with the following cycling program: Denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 40 sec, annealing for 40 sec and extension at 72°C for 90 sec and a final extension at 72°C for 10 min. The PCR products of the ND1, ND2, ND3, ND4L, ND4, ND5 and ND6 genes were purified using a QIAquick PCR Purification kit (Qiagen). The PCR products were then directly sequenced in both directions using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems). Sequencing products were run on 3130XL Genetic Analyzer (Applied Biosystems) and analyzed using SeqScape Software v2.5 (Life Technologies; Thermo Fisher Scientific, Inc.).

Statistical analysis. SPSS software version 21 was used for data analysis. Differences in clinical variables between patients and controls were evaluated according to the variable distribution. First, the Kolmogorov-Smirnov test was used to evaluate the normal distribution of the data. Accordingly, comparisons of variables between the 2 groups were carried out using a Chi-square test for categorical variables and the equivalent non-parametric Wilcoxon signed-rank test and Mann-Whitney test for normally distributed variables. Data are presented as the means ± standard deviation (SD). A P-value <0.05 was considered to indicate a statistically significant difference.

Bioinformatics analysis. *Homo sapiens* mitochondrion complete genome, the Revised Cambridge Reference Sequence (GenBank no. NC_012920.1.) was used as a reference sequence. Raw sequences obtained from DNA sequencing were first examined using Chromas software, version 2.6.6 (Technelysium Ltd.). Variations were identified and listed according to their corresponding positions on the reference genome. Artemis was used to annotate base variations and locate their positions on the annotated reference sequence (32). The variant sequence of each corresponding coding sequence was translated into amino acid sequence by Translate tool (EXPASy.org) using the vertebrate mitochondrial genetic code. ClustalOmega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) was used to align the obtained sequence to the annotated one. To predict the effects of mutations on protein function, Site Directed Mutator (SDM) server was used (33). SDM is a computational method that analyses the variation of amino acid substitutions occurring at specific structural environment that are tolerated within the family of homologous proteins of known 3-D structures, and convert them into substitution probability tables. SMD is considered more effective than other published methods in the task of classifying mutations as stabilizing or destabilizing (33); mutations can be analyzed, either separately or in a cumulative manner, by incorporating all mutations in one single file. The results are expressed in Del Del G (Enthalpy).

Results

Characteristics of the study subjects. In this study, 60 patients with RRMS and 64 healthy control individuals were included. The baseline characteristics of the participants are presented in Table II. The MS group included 14 males and 46 females, and the group of healthy subjects included 22 males and 42 females. No significant differences were observed in sex distribution between the MS group and the control group (P=0.679). The mean age in the MS group was 30±8.9 years and ranged between 18 to 50 years. The mean age in the control group was 36.7±12.2 years and ranged between 19 to 60 years. The mean age differed significantly between the patient group and the control group (P=0.003). However, no significant differences between the 2 groups were found as regards BMI (P=0.140) or mean blood pressure (P=0.089). The degree of disability of the patients as evaluated by the

Table II. Baseline characteristics of participants.

Characteristics	RRMS (n=60)	Control (n=64)	P-value
Sex (male/female)	14/46	22/42	0.679
Age (years)	30.4±8.9	36.7±12.2	0.003
BMI (kg/m ²)	26.4±5.31	29.5±5.8	0.140
Mean blood pressure (mmHg)	107.2±10.38	96.6±16.14	0.089
Disease duration (years)	6.33±4.5	-	
EDSS	4.8±7.1	-	
Medication			
Rebif	12		
Glienya	13		
Betaferon	8		
Avonex	15		
Tysabri	12		

Data are presented as number for categorical data or the means ± standard deviation (SD) for parametrically distributed data. RRMS, relapse-remitting multiple sclerosis; BMI, body mass index; EDSS, expanded disability status scale.

EDSS score ranged from 1-8 (4.8±7.1). The patients had a different duration of disease ranging from 1 to 20 years with a mean of 6.33±4.5 years. The patients were undergoing treatment with Rebif (n=12), Gilenya (n=13), Betaferon (n=8), or Avonex (n=15) and Tysabri (n=12).

Variants in mtDNA-encoded complex I genes in MS patients and healthy individuals. DNA sequencing of mtDNA-encoded complex I revealed successful results for ND genes (*ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6*). Overall, in the patients and healthy individuals, sequence analysis revealed several variants in the *ND1*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6* genes, and no variants were found in the *ND2* gene (Table III). In the patients with MS, variants were found in different positions of complex I genes; of these, 79 in *ND1*, 6 in *ND3*, 15 in *ND4L*, 119 in *ND4*, 127 in *ND5* and 8 in *ND6*. In the healthy subjects, variants were also found in different positions of complex I genes; of these 65 in *ND1*, 32 in *ND3*, 16 in *ND4L*, 84 in *ND4*, 139 in *ND5* and 16 in *ND6*. Moreover, a number of variants in the *ND4* and *ND5* genes were commonly observed at high frequencies in the patients with MS and the healthy subjects. Of the observed variants in the *ND4* gene, m.11719G>A was found in 53.33% (n=32) of patients and 45.31% (n=29) of healthy individuals; m.11251A>G was found in 28.33% (n=17) of patients and 25% (n=16) of healthy individuals. Of the observed variants in the *ND5* gene, m.12372G>A was found in 21.66% (n=13) of patients and 17.19% (n=11) of healthy individuals; m.13708G>A was found in 21.66% (n=13) of patients and 14.06% (n=9) of healthy individuals; m.12612G>A was found in 25% (n=15) of patients and 12.5% (n=8) of healthy subjects, and m.12705 C>T was found in 21.66% (n=13) of patients and 21.88% (n=14) healthy individuals.

Variants in mtDNA-encoded complex I genes identified only in patients with MS. Sequence analysis revealed a number of variants only in patients with MS, while none of the healthy subjects carried these variants. These variants were found in the *ND2*, *ND3*, *ND4L*, *ND4*, *ND5* and *ND6* genes. Of these, 22 were observed in the *ND1* gene, 2 in the *ND3* gene, 7 in the *ND4L* gene, 27 in the *ND4* gene, 31 in the *ND5* gene and 19 in the *ND6* gene (Table IV). Using bioinformatics analysis, it was revealed that the majority of the variants were synonymous and did not cause any amino acid changes. Therefore, they were not considered to be significant, but rather simple polymorphisms and were excluded from further analysis.

Of note, bioinformatics analysis indicated 4 variants as missense mutations which were found in the coding sequence of the *ND4* gene (Tables IV and V). These included m.11150G>A, which exhibited an alanine to threonine alteration (p.A131T), m.11519A>C which exhibited a threonine to proline alteration (p.T254P), m.11523A>C, which exhibited a lysine to threonine alteration (p.K255T) and m.11527C>T, which exhibited a histidine to leucine alteration (p.H256L). Notably, the m.11150G>A variant was observed in 1 patient (no. 48), whereas the other 3 variants, m.11519A>C, m.11523A>C and m.11527C>T, were all observed in another patient (no. 44). The clinical characteristics of these patients, including age, sex, years since diagnosis, number of relapses per year, main neurological dysfunction and the severity of disease, MRI and lumbar puncture (LP) findings, as well as previous and current medications are presented in Table VI. Patient no. 44, who carried the mutations, m.11519A>C, m.11523A>C and m.11527C>T, was a 31-year-old female and presented with blurry vision, pain on ocular movement, sensory ataxia, weakness, numbness and imbalance. Patient no. 48, who carried the mutation, m.11150G>A, was a 25-year-old female and presented with imbalance, incoordination of movement, weakness on lower limbs, numbness and imbalance.

Predicting the functional impact of mutations. The functional context of the 4 missense mutations identified in the patients with MS was predicted using the SDM server (33). As indicated by their scores (Table V), m.11150G>A, m.11519A>C and m.11523A were revealed to be destabilizing mutations in terms of protein stability with Del Del G of -2.05, 1.54 and -0.95, respectively. However, the m.11527C>T mutation exhibited no effect on protein stability (Del Del G of 0.99). Since the mutations, m.11519A>C, m.11523A>C and m.11527C>T, were observed in 1 patient (Table V), they were further analyzed for their cumulative functional effect on *ND4* protein. The results predicted a cumulative effect of the 3 mutations together, causing an overall destabilizing effect on *ND4* protein.

Discussion

In the current study, we carried out sequence analysis for the mtDNA-encoded complex I (NADH dehydrogenase) genes in 164 Saudi subjects, 60 patients with RRMS and 64 healthy controls, in order to identify mutations relevant to MS. We found several variants in different positions of the *ND1*, *ND3*, *ND4L*, *ND4*, *ND5* and *ND6* genes both in the patients with MS and the healthy individuals. However, no variants were found in the *ND2* gene in the 2 groups. Some of the identified

Table III. Variations in mtDNA-encoded complex I identified in patients with multiple sclerosis and healthy controls.

Gene	Patients with multiple sclerosis			Healthy controls		
	Nucleotide change	No. of nucleotide changes	Frequency (%)	Nucleotide change	No. of nucleotide changes	Frequency (%)
<i>ND1</i>	m.3316G>A	1	1.66	m.3316G>A	1	1.56
	m.3438G>A	4	6.66	m.3421G>A	2	3.13
	m.3531G>A	3	5	m.3438G>A	1	1.56
	m.3834G>A	4	6.66	m.3531G>A	1	1.56
	m.3915G>A	1	1.66	m.3666G>A	1	1.56
	m.3480A>G	1	1.66	m.3693G>A	1	1.56
	m.3720A>G	3	5	m.3705G>A	1	1.56
	m.3537A>G	1	1.66	m.3834G>A	1	1.56
	m.579A>G	3	5	m.3915G>A	1	1.56
	m.3584A>G	1	1.66	m.4048G>A	1	1.56
	m.3865A>G	2	3.33	m.3384A>G	1	1.56
	m.3948A>G	1	1.66	m.3480A>G	5	7.813
	m.4104A>G	5	8.33	m.3505A>G	1	1.56
	m.4188A>G	1	1.66	m.3537A>G	3	4.69
	m.14340A>G	1	1.66	m.3768A>G	1	1.56
	m.3513C>T	1	1.66	m.4093A>G	1	1.56
	m.3533C>T	6	10	m.4104A>G	3	4.69
	m.3594C>T	1	1.66	m.4188A>G	2	3.13
	m.4059C>T	1	1.66	m.4225A>G	1	1.56
	m.4312C>T	3	5	m.4231A>G	1	1.56
	m.3516C>A	4	6.66	m.4340A>G	1	1.56
	m.3847T>C	10	16.66	m.4316A>G	1	1.56
	m.3866T>C	4	6.66	m.3336T>C	1	1.56
	m.3944T>C	1	1.66	m.3350T>C	1	1.56
	m.4216T>C	16	26.66	m.3423T>C	1	1.56
			m.3847T>C	7	10.94	
			m.4216T>C	13	20.31	
			m.4232T>C	1	1.56	
			m.4336T>C	1	1.56	
			m.3429T>C	1	1.56	
			m.3594T>C	4	6.25	
			m.4312T>C	1	1.56	
			m.3516C>A	1	1.56	
			m.3546C>A	1	1.56	
<i>ND3</i>	m.10373T>C	1	1.66	m.10172T>C	1	1.56
	m.10289A>G	1	1.66	m.10325T>C	1	1.56
	m.10115T>C	2	3.33	m.10217A>G	1	1.56
	m.10238T>C	1	1.66	m.10289A>G	1	1.56
	m.10355C>T	1	1.66	m.10295A>G	1	1.56
				m.10398A>G	20	31.25
				m.10238T>C	2	3.13
				m.10410T>C	1	1.56
				m.10115T>C	1	1.56
				m.10343C>T	1	1.56
<i>NDL4</i>	m.10586G>A	1	1.66	m.10589G>A	1	1.56
	m.10589G>A	2	3.33	m.10685G>A	1	1.56
	m.10688G>A	2	3.33	m.10688G>A	2	3.13

Table III. Continued.

Gene	Patients with multiple sclerosis			Healthy controls		
	Nucleotide change	No. of nucleotide changes	Frequency (%)	Nucleotide change	No. of nucleotide changes	Frequency (%)
<i>ND4</i>	m.10499A>G	4	6.66	m.10550A>G	5	7.813
	m.10550A>G	1	1.66	m.10463T>C	7	10.94
	m.10664C>T	2	3.33			
	m.10463T>C	3	5			
	m.11150G>A	1	1.66	m.10810T>C	10	15.63
	m.11176G>A	3	5	m.10810T>C	1	1.56
	m.11377G>A	3	5	m.10915T>C	1	1.56
	m.11440G>A	4	6.66	m.11025T>C	1	1.56
	m.11719G>A	32	53.33	m.11299T>C	6	9.38
	m.10819A>G	2	3.33	m.10822C>T	1	1.56
	m.10876A>G	3	5	m.11332C>T	1	1.56
	m.10895A>G	1	1.66	m.11674C>T	1	1.56
	m.11002A>G	4	6.66	m.10876A>G	1	1.56
	m.11172A>G	2	3.33	m.11251A>G	16	25
	m.11251A>G	17	28.33	m.11467A>G	10	15.63
	m.11337A>G	1	1.66	m.11530A>G	1	1.56
	m.11380A>G	1	1.66	m.11641A>G	1	1.56
	m.11467A>G	14	23.33	m.11671A>G	1	1.56
	m.11530A>G	1	1.66	m.11708A>G	1	1.56
	m.11641A>G	3	5	m.11719G>A	29	45.31
	m.10822C>T	4	6.66	m.10984C>A	1	1.56
	m.11332C>T	1	1.66	m.11260T>G	1	1.56
	m.11527C>T	1	1.66			
	m.10810T>C	1	1.66			
	m.10810T>C	2	3.33			
	m.10873T>C	11	18.33			
	m.10915T>C	3	5			
	m.11299T>C	1	1.66			
	m.11518G>T	1	1.66			
	m.11519A>C	1	1.66			
	m.11523A>C	1	1.66			
	<i>ND5</i>	m.12372G>A	13	21.66	m.12372G>A	1
m.12771G>A		2	3.33	m.12372G>A	11	17.19
m.13316G>A		1	1.66	m.12501G>A	2	3.13
m.13368G>A		3	5	m.12771G>A	1	1.56
m.1356G>A		2	3.33	m.13368G>A	7	10.94
m.13708G>A		13	21.66	m.13194G>A	1	1.56
m.13813G>A		4	6.66	m.13708G>A	9	14.06
m.13803G>A		2	3.33	m.12397G>A	1	1.56
m.12530G>A		4	6.66	m.12530G>A	2	3.13
m.12612G>A		15	25	m.12612G>A	8	12.5
m.12693G>A		2	3.33	m.12654G>A	2	3.13
m.12720G>A		1	1.66	m.12693G>A	1	1.56
m.12753G>A		1	1.66	m.12720G>A	1	1.56
m.12720G>A		2	3.33	m.12810G>A	1	1.56
m.12720G>A		1	1.66	m.12937G>A	1	1.56
m.13104G>A		1	1.66	m.12950G>A	1	1.56
m.13105G>A		3	5	m.13105G>A	6	9.38

Table III. Continued.

Gene	Patients with multiple sclerosis			Healthy controls		
	Nucleotide change	No. of nucleotide changes	Frequency (%)	Nucleotide change	No. of nucleotide changes	Frequency (%)
	m.13276G>A	3	5	m.13276G>A	1	1.56
	m.13542G>A	2	3.33	m.13470G>A	1	1.56
	m.13966G>A	2	3.33	m.13542G>A	2	3.13
	m.14007G>A	2	3.33	m.13780G>A	2	3.13
	m.12615C>T	1	1.66	m.13803G>A	2	3.13
	m.12705C>T	13	21.66	m.13927G>A	1	1.56
	m.13188C>T	12	20	m.13966G>A	5	7.813
	m.13188C>T	2	3.33	m.13980G>A	1	1.56
	m.13506C>T	3	5	m.13986A>G	1	1.56
	m.13650C>T	4	6.66	m.14013A>G	1	1.56
	m.13695C>T	1	1.66	m.14028A>G	1	1.56
	m.14100C>T	1	1.66	m.14053A>G	1	1.56
	m.14110C>T	2	3.33	m.12633C>A	2	3.13
	m.14155C>T	1	1.66	m.13880C>A	1	1.56
	m.14167C>T	2	3.33	m.12633C>T	1	1.56
	m.13111T>C	1	1.66	m.12705C>T	14	21.88
	m.14178T>C	1	1.66	m.12741C>T	1	1.56
	m.13392T>C	1	1.66	m.13188C>T	7	10.94
	m.14094T>C	3	5	m.13506C>T	3	4.69
				m.13547C>T	1	1.56
				m.13650C>T	5	7.813
				m.14109C>T	1	1.56
				m.14167C>T	4	6.25
				m.12705C>G	1	1.56
				m.12738T>G	1	1.56
				m.12950A>C	2	3.13
				m.12696T>C	1	1.56
				m.12793T>C	1	1.56
				m.12903T>C	1	1.56
				m.12945T>C	1	1.56
				m.13111T>C	1	1.56
				m.13215T>C	1	1.56
				m.13392T>C	2	3.13
				m.13743T>C	2	3.13
				m.13752T>C	3	4.69
				m.13789T>C	1	1.56
				m.13879T>C	2	3.13
				m.13965T>C	1	1.56
				m.14094T>C	1	1.56
				m.14110T>C	2	3.13
<i>ND6</i>	m.14139A>G	2	3.33	m.14139A>G	2	3.13
	m.14233A>G	2	3.33	m.14203A>G	1	1.56
	m.14308T>C	2	3.33	m.14233A>G	5	7.813
	m.14212T>C	1	1.66	m.14323G>A	1	1.56
	m.14305G>A	1	1.66	m.14239C>T	1	1.56
				m.14153T>C	1	1.56
				m.14178T>C	3	4.69
				m.14212T>C	2	3.13

Table IV. Variations in mtDNA-encoded complex I genes identified only in patients with multiple sclerosis.

Gene	Nucleotide change	No. of nucleotide changes	Amino acid change	
<i>ND1</i>	m.3513C>T	1	-	
	m.3533C>T	1	-	
	m.3594C>T	5	-	
	m.4059C>T	1	-	
	m.4312C>T	3	-	
	m.3944A>G	1	-	
	m.3720A>G	3	-	
	m.3948A>G	1	-	
	m.3865A>G	3	-	
	m.3866T>C	1	-	
	m.3944T>C	2	-	
	<i>ND3</i>	m.10343C>T	1	-
		m.10355G>A	1	-
<i>NDL4</i>	m.10499A>G	4	-	
	m.10586G>C	1	-	
	m.10664C>T	2	-	
<i>ND4</i>	m.10819A>G	2	-	
	m.10895A>G	2	-	
	m.11002A>G	1	-	
	m.11172A>G	4	-	
	m.11337A>G	2	-	
	m.11380A>G	1	-	
	m.11143C>T	2	-	
	m.11150G>A	1	A131T	
	m.11377G>A	4	-	
	m.11440G>A	4	-	
	m.11518G>T	1	-	
	m.11519A>C	1	T254P	
	m.11523A>C	1	K255T	
m.11527C>T	1	H256L		
<i>ND5</i>	m.12570A>G	4	-	
	m.12615A>G	1	-	
	m.12753A>G	1	-	
	m.13104A>G	1	-	
	m.14007A>G	2	-	
	m.14070A>G	1	-	
	m.12843T>C	1	-	
	m.12879T>C	1	-	
	m.13020T>C	3	-	
	m.13174T>C	2	-	
	m.13734T>C	3	-	
	m.13116C>T	2	-	
	m.13695C>T	1	-	
	m.13317G>A	1	-	
	m.13590G>A	2	-	
	m.13813G>A	4	-	
	m.14100C>T	1	-	
<i>ND6</i>	m.14155C>T	1	-	
	m.14305G>A	1	-	
	m.14308T>C	2	-	
	m.13116C>T	2	-	
	m.13695C>T	1	-	
	m.13317G>A	1	-	
	m.13590G>A	2	-	
	m.13813G>A	4	-	
	m.14100C>T	1	-	
	m.14155C>T	2	-	
m.14305G>A	1	-		
m.14308T>C	1	-		

variants in the *ND1*, *ND3*, *NDL4*, *ND4*, *ND5* and *ND6* genes were commonly observed at high frequencies in the patients with MS and the healthy individuals. These included variants in the *ND4* gene, namely m.11719G>A and m.11251A>G, and variants in the *ND5* gene, namely m.12372G>A, m.13708G>A, m.12612G>A and m.12705C>T. Moreover, numerous variants were identified which were observed only in patients with MS, whereas none of the healthy subjects carried any of these variants. These were found to be located in different positions of the *ND1*, *ND3*, *NDL4*, *ND4*, *ND5* and *ND6* genes. Bioinformatics analysis revealed that the majority of these variants were synonymous and did not cause any amino acid changes, and were thus considered as simple polymorphisms. Importantly, the significant finding in the group of MS patients was the identification of 4 mutations in the *ND4* gene that were revealed to encode missense mutations. Of these, m.11150G>A exhibited an alanine to threonine alteration, m.11519A>C a threonine to proline alteration, m.11523A>C a lysine to threonine alteration and m.11527C>T a histidine to leucine alteration. It was also noted that 1 patient (patient no. 48, Table V) carried the m.11150G>A mutation, whereas another patient (patient no. 44, Table V) carried the m.11519A>C, m.11523A>C and m.11527C>T mutations.

MS has traditionally been considered as an immune-mediated neurological inflammatory demyelinating disease of the central nervous system with axonal degeneration (1,2). Recent studies have suggested that mitochondrial dysfunction occurs early in MS and plays an important role in disease development and progression (15-17). Targeting mitochondrial pathways along with neuroprotection and immunomodulation are a potential therapeutic target in MS (16-18). The mitochondria are the most efficient producers of cellular energy in the form of adenosine ATP and are also the major source of ROS. The mitochondrial respiratory chain is located in the inner mitochondrial membrane and consists of 5 multimeric protein complexes I-V (19). While complex II subunits are entirely encoded by nuclear DNA (nDNA), the subunits of complexes I, III, IV and V are encoded by both nDNA and mtDNA. Complex I is the largest enzyme complex of the mitochondrial respiratory chain, which is responsible for electron transport and the generation of protons across the mitochondrial inner membrane to drive energy production (20). It is a multi-subunit complex consisting of 44 subunits, of which 7 subunits, including *ND1*, *ND2*, *ND3*, *NDL4*, *ND4*, *ND5* and *ND6* are encoded by the mitochondrial genome (20).

In this study, numerous synonymous variants were found in different genes of mtDNA-encoded complex I in the patients with MS and the healthy individuals, as well in patients with MS only. Synonymous mutations, which do not result in changes in amino acid sequences and are considered biologically silent, have been shown to directly affect gene expression and function through diverse mechanisms, and may thus be implicated in human diseases (34-36). The presence of rare codons may lead to a premature arrest of the translation process, hence resulting in a truncated form of a protein. Although in the present study, we did not analyze the function of the identified polymorphisms, studies in our laboratory are ongoing to investigate the effects of these variant on gene expression, where silent mutations can lead to the generation of rare codons.

Table V. Missense mutations in ND4 gene identified in patients with multiple sclerosis.

Gene	Patient no.	PDB File	Chain ID	Nucleotide change	Amino acid change	Predicted $\Delta\Delta G$	Outcome
ND4	48	ND4.pdb	r	m.11150G>A	A131T	-2.05	Reduced stability
ND4	44	ND4.pdb	r	m.11519A>C	T254P	-1.54	Reduced stability
ND4	44	ND4.pdb	r	m.11523A>C	K255T	-0.95	Reduced stability
ND4	44	ND4.pdb	r	m.11527C>T	H256L	0.99	Neutral

Table VI. Characteristics of patients with RRMS with missense mutations in the ND4 gene.

Characteristics	Patient no. 44	Patient no. 48
Sex	Female	Female
Age (years)	31	25
Disease duration (years since diagnosis)	6	4
Number of relapses per year	1	1
Main neurological dysfunction	Blurry vision, pain on ocular movement, sensory ataxia, weakness, numbness, imbalance	Imbalance, incoordination of movement, weakness on lower limbs, numbness, imbalance
EDSS	3.7	2.4
MRI and LP findings	Mainly paraventricular and cervical hyperintensity representing demyelination lesions	Mainly paraventricular and cervical hyperintensity representing demyelination lesions
Previous medication	Rebif	Avonex
Current medication	Gilenya	Avonex

RRMS, relapse-remitting multiple sclerosis; EDSS, expanded disability status scale; MRI, magnetic resonance imaging; LP, lumbar puncture.

A deficiency in complex I is the most frequently enzyme deficit in mitochondrial diseases (37) and degenerative diseases (38), as well as in other pathological conditions (39), and contributes to neurodegeneration in MS (15,40). Moreover, ROS generated by mitochondrial complex I are considered the main source of cellular oxidative stress (41), and impaired complex I activity mediated by mtDNA oxidative damage has been shown in chronic active plaques in MS (42) and is also implicated in axonal degeneration (15).

It has been demonstrated that ND genes of complex I encoded by mtDNA are hotspots for pathological mutations (43). These mutations in complex I cause a deficiency in NADH ubiquinone oxidoreductase enzyme activity and can lead to mitochondrial dysfunction with increased ROS production (37). In particular, mutations in the ND4, ND5 and ND6 genes have been shown to affect complex I assembly and activity, leading to complex I dysfunction (44-47). Previous studies have confirmed a possible implication of several variations in mtDNA-encoded complex I genes in the pathogenicity of MS in different populations. For example, specific variants in the ND2 gene of complex I have been strongly linked to MS in Caucasians (28). Several other variants in different genes of complex I have been shown to be risk factors in the pathogenicity of MS in a Filipino population (29). The study by Yu *et al* (48) demonstrated an association between the m.13708G>A variant in the ND5 gene and an increased risk

of MS in European cohorts. However, Kellar-Wood *et al* (49) demonstrated that this base change at position 13708 of the mtDNA-encoded ND5 gene does not contribute to genetically determined susceptibility in typical MS patients. Moreover, in LHON, a rare maternally inherited mitochondrial disease with clinical features associated with an MS-like illness (26,27), the m.13708G>A variant in the ND5 gene has been reported as a secondary mutation when occurring in patients with LHON (50-52) and does not functionally impair mitochondrial oxidative metabolism *in vivo* or determine the deficit of energy metabolism in LHON (52). In this study, the m.13708G>A variant in the ND5 gene was observed at high frequencies in both patients with MS and healthy individuals. These inconsistencies between studies suggest that the contribution of specific mtDNA variants to disease risk and susceptibility varies among different ancestral and ethnic groups.

In the current study, 4 missense mutations, including m.11150G>A, m.11519A>C, m.11523A>C and m.11527C>T in the ND4 gene of complex I were found in 2 Saudi patients with MS. As indicated by functional analysis, the identified mutations, m.11150G>A, m.11519A>C and m.11523A, in this study were predicted to be deleterious and to directly cause ND4 protein instability, which may render the protein non-functional. As mentioned above, 3 mutations (m.11519A>C, m.11523A>C and m.11527A>C) were observed in 1 patient (patient no. 44, Table V) and 2 of them (m.11519A>C and m.11523A>C)

caused protein instability, whereas the m.11150G>A mutation was found in another patient (patient no. 48, Table V). Although the m.11527C>T mutation in patient no. 44 had no effect on protein function, it may cause a cumulative destabilizing effect, and may thus disrupt ND4 protein function. Indeed, when these 3 mutations in patient 44 were analyzed for their cumulative destabilizing effect, they were found to affect ND4 protein stability. ND4 subunit is part of a large complex, and these mutations, if not affecting ND4 stability directly, may perturb the protein-protein interaction responsible for maintaining the complex and may consequently affect complex I function. The missense mutations in the *ND4* gene observed in Saudi patients with MS in the present study have not been reported in previous studies, at least to the best of our knowledge, and may therefore be considered novel. Since all previous mtDNA studies have revealed a number of variations in MS patients of different populations, such as Caucasians and Filipinos (28,29,48), the identified novel mutations in Saudi patients with MS in the present study could be ethnic-related and may be important to personalized treatment. This study also confirmed the implication of mtDNA mutations in the pathogenicity of MS and the newly identified mutations may serve as a reference for future studies on the mitochondrial genome in MS.

The polyploid nature of the mitochondrial genome with the presence of up to several thousand copies per cell gives rise to an important feature of mitochondrial genetics, homoplasmy and heteroplasmy. Homoplasmy is when all copies of mtDNA are identical, which may be normal or mutated. Heteroplasmy is when there is a mixture of normal and mutated mtDNA (43). The level of heteroplasmic mutations is important for both the clinical expression of the disease and for biochemical defects (53). In most mtDNA disorders, when the percentage of mutant mtDNA exceeds a certain threshold level, mitochondrial dysfunction becomes clinically apparent (54). Due to organ-specific energetic requirements, the proportion of mutant mtDNA in any cell or tissue may be extremely variable, giving rise to variable disease severity (54). It is generally accepted that high levels of heteroplasmy are associated with severe clinical presentations (55). On the other hand, lower levels of heteroplasmy have been commonly detected in maternally inherited diseases (56,57). Moreover, low-level heteroplasmic sequence changes are important features of the pathology of several diseases, including neurodegenerative diseases, which can lead to mtDNA damage and consequently may induce alterations of OXPHOS enzymes (58).

For a DNA alteration to be classified as a deleterious mutation, it should not be present in healthy individuals, and must occur in a structurally and functionally important region (53). While in our results no heteroplasmic mutations were indicated, the identified mutations in the *ND4* gene of complex I, which is a key element in cellular energy production, were observed in only patients with MS and were not found in healthy individuals, suggesting that they are pathogenic mutations and may play an important role in the pathogenicity MS. Nevertheless, further studies are required to predict the energetic effects of amino acid changes caused by these mutations.

A limitation of the current study was the relatively low number of subjects, which affected the statistical differences in age and sex distribution between the patients and controls.

Theoretically, the age and sex distribution between cases and controls in a high number of participants should be very similar (Gaussian distribution). Moreover, further studies are warranted with larger cohorts to analyze the possible association between these variants and the risk of and/or susceptibility to MS.

In conclusion, in this study, we identified a number of variants as synonymous mutations in both patients with MS and healthy individuals in the mtDNA-encoded *ND1*, *ND3*, *NDL4*, *ND4*, *ND5* and *ND6* genes of complex I. Some of these variants in the *ND4* and *ND5* genes were commonly observed at high frequencies in the investigated groups. We also identified numerous variants in patients with MS only, which were absent in healthy individuals. While the majority of these variants in patients with MS were synonymous, 4 variants in the *ND4* gene were missense mutations and could lead to complex I dysfunction. This study, to the best of our knowledge, is the first to report novel mutations in mtDNA-encoded ND4 gene of complex I in Saudi patients with MS, which may prove to be of importance in personalized treatment.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MAA, GAK and MB conceived and designed the study and edited the manuscript; NBK, SAM, ST and AD collected the data; all authors performed data analysis, managed the data and wrote the manuscript. The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work.

Ethics approval and consent to participate

The study received ethical approval from both the Scientific and Ethics Committee in King Saud University, College of Medicine, Kingdom of Saudi Arabia and from the Medical Research and Ethics Committee in the College of Medicine and Medical Sciences, Arabian Gulf University, Kingdom of Bahrain. The participants were given a complete description of

the study. Informed consent was obtained from all individual participants included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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