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Assessment of Stx-1A gene polymorphism (rs1569061) in relation to the development of multiple sclerosis in Egyptian patients

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Abstract

Multiple sclerosis (MS) is a multifactorial polygenic disease; results from autoimmune and neurodegenerative processes which lead to multifocal lesions of the central nervous system. Axonal degeneration was found to be prominent in the inflammation period of MS and contribute to the progression of disability. Soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) complex plays a vital role in the release of neurotransmitter by synaptic vesicle fusion. Stx-1A protein (Stx-1A), a major component of the SNARE complex, is widely expressed in brain tissue. This study intended to evaluate the prevalence of the Stx-1A gene polymorphism (rs1569061) in the Egyptian population with MS and to investigate its association with various clinical factors. This study included 65 adult Egyptian MS patients and 35 age- and sex-matched normal control subjects. Diagnosis of MS was made by an experienced neurologist according to revised McDonald criteria. All Patients underwent full history taking, included Age of onset of MS, disease duration, disease course and degree of disability according to the Expanded Disability Status Scale (EDSS) and family history of neurological diseases. Stx-1A gene polymorphism (rs1569061) genotyping was performed by TaqMan assay based quantitative real time (qPCR) and verified by sanger sequencer. Genotype and allele frequencies of (rs1569061) did not differ significantly between case and control groups. No difference was detected when comparing the genotype frequency and the allele frequency to different disease parameters. Discrepancy of the minor allele frequency (MAF) of Stx-1A gene (rs1569061) between different populations was noted. In conclusion, our study in Stx-1A gene polymorphism (rs1569061) and MS showed that no difference between the patient and control as regards gene frequency and allele frequency. Predicting no association between the studied polymorphism and MS in the Egyptian population. However, discrepancy between different population was noted as regards the MAF for Stx-1A gene (rs1569061).

Keywords: Multiple Sclerosis, Stx-1A, Polymorphisms, rs1569061, qPCR, Sanger sequencing

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Introduction

Multiple sclerosis (MS) is a debilitating autoimmune disease of the central nervous system that mostly occurs in young adults and more prevalent in females. Depending on different clinical courses, the inflammatory phenotypes of MS are expressed as relapsing remitting (RRMS), secondary progressive (SPMS), primary progressive (PPMS), and progressive relapsing (PRMS*)*.[1]

Multiple Sclerosis is a growing global health problem, it affected nearly 3 million people causing significant public health and economic impact.[2] Young women are most commonly affected with a female to male ratio of at least 2:1.[3] Its prevalence ranges from 1 to 100 per 100,000 between countries.[4] Limited data is available as regards the prevalence of MS in Egypt. In 2013, a single study in a small city located south of Egypt reported a low prevalence of 13.7/100,000, possibly reflecting the lower prevalence rates seen in African countries.[5] The cause of MS is still elusive but both genetic and environmental factors play an important role in the pathogenesis. Sun exposure and resulting vitamin D levels, smoking, obesity, and Epstein-Barr virus (EBV) infection are the most corroborated environmental risk factors out of several suggested.[6]

The EDSS is a scoring system that intends to capture disability of MS patients, an overall score can be given on an ordinal scale ranging from 0 (normal neurological examination) to 10 (death due to MS). EDSS score up to 4 is essentially a measure of impairment (fully ambulatory patients), while scores higher than 4.0 basically address disability.[7] Recent studies reported that axonal degeneration may be prominent in the beginning of the inflammation period of MS, as well as being one of the major determinants of permanent neurological impairment.[8,9]

Synaptopathy, which is defined as the alteration of the synaptic structure and function, has been associated with various neurological diseases, including epilepsy, autism, Alzheimer's disease, and recently MS. Inflammatory-dependent synaptopathy is of particular interest because it is potentially reversible and may represent a novel therapeutic target for MS.[10] At the presynaptic terminal, the membrane fusion process is primarily mediated by the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex.[11]

The SNARE complex is mainly composed of three proteins: VAMP-2, syntaxin-1, and SNAP-25. SNAP-25 and syntaxin-1 are categorized as t-SNAREs due to their location in the presynaptic membrane, whereas VAMP-2 is a v-SNARE anchored on the vesicle membrane.[11] To allow exocytosis the amino terminal of SNAP25 binds to Stx-1A and the carboxy-terminal binds to VAMP2, forming the four-helical bundle that brings secretory granules in close contact with the plasma membrane, thus enabling fusion to occur.[12] The SNARE complex mediate exocytosis by forming a bundle that brings the two membranes together and forces their fusion.[13] Stx-1A protein, in particular, is widely expressed in the brain, in the endocrine system and in the heart.[14]

Therefore, MS is an increasingly prevalent disease and usually presents at a highly productive stage of life when people are planning families and building careers and as such MS can have a significant impact on affected individuals, their families and society. In our study we aimed to evaluate the association of the Stx-1A gene polymorphism (rs1569061) in the Egyptian population with MS.

Patients and Methods

Study participants

This study was a case-control study, conducted at the Main Laboratory, Clinical Pathology Department, Ain Shams University Hospitals. Subjects enrolled in the study were divided into two groups. Group I: Patient Group, involved 65 MS patients recruited from MS Unit, Department of Neurology, Faculty of Medicine, Ain Shams University. The diagnosis of MS was made by an experienced neurologist, in accordance to the revised McDonald criteria.[4] It was further divided into three subgroups: (Subgroup I, included 45 patients with RRMS, Subgroup II, included 12 patients with PPMS

and Subgroup III, included 8 patients with SPMS). All Patients underwent full history taking, included age of onset of MS, disease duration, disease course and degree of disability according to the Expanded Disability Status Scale (EDSS) [7] and family history of neurological diseases). Group II: Control Group, included 35 age- and sex- matched normal subjects. The exclusion criteria included subjects with any of the following conditions were excluded from the study; clinically isolated syndrome, neuromyelitis Optica and other demyelinating diseases.

Sampling

A venous blood sample (5 mL) was withdrawn from each subject in the study under complete aseptic conditions. Collected venous blood samples were put on tri-potassium ethylene diamine tetra acetate "k3 EDTA" vacutainers and the tube was inverted several times and inspected to exclude the possibility of clots. The samples were stored at -20˚C as whole blood till the time of analysis. Repeated freezing/thawing of samples was avoided until DNA extraction and detection of Stx-1A gene Polymorphism (rs1569061) (C/T).

Genotyping

Genomic DNA was extracted from peripheral blood samples using DNA purification mini kits (ThermoFisher® Fisher Scientific, 168 Third Avenue Waltham, MA USA 02451), according to the manufacturer's instructions. The Stx-1A polymorphism (rs1569061) was analyzed by real time qPCR on DTlite real time PCR System (DNA-Technology, Research & Production", LLC) using the readymade genotyping assay kit supplied by ThermoFisher® containing sequence-specific Forward primer 5'-CACCTCTCCCCTAACCTGAA-3' and Reverse primer 5'-TCACCTTCCCCTGCTT GGAT-3'; and two fluorescents (HEX/FAM) labeled TaqMan probes CTGGCGGCCCTGCCT GGGTCTGCTC[C/T]TCGCTGTGCACACTGCATCAC GCCC for distinguishing between the two alleles.

The presence or absence of the Stx-1A gene (rs1569061) and the allelic discrimination was established according to the type of the emitted fluorescence of either of the reporter dyes or both at the same time as in Table 1.

To obtain exact nucleotide sequencing, random samples with CT genotypes were validated by sanger sequencing using an automated ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). The real time PCR end product of the selected samples were subjected to amplification by specific primers mediated conventional PCR method supplied by Thermo Scientific.

These PCR products were purified by QIA quick Gel Extraction Kits (QIAGEN, Strasse 1, 40724 Hilden, Germany), according to the manufacturer's instructions. The purified PCR products were then subjected to cycle sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Thermo-scientific; Singapore), according to the manufacturer's instructions.

The product from cycle sequencing was purified using Centri-step Spin Columns Kits (Invitrogen™ Centri-Sep™ Spin Columns), according to the manufacturer's instructions. Then the purified sequence product was subjected to sequencing by capillary electrophoresis using an automated ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). Data analysis was done using https://blast.ncbi.nlm.nih.gov/Blast.cgi website.

The sequencer analysis resulted in a case with a genotype CT Figure (1A, 1B and 1C) and another case with a genotype CC as shown in Figure (2).

Statistical analysis

The IBM SPSS statistics program (V. 26.0, IBM Corp., USA, 2019) was used for data analysis. Data were expressed as Mean±SD for quantitative parametric measures in addition to median and percentiles for quantitative non-parametric measures and both number and percentage for categorized data. Student t test and Wilcoxon Rank Sum test were used in the comparison between two independent groups for parametric data and non-parametric data, respectively. Kruskall Wallis test was used in the comparison between more than 2 patient groups for non-parametric data. Chi-square test was used to study the association between each

2 variables or comparison between 2 independent groups as regards the categorized data. The probability of error at 0.05 was considered significant.

Table 1. Relation between fluorescence signals and sequences in a sample.

Fluorescence Increase	Indication
HEX dye fluorescence only (green)	Homozygosity for allele 1(C)
6FAM dye fluorescence only (blue)	Homozygosity for allele 2(T)
Fluorescence signals for both dyes	Heterozygosity for allele 1 (C) +allele 2 (T)

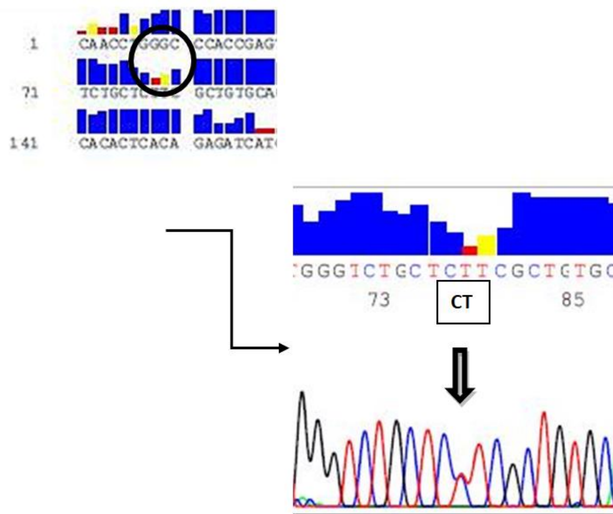

Figure 1A. Sanger Sequencer result of heterozygous (CT) genotype of Stx-1A gene polymorphism (rs1569061).

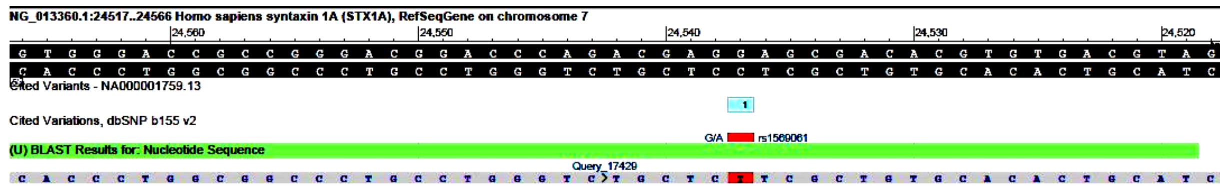

Figure 1B. Data Analysis by https://blast.ncbi.nlm.nih.gov/Blast.cgi website for nucleotide sequence.

```
>Homo sapiens syntaxin 1A (STX1A), RefSeqGene on chromosome 7
Sequence ID: NG_013360.1 Length: 27483
Range 1: 24460 to 24671

Score:387 bits(209), Expect:4e-106,
Identities:211/212(99%), Gaps:0/212(0%), Strand: Plus/Minus

Query  1      CTGGAGAGGACAGGGCAGGTCAGCTGGAGGCGAGGTTAGGGTCCCCAGGGAAGAGCAGAA  60
Sbjct  24671  ............................................................  24612

Query  61     CCTGGGCCCACCGAGTTACTGAAGGCAAGGAAGGGTGGCCTGTGTCACCCTGGCGGCCCT  120
Sbjct  24611  ............................................................  24552

Query  121    GCCTGGGTCTGCTCTTCGCTGTGCACACTGCATCACGCCCCGCTGGCTGCCTCCCTCTGC  180
Sbjct  24551  .............C..............................................  24492

Query  181    CTCTTCCCGTACAGACGCACACTCACAGAGAT  212
Sbjct  24491  ................................  24460
```

Figure 1C. Alignment between our query nucleotide sequence and Stx-1A nucleotide sequence showing identity of 99%.

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>Homo sapiens syntaxin 1A (STX1A), RefSeqGene on chromosome 7
Sequence ID: NG_013360.1 Length: 27483
Range 1: 24465 to 24675

Score:390 bits(211), Expect:2e-104,
Identities:211/211(100%),  Gaps:0/211(0%), Strand: Plus/Minus

Query  1      ACAGCTGGAGAGGACAGGGCAGGTCAGCTGGAGGCGAGGTTAGGGTCCCCAGGGAAGAGC  60
Sbjct  24675  ............................................................  24616

Query  61     AGAACCTGGGCCCACCGAGTTACTGAAGGCAAGGAAGGGTGGCCTGTGTCACCCTGGCGG  120
Sbjct  24615  ............................................................  24556

Query  121    CCCTGCCTGGGTCTGCTCCTCGCTGTGCACACTGCATCACGCCCCGCTGGCTGCCTCCCT  180
Sbjct  24555  ............................................................  24496

Query  181    CTGCCTCTTCCCGTACAGACGCACACTCACA  211
Sbjct  24495  ...............................  24465
```

Figure 2. Alignment between the tested sample nucleotide sequence and Stx-1A nucleotide sequence showing identity of 100%.

Results

The study included 65 MS patients, with mean age of 34.08 ± 8.896 years and 79% of them were females. In addition to 35 normal subjects were included as controls, with mean age of 31.11 ± 6.425 years and 80% of them females.

The Stx-1A gene polymorphisms (rs1569061) was not different between patient and control groups, regarding genotype frequencies (*p*=0.473) and allele frequencies (*p*=0.308) Table 2. Furthermore, there was no difference in Stx-1A Gene Polymorphism (rs1569061) genotype frequency (*p*=0.646) and the allele frequency (*p*=0.597) between the three subgroups depending on the disease course to RRMS, PPMS and SPMS. Table 3.

Table 2. Descriptive and comparative statistics of Stx-1A Gene Polymorphism (rs1569061) genotype frequency (A) and Allele frequency (B) between the patient and control groups.

		Control N =35 (%)	Patient N=65 (%)	Total	*p* value
Genotype frequency (A)					
Genotype	CC	26 (74.3%)	43 (66.2%)	69	NS
	CT	9 (25.7%)	20 (30.8%)	29	
	TT	0 (0%)	2 (3%)	2	
Allele frequency (B)					*p* value
Allele	C	61 (87.1%)	106 (81.5%)	167	NS
	T	9 (12.9%)	24 (18.5%)	33	

P > 0.05 is not significant (NS).

Table 3. Descriptive and comparative statistics between RRMS, PPMS and SPMS groups as regards Stx-1A Gene Polymorphism (rs1569061) genotype frequency (A) and allele frequency (B).

		RRMS	PPMS	SPMS	Total	*p* value
Genotype frequency (A)						
Genotype	CC N=43 (%)	30 69.8%)	9 (20.9%)	4 (9.3%)	43	NS
	CT N=20 (%)	13 (65%)	3 (15%)	4 (20%)	20	
	TT N=2 (%)	2 (100%)	0 (0%)	0 (0%)	2	
Allele frequency (B)						*p* value
Allele	C N=106 (%)	73 68.9%)	21 (19.8%)	12 (11.3%)	106	NS
	T N=24 (%)	17 (70.8%)	3 (12.5%)	4 (16.7%)	24	

P > 0.05 is not significant (NS). χ2= Chi-Square test. RRMS= Relapsing Remitting Multiple Sclerosis
PPMS= Primary Progressive Multiple Sclerosis. SPMS= Secondary Progressive Multiple Sclerosis
CC, CT, TT= Stx-1A genotypes. C= C Allele, T= T Allele.

In addition, there was no difference in the genotype frequency and the disease most important clinical characteristic, included age of onset of the disease, duration of the disease, number of relapses, EDSS and family history (Tables 4 and 5).

Table 4. Clinical Data of the disease in the three patient subgroups: RRMS, PPMS and SPMS.

Parameter	Subgroup I: RRMS (n=45)	Subgroup II: PPMS (n=12)	Subgroup III: SPMS (n=8)
Age of Onset			
<30 years old (%)	25 (55.6%)	4 (33.3%)	4 (50%)
≥30 years old (%)	20 (44.4 %)	8 (66.7%)	4 (50%)
Duration			
2 months-5 Years (%)	23 (84.5%)	45 (33.3%)	1 (12.5%)
6-20 Years (%)	22 (15.5%)	8 (66.7%)	7 (87.5%)
EDSS			
1-4 (%)	38 (55.6%)	1 (33.3%)	1 (50%)
4.5-10 (%)	7 (44.4 %)	11 (66.7%)	7 (50%)
Family History			
Present (%)	4 (8.9%)	1 (8.3%)	4 (50%)
Absent (%)	41 (91.1%)	11 (91.7%)	4 (50%)

Table 5. Comparison between the genotype frequencies of Stx-1A gene polymorphism (rs156906) and disease studied parameters.

Studied parameters	Genotype			*p* value
	CC Number Median (IQR)	CT Number Median (IQR)	TT Number Median (IQR)	
Age Of Onset	43 29 (22-35)	20 25.5 (20.5-38)	2 18 (15-22)	NS
Duration of the Disease	43 5 (2-8)	20 6.5 (9-4.125)	2 4 (4-4)	NS
EDSS	43 4 (5-2.5)	20 4 (3-5.375)	2 2.5 (1.5-3.4)	NS
Number of Relapses	38 2 (1.75-4)	19 4 (2-6)	2 2 (2-2)	NS
Family History				NS
Present	6	2	1	
Absent	37	18	1	

P > 0.05 is not significant (NS). IQR= Interquartile range. H= Kruskall Wallis Test. CC, CT, TT= Stx-1A genotypes.

Table 6 shows a comparison the three genotype frequencies and allele frequencies of the Stx-1A gene polymorphism (rs156906) among the EDSS subgroups (disease impairment, EDSS=<4 and disease disability, EDSS>4).

Table 6. Comparison between the three genotype frequencies (A) and allele frequencies (B) of Stx-1A gene polymorphism (rs156906) among the EDSS subgroups.

		EDSS 1-4 (N)	EDSS 4.5-10 (N)	Total	*p* value
Genotype frequencies (A)					
Genotype	CC	26	17	43	NS
	CT	12	8	20	
	TT	2	0	2	
Allele frequencies (B)		EDSS 1-4 (N)	EDSS 4.5-10 (N)	Total	*p* value
Allele	C	64	42	106	NS
	T	16	8	24	

P > 0.05 is not significant (NS).

Discussion

MS is a chronic inflammatory disease of the CNS characterized by demyelination and primary or secondary axonal degeneration. The pathogenetic mechanisms included in the disease development are highly diverse and are closely related to the different phenotypes of MS.[15]

MS has variable clinical course, but most patients will follow a RRMS. A small percent of patients will follow a PPMS.[3] Data from natural history cohorts propose that about 85% of patients with RRMS will ultimately advance into SPMS. With the improvement we have with the treatment protocols, recent cohorts suggested lower rates of conversion of RRMS to SPMS.[16]

While the hallmark of MS is the presence of acute inflammatory demyelination, axonal damage is a consistent feature of MS lesions.[17] Axonal degeneration is assumed as one of the major determinants of permanent neurological impairment and disability.[8]

The SNARE complex mediates the membrane fusion process at the presynaptic level. One of these proteins is the Stx-1A.[11] Very few studies assessed the association between Stx-1A gene polymorphism (rs1569061) and MS. One of these is the study by Yalın et al., 2019, carried out in Istanbul, Turkey. This study discussed Stx-1A as one of a panel of multiple polymorphisms in SNARE complex.[8] The association of Stx-1A gene polymorphism (rs1569061) was assessed before with idiopathic generalized epilepsy.[18] and in alcohol dependence and its relationship with impulsivity and temperament.[19]

In our study we found no difference in the genotype frequency between MS patient and control groups ($p<0.05$). On the contrary, Yalin et al., 2019 observed that Stx-1A gene polymorphism (rs1569061) TC and CC genotypes were significantly more frequent in the patients group.[8]

There is discrepancy between our results and those of Yalin et al., 2019 as regards the genotype frequency in the control subjects. In our study, TT genotype was not detected in the control group (0%) and just 3% of patient group. However, in the study by Yalin et al., 2019, TT genotype represented 40% of the control group and 22.8% of the patient group.[8]

In addition, a study by Şenormancı et al., 2021 reported high frequencies of the genotypes CT, CC and TT in the control group as 52.69%, 24% and 23.1%, respectively.[19]

Focusing on the T and C allele frequency, our study found no difference between the control group and the patient group (p=0.308). However, Yalin et al., 2019, revealed statistically significant difference as regards the allele frequency between the patient and the control group.[8]

The results of our study showed that in the control group the T allele was the minor allele with frequency 9/70 (12.9%) and C allele was the major allele with frequency 61/70 (87.1%). This is in accordance to the National Center for Biotechnology Information (NCBI), indicating

that T allele is the minor allele for Stx-1A gene polymorphism (rs1569061) referring to TOPMed (The Trans-Omics for Precision Medicine) with MAF (Minor Allele Frequency) T=28978/264690 (10.9%), gnomAD (Genome Aggregation Database)[19] with MAF T=0.100856 (14134/140140) and ALFA (The Allele Frequency Aggregator) with MAF T=0.087388 (9429/107898).[21]

On the other hand, Yalin et al., 2019 mentioned that T allele was the major allele with frequency in the control group of 229/384 (59.6%) and C allele 155/384 (40.4%).[8]

Additionally, studies by Şenormancı et al., 2021 and Yilmaz et al., 2014 revealed that the T allele frequency was comparable to the C allele frequency in the control groups of both studies, resulting in T allele frequencies of 105/214 (49.1%) and 86/186 (46.2%), respectively.[19,18]

This allelic discrepancy may be attributed to the different population included in each study. Since those three studies were conducted in Turkey and our study was carried out in Egypt, this could indicate presence of different allele frequencies in the normal population in the two countries.

Numerous studies have discussed the significance of estimating gene allele frequency in epidemiological and genetic association studies. Notably, allele frequencies for SNPs can vary by ethnic group.[22] In fact, several studies have shown differences in allele distribution of SNPs; in candidate genes for immune response and lipid metabolism[23], cardiovascular disease[24] and type 2 diabetes.[25] Additionally, Mattei et al., 2009 revealed discrepancy while comparing allele frequency distribution of 101 SNPs associated with several chronic diseases, between Puerto Ricans and non-Hispanic whites.[22]

Moreover, pharmacogenomic relevant markers of drug response and adverse drug reactions are known to vary in frequency across populations. Ramos et al., 2014 examined MAFs, genetic diversity and population structure of 1156 genetic variants in 212 genes involved in drug absorption, distribution, metabolism, and excretion (ADME) in 19 populations. Data from this study demonstrated that ADME genetic variants showed considerable differences in allele frequency among global populations in general as well as among populations that are often grouped together by continental origin, ancestry, or ‘race’.[26]

Comparing the three disease courses (RRMS, PPMS and SPMS) regarding the genotype frequency and the allele frequency, there was no significant difference. This contrasts with Yalin et al., 2019 who observed significant difference in Stx-1A gene (rs1569061) polymorphism in patients with RRMS with p=0.05.[8]

To the best of our knowledge, our research is the first study to assess the genotype frequency and allele frequency of Stx-1A gene polymorphism (rs1569061) as regards different disease characteristics and disability grade. Comparing genotype frequency and allele frequency as regards the age of onset of the disease, duration of the disease, number of relapses, EDSS and family history resulted in statistically non-significant results.

In our study, data obtained from sequencing random samples with CT genotypes by real time PCR yielded a sample with CC genotype. This discrepancy was also detected by Lemos et al., 2010, in their study to assess the involvement of Stx-1A gene in migraine susceptibility.[26]

Finally, our study findings indicated no differences in the genotype and allele frequencies of (rs1569061) between MS cases and controls. Furthermore, no difference was found when comparing the genotype frequency and the allele frequency to different disease parameters. Unlike studies in another county, we observed no association between the studied polymorphism and MS in the Egyptian population.

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Author Contributions

AAE; designed and approved the whole research protocol. SAB; contributed to the protocol design, revised laboratory work, and approved the final paper version to be published. MMF; supervised sample collection according to inclusion criteria.

MAE; interpreted the data, and critically revised the paper. AMA; monitored the laboratory work, and critically revised the paper. CAH; collected the samples and patient's clinical data, carried out the laboratory work, and statistical analysis and wrote the manuscript. All authors read and approved the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Researcher Ethics Committee at Faculty of Medicine, Ain shams University (approval number FMASU MD 390/2019).

Informed consent

Verbal informed consents were obtained from all participants before enrollment in the study.

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