Original Article

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Background: The role of axonal degeneration on disability in patients with multiple sclerosis is well known and the synapthopathy has recently become an important issue.
Aims: To investigate possible roles of selected synaptic and presynaptic membrane protein genetic polymorphisms (VAMP2, SNAP-25, Synaptotagmin, Syntaxin 1A) in patients with multiple sclerosis.
Study Design: Case-control study
Methods: One hundred and twenty-three patients with multiple sclerosis and 192 healthy controls were included. We analyzed the functional polymorphisms of specific SNARE complex proteins (VAMP2, Synaptotagmin XI, Syntaxin 1A and SNAP-25) by polymerase chain reaction.
Results: The study indicated significant differences for the genotype and allele distribution of 26 bp Ins/Del polymorphisms of VAMP2 between multiple sclerosis and control subjects; Del/Del genotype and Del allele of VAMP2 were more frequent in patients with multiple sclerosis (p=0.011, p=0.004), respectively. Similarly, Ddel polymorphism of SNAP-25 gene C/C genotype (p=0.059), Syntaxin 1A T/C and C/C genotypes (p=0.005), and Synaptogtagmin XI gene C allele (p=0.001) observed more frequently in patients with multiple sclerosis. CC, Syntaxin rs1569061 1A gene for 33-bp promoter region TC haplotypes and Synaptotagmin XI gene has been shown to be associated with increased risk for multiple sclerosis (p = 0.012). Similarly, GC haplotype for rs3746544 of SNAP-25 gene and rs1051312 of SNAP-25 gene were found to be an increased risk for multiple sclerosis (p=0.022).
Conclusion: The genetic polymorphisms of SNARE complex proteins, which have critical roles in synaptic structure and communication, may play a role in the development of multiple sclerosis.
Keywords: multiple sclerosis, genetic, SNARE complex, polymorphism

Multiple sclerosis (MS) is an autoimmune, inflammatory disorder related with neuronal structure, and is one of the prominent causes of young and middle-aged adult disability in the community. Several factors have been proposed for the development of MS including genetic and environmental factors. Large-population studies revealed that high incidence of family history is possibly related with genetic factors [1]. Specific genes associated with MS include the human leukocyte antigen (HLA) system locus on chromosome 6,which serves as a major histocompatibility complex. The most consistent associations with MS have been found with DR15 and DQ6 alleles. Genome-wide association studies have also revealed many other susceptible genes other than HLA loci[2, 3].

Clinical presentation of MS can include various typical syndromes including optic neuritis, pyramidal signs and brainstem syndromes. However, there is consensus about various clinical courses of the disease and different types of MS (relapsing and progressive forms) based on clinical and pathological features.
MS traditionally characterized by chronic inflammatory demyelinating lesions [3], but recent studies have revealed that axonal degeneration may be prominent in the beginning of the inflammation period of disease [5, 6]. Axonal damage could be resulted from inflammation, vulnerability of demyelinated axons, or toxic effects of various complex biochemical reactions (7). There is accumulating evidence that axonal degeneration occurs not only as a consequence of inflammatory demyelination but can also progress independently [5]. Furthermore, axonal degeneration is assumed to be one of the major determinants of permanent neurological impairment [8, 9], and more recent studies have been focused on the neurodegenerative component of the disease, which is thought to cause disability.
Experimental autoimmune encephalomyelitis (EAE) is a useful sample for the development of new therapies of MS [10]. Levels of synapsins and syntaxins are decreased in EAE. Synapsins and syntaxins are vesicle proteins that exhibit principal roles in exocytosis, in presynaptic terminals in clinical symptoms of EAE [11].
There is accumulated evidence suggesting the role of synapthopathy in MS. Synaptopathy term defines alterations of synaptic structure and function that have been associated in various neurological disease including epilepsy, autism, Alzheimer's disease, and recently MS [12]. Inflammatory-dependent synapthopathy is of particular interest because it is potentially reversible and may represent a novel therapeutic target for MS [12].
Synapsins are neuronal phosphorylated proteins and have significant roles for vesicular trafficking in synaptic region. and particularly related with the cytoplasmic vesicle membranes [13]. Syntaxin 1A protein is located presynaptic region and forms SNARE complex with SNAP-25 and VAMP2, and formsSNARE complex, SNARE complex is critical in neurotransmission [14]. SNAP-25 have role in the vesicle docking and fusion via mediating neurotransmitter secretion[15]. SNAP-25 is present in the neurons and highly located in synaptic plasticity locations [16]. Vesicle-associated SNARE complex components induce membrane fusion.
Recent studies have been focused on factors that determine disability. Synaptopathy, neuroplasticity, axonal degeneration and accumulated oxidative stressors are some of the proposed mechanisms. Therefore, we examined synaptic vesicle protein (VAMP2) Syntaxin 1A and SNAP-25 genetic variants those are related to synaptogenesis and neuroplasticity in MS patients. We investigated firstly in the literature associations of Syntaxin 1A (Intron 7, rs1569061), SNAP-25 (MnlI, rs3746544 andDdeI, rs1051312), VAMP2 and synaptotagmin XI gene polymorphisms in the MS population.

Method

All patients voluntarily participated the study, consent obtained from all participants. The Ethical Committee of the Istanbul Education and Research Hospital approved this non-profit study. The study was performed between February 2014 and November 2015 in the neurology outpatient and inpatient clinics at the Istanbul Education and Research Hospital. One hundred and twenty-three MS subjects over 18 years and 192 age- and healthy controls admitted. Patients evaluated and underwent a detailed neurologic examination by an experienced neurology specialist. After the final evaluation, the diagnosis of MS was based to the 2010 McDonald criteria [17]. Ultimately 87 RRMS, 32 SPMS, 4 PPMS and totally 123 MS patients were enrolled in the study. Clinically isolated syndrome, neuromyelitis optica, other demyelinating diseases and doubtful cases were excluded.

Genotype Analysis

Blood samples which are taken from patients and healthy volunteers were collected in tubes containing ethylenediamine tetra acidic acid (EDTA). Samples were storaged at -20 ^{0}C until to DNA isolation. DNA isolation were made with Pure Link Genomic DNA Purification Kit (Thermo) from whole blood. PCR studied with 2 mMdNTPs (Thermo Scientific R0242), 10 pmol of each primer, 1.5 mM $MgCl_2$, 1 x PCR buffer with $(NH_4)_2SO_4$ and 2 U Taq DNA polymerase (Thermo Scientific 0402). Amplification was performed on an automated thermal cycler (TechneFlexigene, Cambridge, UK). We used 100bp DNA ladder (Thermo Scientific) for size of standard for each gel lane. The gel was visualized under UV visualizing system (VilberLourmat).

Statistical analysis

Power analysis was performed considering the type I error rate as 0.05 for Vamp2 variable with 0.17 effect size and the power was calculated 0.775. Analysis of Hardy-Weinberg equilibrium performed for control group and patients. The Hardy-Weinberg equilibrium was verified using the chi-square test and estimating the expected genotypic frequencies based on the development of the square of the binomial. For comparison of baseline characteristics for patients and controls, *t* tests were used for continuous variables and it was given as mean±standard deviations (SDs) and χ^2 tests were used for categorical variables. Allelic and genotypic distributions between groups were compared with likelihood-ratio chi-square test or Fisher's exact test. Linkage disequilibrium was tested and haplotype analysis was used to investigate effects of the "linked" genes. Frequencies smaller than 0.03 were unnoticed. Statical analysis performed by SPSS 11.5 for Windows.

Results

One hundred and twenty-three MS patients aged 18 to 65 years old and 192 healthy controls were included in

this study. The patient group comprised 87 RRMS, 32 SPMS and 4 PPMS diagnosed subjects. The mean age of patients was 44.8 ± 10.0/years and the mean of the age of control group was 42.9 ± 11.3/years; there were no significant difference regarding age and gender (p=0.268). Demographic characteristics presented in Table 2. DNA samples collected from all participants were evaluated. We analyzed alleles and genotypes of 26 bp Ins/Del polymorphisms of the VAMP2 gene, Intron 7 rs1569061 polymorphism of the Syntaxin 1A gene, *Mnl*I (rs3746544) and *Dde*l (rs1051312) polymorphisms of the SNAP-25 gene and 33-bp repeats in promoter regions of the Synaptotagmin XI gene.

We observed significant differences for genotype distribution of 26 bp Del/Del polymorphisms of the VAMP2, Del/Del genotype was observed 3.194 (1.463-6.974) times more common in MS patients (p=0.012). We did not observed difference for the SNAP-25 gene *Mnl*I (rs3746544) polymorphism, however, *Dde*l (rs1051312) polymorphism C/C genotype was found more frequently in MS patients (OR 95% Cl 2.137 (1.075-4.248), p=0.059). Intron 7 rs1569061 Syntaxin 1A gene T/C and C/C genotypes polymorphisms were observed significantly more frequently in the MS group. This difference was also observed in RRMS patients (OR 95% Cl 2.127 (1.225-3.693) and 2.544 (1.366-4.738), respectively, p=0.05). The results of genotypes of VAMP2, SNAP-25, Syntaxin 1A genes variants are presented in Table 3.

We investigate alleles of VAMP2, SNAP-25, Syntaxin 1A, Synaptotagmin XI gene polymorphisms. Del allele of VAMP2 gene was observed 1,688 (1.183-2.407) times more frequently in MS patients (p=0.004), similarly C allele of Synaptotagmin XI gene observed more frequently in MS patients (OR 95% Cl 1.711 (1.238-2.363), p=0.001).Other genes allelic distrubutions were similar for both groups. The results are presented in Table 4.

Lastly haplotype analysis showed significant differences between groups. We determined that TC and CC haplotypes for rs1569061 Syntaxin 1A gene;and the 33-bp promoter region of the Synaptotagmin XI gene could be risk factors for development of MS (OR 95% Cl 1.686 (1.049-2.710), 1.588 (1.041-2.420), p= 0.012, respectively). Similarly,the GC haplotype of the SNAP-25 *Mnl*I gene and the SNAP-25*Ddel* gene found 1.948-fold (OR 95% Cl 1.948 (1.251-3.033), p=0.022) increased risk of MS development. Haplotype analysis results are presented in Table 5.

SPMS and PPMS patient analyses were not performed separately due to the small number of patients.

Discussion

In the present study, we evaluated polymorphisms of SNARE complex proteins (VAMP2, SNAP-25, Syntaxin 1A and synaptotagmin XI) in MS. To our knowledge, this is the first study evaluating associations of the SNARE complex genetic polymorphisms and MS. We found associations between MS and VAMP2 Del/Del, SNAP-25 *Dde*l C/C and Syntaxin1A T/C and C/C genotypes. VAMP2 gene *Dde*l allele and C alleles Synaptotagmin XI were also related with increased risk of MS. Moreover, Syntaxin1A/Synaptotagmin XI genes, TC and CC haplotypes, and SNAP-25 *Mnl*l/SNAP-25 *Dde*l GC haplotype were found to be associated with increased risk of MS.

Recently, it has been proposed that not only myelin but also synapses are involved in early phases of MS[18, 19]. Initial autopsy studies of RRMS, PPMS and SPMS showed decreased levels of synaptic proteins including synaptotagmin and synaptophysin. Synaptophysin is a presynaptic vesicle protein, which has a role in synaptic vesicle release [20, 21].

Evidence of early synaptopathy in MS is documented in experimental and autopsy studies [12, 21, 22]. Earlier studies investigating Synapsin III genetic polymorphisms in MS produced conflicting results [23, 24, 25]. SNARE protens exist all parts of the brain tissue. There is accumulated evidence that SNARE proteins are present broadly in brain. SNARE complex proteins play a crucial role in providing and sustaining healthy synaptic structure/functions. Genetic polymorphisms of associated proteins have been found associated with many central nervous system disorders [26, 27, 28, 29]. Synapses are morphologically dynamic structures and dysfunctions can be repaired and new synapses formed. This dynamic structure of synapses could provide novel treatment strategies [12]. The SNARE related genes may have a role in structural expression, or it may contribute to neurological disorders. As a part of the SNARE complex, synaptotagmins are extensively found in the brain and have important roles in membrane trafficking and synaptic plasticity. Synaptotagmin polymorphisms are well studied in the etiology of epilepsy [29].

Synapsins are composed from a group of specific neuro-proteins. Synapsins are responsible from trafficking of synaptic vesicles from presynaptic terminals, particularly related with cytoplasmic surface[30].They play crucial role synaptogenesis and early axonal development. Although SNAP-I and II are thought associated with neuronal development; SNAP-III is lastly discovered and have unique functions in neurotransmitter regulation in mature neurons [31]. The role of SNAP-III polymorphisms investigated in Schizophrenia, Alzheimer Disease, and MS[31-33]. Liquori et al. reported an inverse relation with SNAP-III promoter gene polymorphism and MS[31]. SNAP-25 is another SNARE complex protein studied in psychiatric and neurological diseases. SNAP-25 is thought to have roles in arranging synaptic homeostasis [34]. Syntaxin 1A, particularly,has roles in the synaptic exocytosis process and neuronal plasticity [35]. Although the roles of presynaptic and synaptic membrane proteins are well studied, their exact roles in healthy brain and neurological disorders are still unclear. This study has certain limitations; it not population-based and may not represent the wider population. The study

group was relatively small to assess SPMS and PPMS genetic associations; and assess correlations with disability scores. We are planning to present a broader series of relations of genetic polymorphisms in future papers.

This study provides novel genetic associations of MS related to synapthopathy. We believe future functional and clinical studies on the gene expressions of SNARE complex proteins are needed. Our findings should be confirmed by further studies in different populations.

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Table 1. The PCR-RFLP evaluation method for VAMP2, Synaptotagmin XI, syntaxin 1A and SNAP-25 genes.

Gene	Polymorphism	Primers	Temperature of Annealing	Restriction Endonuclease	PCR products
VAMP2	26 bp Ins/Del polymorphism	P1 P2	57 ^{0}C	-	Ins allele:116 bp Del allele:90 bp
Synaptotagmin XI (Syt11)	33-bp repeats in promoter region	P3 P4	53 ^{0}C	*Hph*I	**T allele:** 510 bp (three repeats), 477 bp (2 repeats), 444 bp (1 repeat), **C allele:** 464 bp (3 repeats), 431 bp (2 repeats),398 bp (1 repeat)
Syntaxin 1A	Intron 7 (rs1569061)	P5 P6	57 ^{0}C	*Tai*I	T allele:312 bp C allele:186,126bp
SNAP-25	*Mnl*I (rs3746544)	P7 P8	58 ^{0}C	*Mnl*I	T allele:256, 5 bp G allele:210, 46, 5 bp
	*Dde*I (rs1051312)	P9 P10		*Dde*I	T allele:261 bp C allele:228, 33 bp

Note:
P1:F5'ACAAAGTGCGCCTTATACGC3' P2: R5' GGGATTTTCCTTGACGACACTC 3'
P3: F5'-TCTACCTATGCTTCTTACCC-3' P4: R5' TGTCGTAATCAGAGGCTGTTGCT-3'
P5: F5' CAATGCTGCTGCTGAACT C 3 P6: R5'CGCTGACATTTATGTGACC 3'
P7: F5' TTCTCCTCCAAATGCTGTCG 3' P8:R5' CCACCGAGGAGAGAAAATG 3'
P9: R5' CCACCGAGGAGAGAAAATG 3' P10: F5' TTCTCCTCCAAATGCTGTCG 3'

Table 2. Demographic and clinical features of the study population.

	Control (n=192)	MS All (n=123)	p	RRMS (n=87)	SPMS (n=32)	PPMS (n=4)	p
Female, n (%)	62.5%	70.7%	0.133	74.7%	59.4%	75.0%	0.268
Age (Mean±SD)	42.9±11.3	44.8±10.0	0.129	44.9±10.6	44.9±8.5	42.3±9.3	0.878

MS: Multiple sclerosis; RRMS: Relapsing-Remitting Multiple Sclerosis; SPMS: Secondary-Progressive Multiple Sclerosis; PPMS: Primary Progressive Multiple Sclerosis

Table 3. Genotypes of VAMP2, SNAP-25, Syntaxin 1A genes

Genotypes n (%)	Control (n=192)	Patients (n=123)	p value	OR (95%-CI)
VAMP2				
Ins/Ins	115 (59.9%)	60 (48.8%)		1
Ins/Del	65 (33.9%)	43 (35.0%)	**p=0.011**	1.268 (0.772-2.082)
Del/Del	12 (6.3%)	20 (16.3%)		**3.194 (1.463-6.974)**
SNAP-25 Mnll				
T/T	79 (41.1%)	44 (35.8%)		1
T/G	92 (47.9%)	56 (45.5%)	p=0.144	1.093 (0.665-1.795)
G/G	21 (10.9%)	23 (18.7%)		1.966 (0.979-3.948)
SNAP-25 Ddel				
T/T	102 (53.1%)	50 (40.7%)		1
T/C	69 (35.9%)	51 (41.5%)	p=0.059	1.508 (0.919-2.47
C/C	21 (10.9%)	22 (17.9 %)		**2.137 (1.075-4.248)**
Syntaxin 1A Int				
T/T	46 (24.0%)	33 (26.8%)		1
T/C	94 (49.0%)	53 (43.1%)	p=0.595	0.786 (0.449-1.376)
C/C	52 (27.1%)	37 (30.1%)		0.992 (0.536-1.834)
Syntaxin 1A				
T/T	77 (40.1%)	28 (22.8%)		1
T/C	75 (39.1%)	58 (47.2%)	p=0.005	**2.127 (1.225-3.693)**
C/C	40 (20.8%)	37 (30.1%)		**2.544 (1.366-4.738)**

Table 4. Alleles of VAMP2, SNAP-25, Syntaxin 1A genes				
Allele				
	Control (n=192)	**Cases (n=123)**	**x2 p value**	**OR (95% CI)**
VAMP2				
Ins	295 (76.8%)	163 (66.3%)		1
Del	89 (23.2%)	83 (33.7%)	**p=0.004**	**1.688 (1.183-2.407)**
SNAP-25 Mnll				
T	250 (65.1%)	144 (58.5%)		1
G	134 (34.9%)	102 (41.5%)	p=0.097	1.322 (0.951-1.837)
SNAP-25 Ddel				
T	273 (71.1%)	151 (61.4%)		1
C	111 (28.9%)	95 (38.6%)	p=0.011	1.547 (1.103-2.171)
Syntaxin 1A Int				
T	186 (48.4%)	119 (48.4%)		1
C	198 (51.6%)	127 (51.6%)	p=0.988	1.003 (0.728-1.381)
Sytg2				
T	229 (59.6%)	114 (46.3%)		1
C	155 (40.4%)	132 (53.7%)	**p=0.001**	**1.711 (1.238-2.363)**

Table 5. Haplotype distributions of Syntaxin 1A and SNAP-25 genes				
	Control (n=192)	**Cases (n=123)**	**x2 p value**	**OR (95% CI)**
Syntaxin 1A / Synaptotagmin				
TT	126 (32.8%)	66 (26.8%)		1
CT	103 (26.8%)	48 (19.5%)		0.890 (0.565-1.401)
TC	60 (15.6%)	53 (21.5%)		**1.686 (1.049-2.710)**
CC	95 (24.7%)	79 (32.1%)	**p=0.012**	**1.588 (1.041-2.420)**
SNAP-25 MnlI / SNAP-25 DdeI				
TT	192 (50.0%)	106 (43.1%)		1
GT	81 (21.1%)	45 (18.3%)		1.006 (0.651-1.554)
TC	58 (15.1%)	38 (15.4%)		1.187 (0.740-1.904)
GC	53 (13.8%)	57 (23.2%)	**p=0.022**	**1.948 (1.251-3.033)**