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Detection of STAT4 in Multiple Sclerosis patients by polymerase chain reaction and flowcytometry

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Abstract

Multiple sclerosis (MS) is a disease of the central nervous system, characterized by progressive demyelination and inflammation. MS is characterized by immune system attacks on the myelin sheath surrounding nerve fibers. Genome-wide association studies revealed a polymorphism in the signal transducer and activator of transcription 4 (STAT4) gene that increases risk for MS. This polymorphism affects the T helper1 (Th1) cells to secrete the cytokine interferon-gamma when stimulated by interleukin (IL)-12. This study aimed to determine the association of MS active disease with STAT4 genotypes, detected by the polymerase chain reaction (PCR) and STAT4 protein level detected by flowcytometry. The study included 80 MS patients, and 70 controls matched for age and gender. We used the restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) and flowcytometry to detect STAT4 gene polymorphism. Our results showed that, in MS patients, STAT4 genotypes of GC and CC as detected by PCR, were more common when compared to controls. The C allele of the STAT4 gene was also more common in MS patients than controls. The STAT4 GC genotype was associated with MS disease activity. Active MS patients also had a much greater frequency of the STAT4 C allele than the G allele or the control group. The STAT4 proteins level by flowcytometry among the active MS studied patients was higher than its level in the inactive patients and controls. In conclusion, this study demonstrated that both techniques are complementary to each other to detect STAT4 level in MS patients and its association with the disease activity. STAT4 proteins expression detected by flowcytometry in the peripheral blood leukocytes could be used as a biomarker for monitoring disease activity in MS patients.

Keywords: MS, STAT4, PCR, Flowcytometry.

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Introduction

Multiple sclerosis (MS) is a debilitating illness of the central nervous system (CNS) that often affects young and middle-aged adults .[1] MS is a chronic inflammatory disorder that causes damage to myelin sheaths and oligodendrocytes in the CNS, but less so to axons and nerve cells.[2] As an immune-mediated disease, inflammation characterizes white matter lesions, and T and B cells infiltrate the zones of demyelination, axonopathy, microglial activation, and astrogliosis that serve as the diagnostic hallmark of multiple sclerosis.[3] The key etiological causes in MS include a complex interplay between environmental factors, genetic factors, and lifestyle choices .[4,5]

The signal transducer and activator of transcription (STAT) dysregulation has been linked to harmful biological processes including chronic inflammation, cancer, and immunological diseases. To regulate physiological and pathological processes, seven distinct STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6) have been found.[4]

Gene studies revealed that there are three distinct clusters of chromosomes that contain the human STATs genes. STAT 1 and STAT 4 map to chromosome 2, STAT 2 and STAT 6 to chromosome 12, and STAT 3 and STAT 5a and 5b to chromosome 17.[5]

Having CD4 T lymphocytes in CNS inflammation lesions is a hallmark of multiple sclerosis.[6] Interferon-gamma (IFN- γ) production is induced by STAT4, which is required for interleukin (IL)-12 signaling. STAT1 and STAT4 play a role in the interferon signaling pathway.[12] Cytokines belonging to the IL-12 family (IL-12, IL-23, IL-27, and IL-35) play an important role in promoting immunological responses and mediating communication between the innate and adaptive arms of the immune system. STAT4 is an obvious potential location for genetic vulnerability to autoimmune illnesses like MS because of its central role in the continuous generation of type I and type II IFNs.[12]

Several susceptibility loci linked with MS identified by genome-wide association studies are represented by single nucleotide polymorphisms (SNP), indicating that the majority of these genetic differences are associated with adaptive immunity genes.[12,13,18]

One additional argument implicates the adaptive immune system in MS etiology. This is the therapeutic response seen when methods directed at T and B cells are used.[4] In sum, genome-wide association studies lend credence to the notion that MS susceptibility may be regulated by the action of common allelic variations in numerous genes defined by their size effect.[14,23] The identification of a genetic profile in MS-prone people has far-reaching consequences. It also aids in reducing the window of time between initial symptoms and therapy.[16]

This study aimed to determine the level of STAT4 gene expression in different cases of multiple sclerosis. And to assess the association of MS active disease with both different STAT4 genotypes as detected by the polymerase chain reaction (PCR) and various STAT4 protein levels as determined by flowcytometry.

Subjects and Methods

This case-control study included 80 patients with multiple sclerosis recruited from the Neurology outpatient clinics of Assiut University Hospital, Assiut, and AL-Demerdash Hospital, Ain Shams University, Cairo, Egypt, during the period from August 2020 till February 2021. Also, the study included 70 apparently healthy, age and sex matched individuals as the control group.

Patients were divided according to the stage of the disease progression into relapsing-remitting multiple sclerosis (RRMS, 66 patients), secondary progressive multiple sclerosis (SPMS, 7 patients), primary progressive multiple sclerosis (PPMS, 3 patients) and clinically isolated syndrome (CIS, 4 patients). Patients were also divided, according to the clinical and radiological assessments, into an active disease state (36 patients) and inactive disease state (44 patients).

The exclusion criteria included people who were not diagnosed with MS at any point during

the study, and individuals with a pre-existing history of an autoimmune disease or other neurological problem

All patients were subjected to complete history taking, including age, gender, duration of disease, and family history. Also, to thorough clinical examination, thorough neurological examination, and expanded Disability Status Scale (EDSS).

Venous blood samples (4 ml) were collected from each study subject under aseptic conditions into a tube containing anticoagulant (K3 EDTA). Each sample was divided into two parts. The first part (2 ml) was: stored frozen at -20°C to be used later for DNA extraction. The second part (2 ml) was used freshly for flowcytometry.

PCR Analysis of STAT4 Gene

Genomic DNA preparation:

DNA extraction from blood samples was carried out using commercial Kits (Catalog no. K0781, Gene JET Whole Blood Genomic DNA Purification Mini Kits, Thermo Scientific, USA), according to the manufacturer's instructions.

Genotyping of MS for the studied subjects

The SNP rs7582694 of STAT4 gene was genotyped utilizing the restriction fragment length polymorphism-polymerase chain reaction- (RFLP-PCR). The sequence of the primers used in PCR was as follow; Forward primer: 5' ATCCAACTCTTCTCAGCCCTT3', Reverse primer: 5' TCATAATCAGGAGAGAGG AGT3' (Biosearch technologies-LGC, USA).

The 338 bp PCR products were isolated and digested with restriction enzymes (Catalog no. FD1364, FsatDigest TAAI, Thermo Scientific, USA), according to the manufacturer's instructions.

Then, to separate the restriction fragments, 10 µl of the digest mixture were loaded into 2% agarose gel followed by electrophoresis. The molecular size of product was estimated by comparing it with the migration distance of DNA marker (50-1000 bp ladder). The G allele could be detected at 338 bp, and the C allele was detected at 300 bp length (Figure 1).

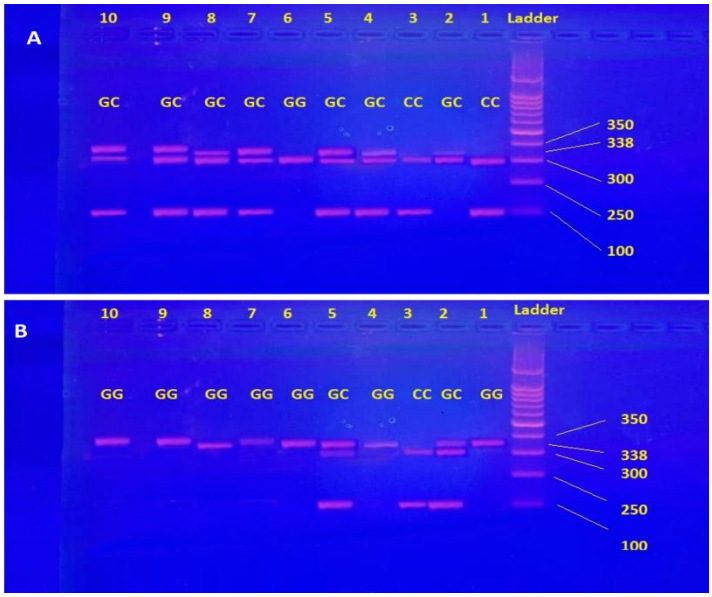

Figure 1. Restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) analysis of the genotype pattern of STAT4 gene polymorphism. (A) multiple sclerosis patients. Lane 1 and 3: bands of 300 and 100 bp were categorized as CC genotype; lane 6: band of 338 bp was categorized as GG genotype and lanes 2,4,5,7,8,9,10: bands of 338, 300 and 100 were categorized as GC genotype. (B) Controls. Lane 3: bands of 300 and 100 bp were categorized as CC genotype; lanes 1,4,6,7,8,9,10: bands of 338 bp were categorized as GG genotype and lanes 2 and 5: bands of 338, 300 and 100 were categorized as GC genotype.

Flowcytometry analysis of STAT4 protein

A multicolor benchtop flowcytometry system (BD FACS Canto ll of Becton Dickinson Company, CA, USA), capable of both analyzing and sorting was used for STAT4 protein analysis.

The following monoclonal antibodies were used for flowcytometry; PerCP conjugated anti CD4 (Clone SK3, BD-Bioscience, USA,), PECy7 conjugated anti CD8 (Clone RPA/T8, BD-Bioscience, USA,), FITC conjugated anti CD36 (Clone TR9, Beckman Coulter, France,), and PE conjugated anti STAT4 (Clone 38/p, BD-Bioscience, USA,), The gating strategy is illustrated in Figure 2.

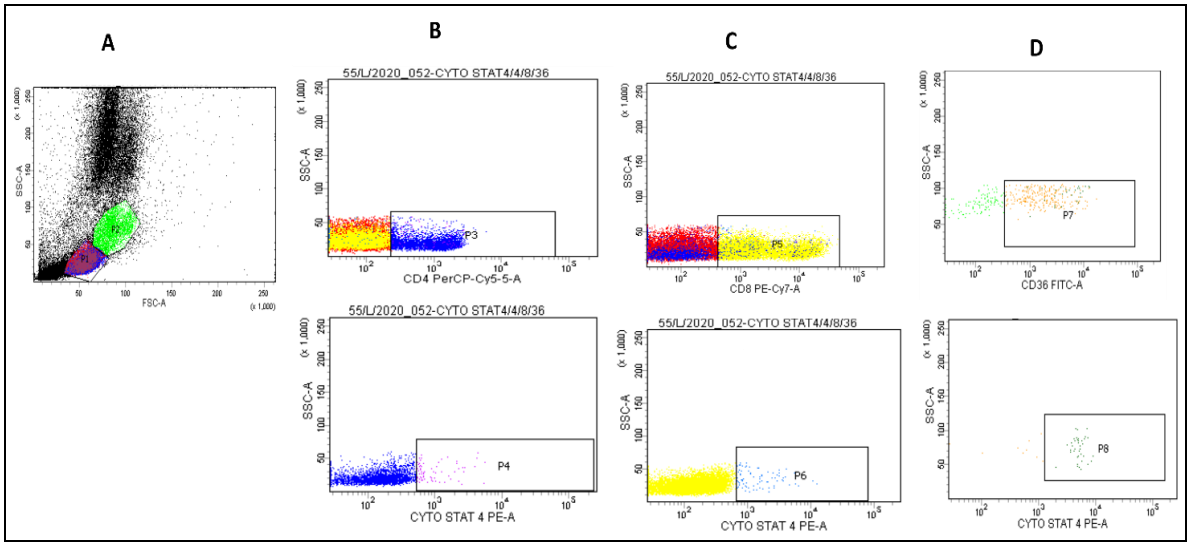

Figure 2. STAT-4 expression in patients with active multiple sclerosis disease. Dot blot (a) shows the gating strategy for flowcytometry of STAT4. Lymphocytes (p1) and Monocytes (p2) were gated on a forward (FSC) and side scatter (SSC). Dot plot (b) shows the expression of STAT4 on CD4+ T lymphocytes. Dot plot (c) shows the expression of STAT4 on CD8+ T lymphocytes. Dot plot (d) shows the expression of STAT4 on CD36+ monocytes.

Statistical Analysis

The statistical package for the social science (SPSS) IBM Inc., Chicago, IL, USA) version 21 was used for the statistical analysis. When applicable, numerical information is presented as the mean standard deviation (±SD) or median (range), and frequency (%) descriptions. The T-test, the Mann-Whitney U-test, and the analysis of variance (ANOVA) were employed to identify the association between quantitative variables (comparing two groups and three groups, respectively). In order to compare categorical data, the Chi-square ($\chi 2$) or Fisher Exact test were used. Risk was estimated using odds ratios (OR) with 95% confidence intervals (CI) and logistic regression analysis. The receiver operating characteristic (ROC) curve analysis was used to determine the sensitivity and specificity for detection of disease activity in MS patients. The area under the curve (AUC) was also determined. A $p< 0.05$ value was considered significance.

Results

The EDSS score was significantly increased among MS active diseased patients as compared to inactive MS diseased patients [median (range) 4 (1.0 – 6.5) vs 2.0 (1.0 – 6.5), p<0.001, respectively]. The median STAT4 level in the peripheral blood lymphocytes, monocytes and in both cells by flowcytometry were significantly increased in the active MS diseased patients than in the inactive disease patients (6.1 vs 2.7, 5.2 vs 2 and 11.6 vs 5.2, p<0.001, for all). Table 1 shows the demographic and clinical details of the 80 MS patients.

Table 1. Demographic and clinical details of the 80 multiple sclerosis (MS) patients according to disease activity.

Variable	Active group (n=36)		Inactive group (n=44)		*p value*
Age (yrs), Median (range)	34 (24 – 55)		32 (20 – 45)		NS
Age of onset (yrs), Median (range)	28.5 (17 – 49)		26.5 (17 – 42)		NS
Age of onset groups, n (%)					NS
18 -< 40	33	(91.7)	41	(93.2)	
≥ 40	3	(8.3)	3	(6.8)	
Disease duration (yrs), Median (range)	4 (1 – 21)		3.5 (1 – 21)		NS
Gender, n (%)					NS
Female	29	(80.6)	39	(88.6)	
Male	7	(19.4)	5	(11.4)	
Marital status, n (%)					NS
Married	23	(63.9)	22	(50.0)	
Single	13	(36.1)	22	(50.0)	
Residence, n (%)					NS
Cairo + Giza	27	(75.0)	28	(63.6)	
Delta	8	(22.2)	11	(25.0)	
Upper Egypt	1	(2.8)	5	(11.4)	
Occupation, n (%)					NS
Physical	7	(19.4)	12	(27.3)	
Mental	29	(80.6)	32	(72.7)	
Status of treatment, n (%)					NS
Non-compliant	14	(38.9)	13	(29.5)	
Compliant	22	(61.1)	31	(70.5)	
EDSS/ T, Median (range)	4.0 (1.0 – 6.5)	3 (1.0 – 6.5)	2.0 (1.0 – 6.5)		0.001

EDSS: Expanded Disability Status Scale. Quantitative data are presented as median (range); qualitative data are presented as n (%). Chi-square test was used to compare categorical data. $p > 0.05$ is not significant (NS).

Using the univariate logistic regression analysis, STAT4 genotype by PCR was detected to be the only significant risk factor associated with MS disease (Table 2). As patients with GC variant were two times more likely associated with MS disease (OR=2.857, 95%CI: 1.364 – 5.985, p=0.005), and patients with CC variant were six times more likely associated with MS disease (OR=4.643, 95%CI: 1.397 – 15.431, p=0.012) than patients with GG variant.

Table 2. Logistic regression analysis for multiple sclerosis (MS) risk factors.

Variables	No.	Univariate analysis		
		OR	*p* value	95% CI
Age (years)				
18 - < 40	112	Ref		
≥ 40	38	0.836	NS	0.400 – 1.746
Gender				
Female	126	Ref		
Male	24	0.853	NS	0.356 – 2.043
Marital status				
Married	83	Ref		
Single	67	0.924	NS	0.484 – 1.761
Occupation				
Physical	33	Ref		
Mental	117	0.803	NS	0.368 – 1.751
Consanguinity				
Negative	142	Ref		
Positive	8	0.506	NS	0.117 – 2.201
STAT4 genotype (PCR)				
GG	85	Ref		
GC	48	2.857	0.005	1.364 – 5.985
CC	17	4.643	0.012	1.397 – 15.431

No: the number of the whole studied populations (150); STAT: Signal transducer and activator of transcription. OR= odds ratio, CI =confidence interval, $p > 0.05$ is not significant (NS).

Flowcytometric analysis of the STAT4 proteins among MS studied patients were found in lymphocytes, monocytes and on both cells ranged from (1.1 to 13.01%, 0.4 to 12.6% and 1.9 to 25.1%, respectively). However, the STAT4 proteins were not detected among controls (Table 3).

The distribution of genotypes and allele rates of SNP rs7582694 of STAT4 gene was detected by PCR. The genotype frequencies of CC and GC were significantly increased in MS patients compared to the GG genotype which was increased in control group (p=0.002). And the STAT4 gene C allele was significantly increased in MS patients as compared to the G allele which increased in the control group (p< 0.0001) (Table 3).

Table 3. Detection of signal transducer and activator of transcription 4 (STAT4) by flowcytometry and by the polymerase chain reaction (PCR) among multiple sclerosis (MS) patients and control groups.

Variable name	MS (n=80)		Controls (n=70)		*p value*
STAT4 by flowcytometry					
Lymphocytes	4.05 (1.1 – 13.01)		0		-----
Monocytes	2.7 (0.4 – 12.6)		0		-----
Both	6.5 (1.9 – 25.1)		0		-----
STAT4 by PCR					0.002
GG	35	(43.8)	50	(71.4)	
CG	32	(40.0)	16	(22.9)	
CC	13	(16.2)	4	(5.7)	
Alleles					<0.0001
G	102	(63.8)	116	(82.9)	
C	58	(36.2)	24	(17.1)	

STAT4: Signal transducer and activator of transcription 4. Quantitative data are presented as median (range), and qualitative data presented as n (%). The Chi-square test or Fisher Exact test were used to compare categorical data. Significance defined at $p < 0.05$.

The predictive ability of STAT4 protein level detected by flowcytometry in the peripheral blood lymphocytes, monocytes and on both of the cells in MS patients was demonstrated using the ROC curve analysis (Table 4 and Figure 3). The AUC for them were 90.2%, 76.4% and 85.7%, respectively. STAT4 level in lymphocytes was observed to be a significantly better predictor of MS disease activity with higher AUC than the other predictors (p<0.001).

Table 4. The best cut off, sensitivity and specificity for multiple sclerosis (MS) disease activity by detecting signal transducer and activator of transcription 4 (STAT4) proteins on different blood cells by Flowcytometry.

STAT4 by Flowcytometry	Cut off	95%CI	Sensitivity	Specificity	AUC	*p value*
Lymphocytes	3.5	0.831 - 0.973	88.9%	72.7%	0.902	<0.0001
Monocytes	2.3	0.651 – 0.877	80.6%	65.9%	0.764	<0.0001
Lymphocytes Monocytes	6.6	0.772 – 0.942	75.0%	72.7%	0.857	<0.0001

AUC, Area under the curve; CI, confidence interval. Significance defined at p <0.05

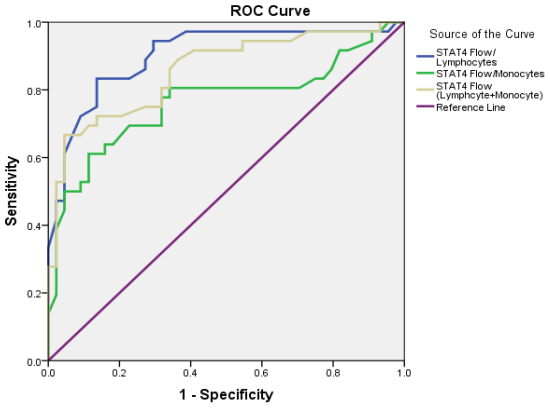

Figure 3. The receiver operating characteristic (ROC) curve analysis for detection of disease activity in MS patients. STAT4 Flow/Lymphocytes (blue). STAT4 Flow/Monocytes (green), STAT4 Flow Lymphocytes+ Monocytes (brown) and Reference line (purple). curve = 0.902 (0.831 to 0.973), p <0.0001, p=0.764 (0.651 to 0.877) and p=0.857 (0.772 to 0.942), p < 0.0001, respectively.

The distribution of alleles and genotypes of the selected SNP rs7582694 of STAT4 gene was detected by PCR (Table 5). Patients with active disease state had increased frequency of CC genotype as compared to patients with inactive disease state (36.1% versus 0.0%, respectively) (p<0.05). And the frequency of the C allele increased in patients with active disease state than that in patients with inactive disease state (65.3% versus 12.5%, respectively) (p<0.001).

Table 5. Signal transducer and activator of transcription 4 (STAT4) detection by flowcytometry and by the polymerase chain reaction (PCR) among active and inactive multiple sclerosis (MS) diseased patients.

STAT4 by flowcytometry	Active group (n=36)		Inactive group (n=44)		*p* value
Lymphocytes					
< 3.5	4	(11.1)	32	(72.7)	<0.0001
≥ 3.5	32	(88.9)	12	(27.3)	
Monocytes					
< 2.3	7	(19.4)	29	(65.9)	<0.0001
≥ 2.3	29	(80.6)	15	(34.1)	
Lymphocytes + Monocytes					
< 6.6	9	(25.0)	32	(72.7)	<0.0001
≥ 6.6	27	(75.0)	12	(27.3)	
STAT4 by PCR					
GG	2	(5.6)	33	(75.0)	
GC	21	(58.3)	11	(25.0)	<0.0001
CC	13	(36.1)	0	(0.0)	
GG vs GC					
GG	2	(8.7)	33	(75.0)	<0.0001
GC	21	(91.3)	11	(25.0)	
GG vs CC					
GG	2	(13.3)	33	(100.0)	<0.0001
CC	13	(86.7)	0	(0.0)	
GC vs CC					
GC	21	(61.8)	11	(100.0)	0.019
CC	13	(38.2)	0	(0.0)	
Allele (G vs C)					
G	25	(34.7)	77	(87.5)	<0.0001
C	47	(65.3)	11	(12.5)	

Quantitative data are presented as median (range); qualitative data are presented as n (%). The Chi-square test or Fisher Exact test were used to compare categorical data. Significance defined at $p < 0.05$.

A univariate analysis showed association of active MS disease with both the level of STAT4 protein detected by flowcytometry and STAT4 genotype of the selected SNP rs7582694 by PCR (Table 6). Patients with STAT4 cut off level of ≥ 3.5 in lymphocytes were about 21 times more likely to be associated with active MS disease (OR=21.33, 95%CI: 6.216 – 73.214, p<0.0001) compared to patients with STAT4 cut off level of ≥ 2.3 of in monocytes which was about eight times more likely to be associated with active MS disease (OR=8.01, 95%CI: 3.847 – 22.532, p<0.0001). Patients with GC genotype were about 32 times more likely to be associated with active MS disease (OR=31.50, 95%CI: 6.341 – 156.474, p<0.0001).

Table 6. Logistic regression analysis for prediction of MS disease activity according to STAT4 detection by flowcytometry and by PCR.

Variables	n	Univariate analysis		
		OR	*p* value	95% CI
STST4 Flow/Lymphocytes				
< 3.5	36	ref		
≥ 3.5	44	21.33	<0.0001	6.216 – 73.214
STAT4 Flow/Monocytes				
< 2.3	36	ref		
≥ 2.3	44	8.01	<0.0001	2.847 – 22.532
STAT4 genotype (PCR)				
GG	55	ref		
GC	32	31.50	<0.0001	6.341 – 156.474
CC	13	NA	NS	NA

Logistic regression analysis was used. N= number, OR= odds ratio, CI =confidence interval,
p > 0.05 is not significant (NS).

Discussion

MS is defined as an inflammatory disorder that results in damage in the central nervous system and the most common cause of non-traumatic disability in young adults.[7] The underlying cause of MS remains opaque, although complex gene–environment interactions play a significant role. Changes in diagnostic methods and criteria mean that people with MS can be diagnosed increasingly early in their disease trajectory.[9]

Abnormal levels of the STAT family in the peripheral blood mononuclear cells of MS patients were associated with a number of clinically relevant phenotypes. [10] Signal transmission from the interferons involves STAT1 and STAT4. The extensive involvement of type I and type II interferons in the pathogenesis of MS made STAT4 an obvious candidate region for genetic predisposition to these autoimmune diseases.[15] Moreover, the requirement of STAT4 in IL-23-induced IL-17 production has been suggested.[17]

The present study was a case control study in Egyptian population, aimed to determine the level of STAT4 in different cases of MS using both PCR and flowcytometry and its association with disease activity.

In the current study, using flowcytometry, a range of the STAT4 proteins level was detected in the peripheral blood leukocytes. In the lymphocytes it ranged from (1.1 – 13.01%), in the monocytes it ranged from (0.4 – 12.6%) and in both lymphocytes and monocytes it ranged from (1.9 – 25.1%). STAT4 proteins were not detected in the study controls by the flowcytometry. The current study also assessed the predictive ability of STAT4 protein level to MS active disease with cutoff values in the lymphocytes (3.5), monocytes (2.3) and in both cells (6.6). In MS patients through performance of these cells, the ROC curve analysis showed

high sensitivity as 88.9%, 80.6% and 75.0% respectively.

Moreover, by a univariate analysis, we found patients with STAT4 protein cutoff level in lymphocytes ≥ 3.5 were about 21 times more likely to be associated with active MS disease compared to patients with STAT4 protein cutoff level in monocytes ≥ 2.3 were about eight times more likely to be associated with active MS disease.

In the same line, by using RFLP-PCR, the distribution of genotypes and alleles rate of the selected SNP rs7582694 of STAT4 gene in all MS patients and controls showed significant increase in the frequencies of GC and CC genotypes in MS patients compared to age and sex matched controls (p=0.002). The frequency of the C allele was higher in MS patients than that in controls (p=0.000). The same relations were found in its subtypes RRMS and SPMS. These findings were confirmed by a univariate logistic regression analysis and considered the risk for MS. They were associated with CG and CC genotypes as compared to GG genotype which appear as a protective variant. Meanwhile other factors namely age, sex, marital status, occupation and consanguinity have no role for prediction of MS disease development (p>0.05). In addition, this study demonstrated that patients with GC genotype were about 31 times more likely to be associated with active MS disease. The frequency of the C allele was higher in the active disease group (p<0.0001).

These findings were in a strong agreement with a study which assessed STAT4 gene polymorphism and its relation to disease severity in two major autoimmune diseases (40 multiple sclerosis patients, 40 juvenile onset systemic lupus erythematosus patients and 40 controls). They found the STAT4 (rs7582694) gene polymorphism CC genotype and GC genotype frequencies were significantly more detected in MS patients than in controls. The frequency of the STAT4 C allele was significantly higher in patients with MS than in controls (p = 0.01). This was consistent also with another study done in 2001.[12,13]

The current findings were also in harmony with a study conducted on MS patients that demonstrated that IL-17 F CT genotype and C allele may be associated with a susceptibility to MS in Egyptian population by a gender dependent mechanism that contributes to unique predisposition in females.[14]

Also, this study PCR findings are supported by a further study who found that females with a GC/CC genotype showed a significantly elevated MS Severity Score compared to females of GG genotype (p = 0.005).[19]

Also, in line with this study observation, a study of Polish cohort conducted in 2011, found that increased risk of MS was associated with the C allele, particularly with the CC genotype .[11]

The study by Canto et al., 2018, developed a phosphor-flowcytometry protocol to assess the levels of 11 phosphorylated nuclear proteins at baseline and after cell activation in distinct peripheral blood mononuclear cells from 41 treated naïve RRMS subjects and 37 healthy controls, and in a second cohort of 9 untreated RRMS patients and 10 SPMS patients. [32] In contrast with our findings the author reported that monocytes were more susceptible to activation by interferon in MS patients. This exaggerated activation, reflected as an increased phosphorylation of STAT proteins, would lead to an upregulation of human leukocytic antigen expression in this cell type and, as a potential consequence, a dysregulation of the immune responses.[32]

On the opposite side, a study conducted by Martinez et al., 2008, discussed the association of the STAT4 gene with increased susceptibility for some immune-mediated diseases included MS, and reported no significant difference between MS patients and controls.[24] Researchers believe that the demyelinating phenotype and clinical course may be the ultimate expression of different processes of pathogenesis, and that this is due to the phenocopy problem.[34-36] A lack of statistical power in their data made it possible that the STAT4 gene affects disease predisposition in just some MS patients.[22]

Another different study disagreed with our study findings as they found that the C allele was associated with a milder disease course.[21] Moreover, a more recent study reported outcomes in contrast with the current study

findings as there was no significant difference between clinical parameters of MS patients and genotypic pattern.[12] Also, the current results are in contrast to that reported by Shahbazi, et al., 2010, in Iranian cohort, and found an increased risk for MS associated with the G allele of −174.[20] Also, in disagreement with the present findings, another study showed no difference in STAT4 on lymphocytes from patients with RRMS compared to healthy controls.[8]

Whether or if these variations reflect genuine dissimilarities in the underlying cultures of the people being studied, or whether they are the result of chance, is an open question. This is significant because it suggests that the responsiveness and activity of STAT-pathways are influenced by circumstances (environmental factors) beyond the genetic background. They have also been linked to an increased risk of multiple sclerosis and modulation of both innate and adaptive immunity.[22] Our interpretation is hindered by the requirement for larger prospective studies to corroborate it.

In the current study, females were the predominant gender among our patients (86.3%). This is in accordance with many other studies. A study conducted in Egypt, illustrated the clinical characteristics of patients with multiple sclerosis (female represented 72%) [29] and a study by Schiess et al., 2014, study in Abu Dhabi, and another study in Poland by Perwieniec et al., 2021.[26,33]

Female prominent was also found in similar studies, showed the STAT gene in multiple sclerosis patients.[32,12] These results related to the gender predisposition shed more light on well-known differences in the epidemiological and clinical characteristics between sexes in MS. One crucial clinical observation is that MS occurs more frequently in women than men, indicating an impact of sex-related factors on susceptibility to MS.[33]

A further study agreed with this study and mentioned that the sex ratio in MS seems to be increasing. This trend is seen primarily in relapsing-remitting MS and associated with a latitudinal gradient, which strongly indicates that a complex interaction occurs between sex hormones, genetic and epigenetic factors, environmental and cultural factors.[25]

In the present study, patients' age ranged between 20-55 years old, and the age of onset of MS disease ranged from 18-49 years old with a median of 27.5 years old, and the majority of patients (92.5%) had an onset age between 18 and 40 years old. This agreed with a study conducted in 2014 which demonstrated that the mean age at disease onset was 26 years with the peak age of onset of the disease at 20–39 years.[26]

The EDSS gives a total score from 0 to 10. The first levels, 1.0 to 4.5, are for individuals who have a substantial loss of mobility, and the next levels, 5.0 to 9.5, are for people who are losing their mobility.[27] It is the standard way to measure disability in clinical trials for MS.[28]

In the present study, the EDSS score varied from 1 to 6.5 in the total patients and was significantly increased in active MS patients than in the inactive patients (median 4 vs 2 respectively). In the same line, the mean EDSS score among patients included in the study by Zakaria et al., 2016,[29] was 3.6 and in study in Qatar by Deleu et al., 2013,[30] was 2.7. The average EDSS score was 2.8 points in the study by Biernacki et al., 2020.[31] These studies calculated the EDSS for all patients with no stratification to subtypes. The mean EDSS score was 5.92 (±1.46) points in progressive cases and 1.3 (±1.8) in regressive cases according to the Biernacki et al., 2020 study.[31]

In conclusion, in MS patients, STAT4 genotypes of GC and CC were detected by PCR, more common when compared to controls. The C allele of the STAT4 gene was also more common in MS patients than controls. The STAT4 GC genotype was associated with MS disease activity. Flow-cytometric analysis of STAT4 proteins in the peripheral blood leukocytes is expressed only in MS patients. The expression of STAT4 proteins in the peripheral blood leukocytes was markedly increased in the active group. Both techniques are complementary to each other to detect STAT4 level in MS patients and its association with the disease activity.

Author Contributions

SGM, RAE, MMN; performed the laboratory work. AKM, DAM, MAD, MMN; made the statistical

analysis. HNE, DZ; examined the patients. MMN, DZ, SGM; collected samples. All authors participated in writing and reviewing the paper.

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 Institutional Review Board of the Faculty of Medicine, Assiut University, Egypt (approval dated April 2019). The study protocol was registered at Clinical Trials.gov (ID: NCT04557514).

Informed consent

Verbal informed consent was taken from each participant.

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