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The Prevalence of the Genetic Polymorphism of GSTM1, GSTT1 and GSTP1 and Its Relationship with Clinical Criteria of Multiple Sclerosis (MS) Patients in Tehran

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ABSTRACT

BACKGROUND AND OBJECTIVE: Multiple Sclerosis is the chronic inflammation of central nervous system with demyelinated lesions in the brain and spinal cord. The genetic polymorphisms associated with glutathione S-transferase enzymes involved in antioxidant defense in Iranian patients have not been investigated. Therefore, in the present study, the prevalence of the genetic polymorphism of glutathione S-transferase M1, P1 and T1 and its relationship with clinical criteria of MS patients with has been examined.

METHODS: In this case-control study, 69 patients who referred to Sina Hospital in Tehran and had no panic attack within the last three months and 74 healthy subjects were interviewed. After examination by neurologist and blood sampling, DNA extraction was performed using Roche kit. Then, the genotypic variations of the samples were evaluated using RFLP-PCR and its prevalence was analyzed in relation with age, birth weight, malignancy (EDSS) and gender using GraphPad Prism software.

FINDINGS: Most malignancies were observed in men (3.1±5.9) and the highest incidence rate was observed in those born in May (30%). Although the results of genotyping between the studied groups and their gender did not show any significant difference (OR: 2-4, p>0.05), patients with GSTM1 deficiency developed the disease at a lower age (32.8±2.6 years) compared with other patients (29.5±8.9 years) (CI-95%: 20.3–26.4, p=0.009). In addition, people with a rare GSTM1 allele who smoked cigarette had higher EDSS (CI-95%: 2.1–3.7, p=0.03).

CONCLUSION: Based on the results of this study, the effect of GSTM1 on malignancy is indicative of its role in detoxification of tobacco products and can be used as an agent for early diagnosis of disease in people who are susceptible to this disease.

KEYWORDS: *Multiple Sclerosis, Polymorphism, Incidence, Glutathione S-Transferase, GSTT1, GSTP1, GSTM1.*

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Introduction

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (1, 2). The disease is usually diagnosed between the ages of 20 and 45 years (3, 4), and only 5% of those diagnosed with MS are under the age of 10 or over the age of 50 (5). The incidence of this disease is more common in women, and the women to men ratio are reported to be 2 to 1 on average (6). The exact cause of MS has not yet been found. It is likely that the disease is a combination of genetic, environmental, and infectious factor (7). What is certain is the fact that the pathogenicity is caused by inflammatory reactions and myelin breakdown by the immune system (8, 9). Changes in the HLA-DRB1 genetic system on the human chromosome 6 have also been shown to increase the incidence of MS (10). Experts believe that MS patients have inherited the susceptibility to the disease from birth (11). People who are susceptible to MS only develop this disease when they are affected by environmental factors (10, 12). Oxidative stress represents an imbalance between free radical production and the ability of the biological system (antioxidant cascade) to detoxify. Free oxygen radicals can damage all cell biomacromolecules (lipids, sugar, proteins, and polynucleotides) and ultimately lead to the onset of the disease (13). The central nervous system is very sensitive to oxidative stress because of its high rate of oxygen consumption, low antioxidant compounds, and related enzymes, as well as high levels of unsaturated fat. Over the past few years, evidence has shown that the reactive oxygen species (ROS) share several mechanisms in the pathogenesis of MS disease and myelin phagocytosis (14).

It has also recently been shown that oxidative stress plays a major role in the pathogenesis of MS disease. An increase in the level of secondary products of oxidative stress or a decrease in the level of antioxidant enzymes and the presence of low levels of antioxidants in the blood and cerebrospinal fluid (CSF) of patients with MS have been observed during the active phase of the disease. The knowledge about the exacerbation of MS associated with axonal degeneration is increasing, and the obtained information indicates the significant role of oxidative stress in the pathogenesis of this disease (15, 16). Glutathione exists in all cells, and a long interruption or lack of it leads to serious damage to the cell (17). Meanwhile, glutathione S-transferase (GST) enzyme neutralizes their revival by attaching electrophilic compounds to the thiol group of cysteine in glutathione and converts them to water-soluble compounds for better discharge (18). The glutathione S-transferase (GST) is a large family of enzymes discovered for the first time in 1961 (19, 20). The family consists of cytosolic, mitochondrial, and microsomal enzymes (MAPEG) (21). The important role of these enzymes in the detoxification and disposal of drug compounds, carcinogens, xenobiotics and environmental contaminants has given them special attention (22). What is certain is the difference in gene placement, the tendency to substrates, and having a variable amino acid sequence that has led to a variety of isoenzymes in the family (23, 24)(Fig 1).

To date, numerous allelic changes have been reported under the families Alpha, Mu, Pi, Theta and Zeta (24, 25). Removing homozygotes or removing both alleles in these areas results in lack of protein expression for these enzymes, which is also referred to as Null (23). In addition, in the long arm of chromosome No. 11 of GSTP1 gene with 7 exons, (26) it was shown that displacement of adenine to guanine in nucleotide 313 (codon 105) induces the change of isoleucine codon to valine in exon 5. In 2007, a study among 49 patients with multiple sclerosis in Greece reported that the lack of GSTT1 and GSTM1 genotypes was found to be 55.1% and 18% in the case group, respectively. In addition, in a study by Stavropoulou et al., the difference in GSTM1 genotype was also related to gender. To date, the role of GST polymorphism in various pulmonary diseases, such as COPD and α1-antitrypsin deficiency (27), cystic fibrosis (28), lung cancers (29), apoptotic processes in pulmonary fibroblasts (30), and so on have been proved. Considering the role of oxidative stress in this disease, the importance of GST enzymes in antioxidant defense and the fact that GST polymorphism in patients with MS in Iran has not yet been studied, the association between the prevalence of GSTT1, GSTM1 and GSTP1 polymorphism and age of onset and malignancy in patients with MS in Tehran was investigated in this study.

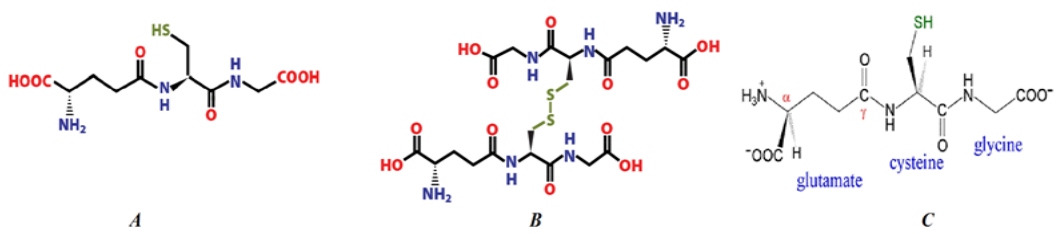

Figure 1. Spatial structure and functional groups in GSH (A), glutathione disulfide (B), and the structure of the peptide components in it (C)

Methods

This case-control study was approved by the ethics committee of the Faculty of Pharmacy, Tehran University of Medical Sciences, with ethics code 22101-33-01-92, and conducted on 69 patients with RR-MS referring to the relevant department in Sina Hospital in Tehran and 74 healthy controls who were randomly selected. After observing the consent form and obtaining permission, an interview was conducted. Prior to entering the study, their disease (RRMS) was confirmed by the neurologist. Having a recent neurological attack within 3 months, lack of defect in major organs (heart, kidney, liver, lung), lack of using dietary supplements and thyroid hormones, lack of metal prostheses in the body, lack of following certain dietary regimens and vegetarianism were among the inclusion criteria. In the control group, the absence of active neurological disease and physical health were among the criteria for choosing healthy people. People who did not reside in the study area or emigrated within the last 10 years, had vegetarian diets or abnormal consumption of products such as seafood or canned food, had contact with contaminated sites and metal melting factories or used pharmaceutical supplements were excluded. Finally, after completing the questionnaire, 4 ml blood was collected from each person using EDTA Vacutainer Tubes (Becton Dickinson, USA) and stored at 2-8 °C until the time of transfer and extraction.

Genotyping: First, the DNA was isolated using the Roche extraction kit (11667327001) based on the manufacturer's instructions. In the next step, a suitable primer pair was designed for each of the desired genes using DNA Star & Oligo7 software (Table 1). Then, using the PCR machine (peqSTAR), the components were multiplied by multiplex PCR using the touchdown temperature program. After performing the PCR, the products were electrophoresed on 2% agarose gel and they were observed and photographed on the Gel doc system using the Safe-Red. The RFLP-PCR method was used to determine the mutation in GSTP1. The primers were designed in such a way that, if there was a mutation, the multiplied piece (300 bp) was cut by the ALW26I (Bsm A1) enzyme and broken into two visible pieces (100 & 200bp). For this reaction, 10 µl of the PCR reaction product, 18 µl of nuclease-free water, 2 µl of tango buffer and 1-2 µl of the ALW26I enzyme (Bsm A1, Fermentas) were poured into 0.5 µl tube and were incubated at 37 °C for 1 – 16 hours. After PCR, products were electrophoresed on 2% agarose gel. Finally, the multiplied bonds were observed and photographed on Gel doc with the presence of Safe-Red.

Table 1. The sequencing primers and the characteristics of the examined genes

Gene name ID	Accession number	Gene location	Aliases	Modified base	Modified amino acids	Variant allele frequency	Sequence of primer pairs (F/R)	Base pair (bp)
GSTM1 2944	NC_000001.11	1p13.3	GST1-1, GSTM1a-1a, GSTM1b-1b, GTH4, GTM1, H-B, MU, MU-1	Gene deletion	No protein	0.5749	5′-GAACTCCCTGAAAAGCTAAAGC-3′ 5′-GTTGGGCTCAAATATACGGTGG-3′	219
GSTT1 2952	NC_000022.10	22q11.23		Gene deletion	No protein	0.4996	5′-TTCCTTACTGGTCCTCACATCTC-3′ 5′-TCACCGGATCATGGCCAGCA-3′	450
β-globin* 3043	NC_000011.10	11p15.5	CD113t-C, beta-globin	-	-	-	5′-GAAGAGCCAAGGACAGGTAC-3′ 5′-CAACTTCATCCACGTTCACC-3′	267
GSTP1 2950	NC_000011.10	11q13	DFN7, FAEES3, GST3, GSTP, HEL-S-22, PI	Exon 5 (A→G)	Ile 105 Val(I 105 V)	0.2256	5′-CTCCCCTCCACCCAACCCCAG-3′ 5′-GCAGGTTGTGTCTTGTCCCAG-3′	Ile/Ile 300 Ile/Val 100 & 200 & 300 Val/Val 100& 200

*** The beta globin is positive control.**

Statistical analysis: After collecting data including genotype and other quantitative and qualitative variables such as month of birth, age, gender, duration of disease, malignancy or EDSS, data were analyzed according to the method of data distribution using statistical software GraphPad Prism and Chi-square tests, Mann-Whitney nonparametric test, while $p < 0.05$ was considered significant. Odds ratio for samples in each section has also been reported.

Results

Demographic Information: According to the recorded data, despite the randomness of the samples, 30% of the total population were born in May (Fig 2). The mean age in the case group was 35.2 ± 10.8 years, which was not statistically significant compared to the control group (32.3±10.3 years) (Table 2).

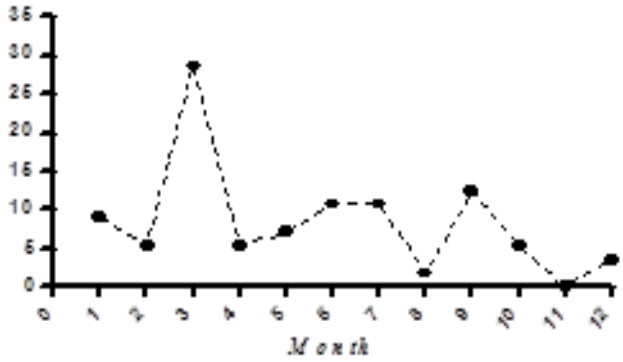

Figure 2. Frequency distribution of patients according to the month of birth

PCR Results: Electrophoresis of the final product in the multiplex PCR model on the gel was detected in GSTT1 and GSTM1 bands in regions 450 and 219, respectively (Fig 3). In addition, the gel electrophoresis of GSTP1 samples after the PCR and after enzyme digest is shown in Fig 4. The samples loaded in wells 1, 5, 6, and 9 were homozygous wild-type, while the sample in well 8 was a homozygous mutant and the remaining samples were heterozygote for GSTP1.

Prevalence of polymorphism: Genotyping studies in both groups indicated that the polymorphism results were within the normal range for the studies population. In addition to high levels of mutant genotypes in the patient group, all these defective enzyme isoforms were found among women. However, in the control group, this genotype was equally distributed among men and women (50%) (Table 3).

Relationship with Clinical Parameters in Patients: There was a significant relationship between age of diagnosis of disease and mutant genotype for GSTM1 ($p=0.009$). There was no statistically significant relationship between the incidence of polymorphism in each of the genes and duration of the disease (95% CI: 20.3 – 26.4) (Table 4).

Comparison of malignancy of patients with their enzyme genotype: The true EDSS values for each genotype were not significant. Individuals without GSTM1 had more malignancies than those with this genotype (CI-95%: 0.67–2.3: OR: 1.84–5.5) (Fig 5).

Comparison of malignancy with regard to the gender of the patients: The division of individuals and the comparison of malignancy with respect to gender indicates that malignancy is significantly higher in males than in females. Although the malignancy was similar in both groups and was observed between 0.5 and 6.5 in both groups, the comparison of means (3.5 in males and 1.9 in females) revealed a difference between the two groups ($p<0.05$).

Table 2. Frequency distribution of the examined criteria in each group according to gender, age, duration of disease, malignancy and smoking

Groups	Parameter	Male N(%)	Female N(%)	Total
Multiple sclerosis patients	Number of participants	11(15.9)	58(84.1)	69
	Age (years) (Mean±SD)	33.12±2.7	35.10±5.6	35.10±2.9
	Smokers	8(30.7%)	18(69.2%)	26
	Non – smokers	3(6.9%)	40(93.0%)	43
	Duration of the disease (year) (Mean±SD)	7.3±6.1 (1–21)	7.4±4.8 (1–23)	7.3±6.1 (1–23)
	Severity of disease (EDSS)	3.5±1.9 (6.0 – 5.5)	1.9±1.2 (6.0 – 0.5)	2.2±1.34 (6.0 – 5.5)
Healthy people	Number of participants	38(52%)	36(48%)	74
	Age(years) (Mean±SD)	29.4±7.9	33.10±8.8	31.10±8.3
	Smokers	4(66.6%)	2(33.3%)	6
	Non – smokers	34(50)	34(50)	68

*** The values are expressed as mean ± standard deviation.**

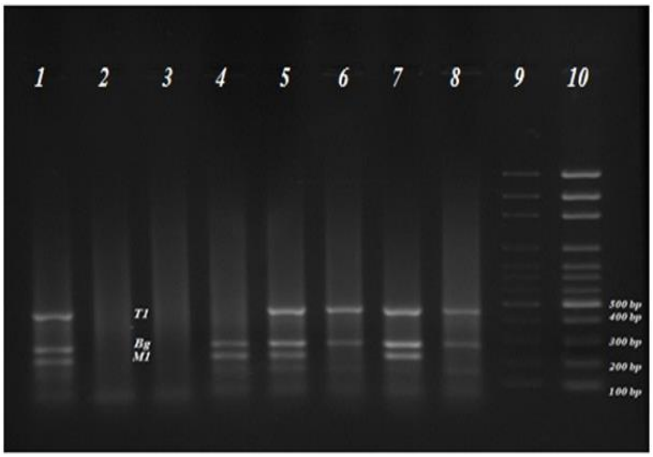

Figure 3. Electrophoresis gel of the final product of Multiplex PCR in which GSTT1 and GSTM1 bands were visible in regions of 450 and 219, respectively. The samples loaded in the wells 9 and 10 contained marker. Samples in wells 1, 5 and 7 contained all three genes, while the sample in well 4 contained only GSTM1.

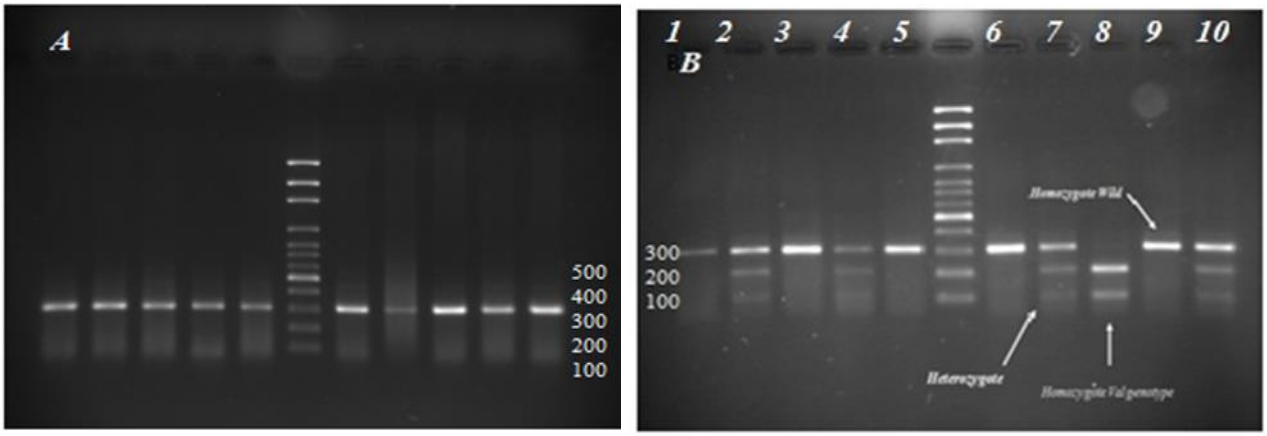

Figure 4. Part A: The results of the gel electrophoresis of the samples after performing the PCR; Part B: The bands obtained from the same samples after digestion by the enzyme Alw26I

Table 3. Frequency distribution of GSTT1 and GSTM1 polymorphisms and its relation with gender of individuals in both groups.

	GSTM1			GSTT1		
	Mutant N(%)	Wild N(%)	OR within groups (P-value)	Mutant N(%)	Wild N(%)	OR within groups (P-value)
Control						
Male	22(50)	14(46.6)	1.14(0.079)	26(48.61)	10(50)	0.9
Female	22(50)	16(53.4)		28(51.8)	10(50)	(0.884)
MS						
Male	30(83.3)	28(84.4)	0.89(0.864)	18(94.7)	40(80)	4.5 (0.135)
Female	6(16.6)	5(15.1)		1(5.3)	10(20)	
Between groups OR	5	2.2		19.3	4	
(P-value)	0.001 *	0.001 *		0.003 *	0.012 *	

Data were analyzed using Chi square statistical test and their odds ratio was calculated. Compared to the control group, the significance level was considered to be 0.05.

Table 4. Comparison of clinical criteria such as age and duration of disease and their relation with genotypes in studied patients

Disease details	GSTM1		GSTT1		GSTP1	
	Wild	Null	Wild	Null	Wild	Mutant
Age of onset (year)						
Min	16	10	14	10	10	14
Max	46	41	55	39	55	46
Mean±SD	29.5±8.9	23.2±8.6	27.3±9.9	22.6±9.7	25.7±10.2	26.9±10.1
P-value	0.009		0.131		0.752	
Disease duration (year) Min	1	1	1	1	1	1
Max	22	23	23	18	23	21
Mean±SD	5.6±5.8	6.4±3.9	6.3±5.2	5.4±3.8	5.9±4.8	6.4±5.1
P-value	0.246		0.172		0.534	

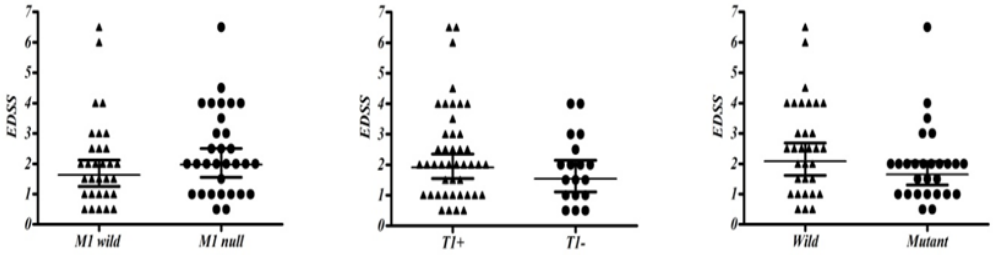

Figure 5. Comparison of patients in each group based on their genotypes and their dispersion in terms of malignancy

Discussion

This study showed that people who do not have the GSTM1 genotype develop the disease at an earlier age, or are more susceptible to the disease for any reason. However, this claim was not proved for any of the other two genotypes, GSTP1 and GSTT1.

Despite the random sampling and insignificance of age-related interference, it was observed that 30% of the patients were born in May. The division and comparison of patients based on month of birth have been considered in several studies. In a study on 6393 people in Sweden, the highest rates in the affected population were observed in the months of May and July, while the lowest rates were in March (31). Similar results were reported by Willer et al., in which 17874 people were surveyed in Canada and 11502 in the UK, and the highest rates were reported in May (32). The only compelling reason for studies that show the birth rate is higher in the spring is the short-term contact of the pregnant mother in winter with sunlight and vitamin D3 deficiency, which was also mentioned in these studies (12). In a study among 49 patients with multiple sclerosis in Greece, the absence of the GSTT1 and GSTM1 genotypes was 55.1% and 18%, respectively, while the genotypes for the control group were 56.5% and 18.9%, respectively, and these numbers were not significant. However, what was considered in this study was the ratio of 3.8 in women without GSTM1 genotype compared to men in the affected group (33). This research confirmed a study conducted in 2000 on the European population that examined this polymorphism in the Caucasian race, and in that study, the results showed lack of association between GSTT1 and GSTM1 and the disease (34). It was observed that differences in the absence of all three genotypes in women were more prevalent compared to men. Although this difference was seen in all the groups and was significant, this difference in the GSTT1 and GSTP1 genotype could be a reason for women's susceptibility to the disease, as mentioned in related articles. However, in a study by Stavropoulou et al., this difference in gender was observed for the absence of the GSTM1 genotype (33). In this study, patients without GSTM1 were diagnosed earlier than those with this genotype. A study by Živković et al. on 455 MS patients in Serbia also suggested that the lack of GSTM1 genotype is a reason for early onset of this disease, and in this study, people without GSTM1 were diagnosed at the age of 27.3 and people with this genotype were diagnosed at the age of 30.6 (35). What can empirically

be mentioned is that people without genotypes GSTM1 and GSTP1 have a higher mean age for the duration of the disease than those with these genes. However, earlier studies have not been able to show a significant correlation between polymorphism in GSTs and duration of the disease (33). In this study, EDSS (The Kurtzke Expanded Disability Status Scale) was used for comparing patients in terms of the clinical criteria, and comparison of the obtained numbers was done according to each genotype, and none of the P values were significant. By examining the obtained information, it can be concluded that those who did not have GSTM1 had more malignancy compared to those with this genotype. Perhaps the small number of samples was the reason for the lack of statistical confirmation of this observation. Evaluation of the effect of cigarette smoke on malignancy and its relation to any genotype showed that the values regarding GSTM1 genotype were significant. People without GSTM1 who were in contact with cigarette smoke had malignancy in a higher range compared to those with GSTM1, which indicates the role of this genotype in the excretion or detoxification of cigarette smoke. Although no significant correlation was found in other genotypes, it can be concluded that although there is no significant correlation between polymorphism in GSTM1, GSTT1 and GSTP1 genes in multiple sclerosis patients, it was found that we can use GSTM1 and its association with early onset of disease in people who are susceptible to the disease.

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