



Combination Effect of *GSTM1*, *GSTT1* and *GSTP1* Polymorphisms and Risk of Systemic Lupus Erythematosus

Saeedeh SALIMI^{1,2}, *Alireza NAKHAE^{1,2}, Mehdi JAFARI², Danial JAHANTIGH¹, Mahnaz SANDOOGHI³, Zahra ZAKERI³, Mahnaz SHAHRAKIPOUR^{4,5}, Anoosh NAGHAVI¹, Farzaneh FARAJIAN-MASHHADI^{1,6}

1. Cellular and Molecular Research Center, Zabedan University of Medical Sciences, Zabedan, Iran
2. Dept. of Clinical Biochemistry, School of Medicine, Zabedan University of Medical Sciences, Zabedan, Iran
3. Dept. of Internal Medicine, School of Medicine, Zabedan University of Medical Sciences, Zabedan, Iran
4. Dept. of Biostatistics and Epidemiology, School of Public Health, Zabedan University of Medical Sciences; Zabedan, Iran
5. Pregnancy Health Research Center, Zabedan University of Medical Sciences, Zabedan, Iran
6. Dept. of Pharmacology, School of Medicine, Zabedan University of Medical Sciences, Zabedan, Iran

*Corresponding Author: Email: alireza_nakhaee@yahoo.com

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Abstract

Background: Progression of systemic lupus erythematosus (SLE) could be due to oxidative stress especially through reactive oxygen species (ROS). Detoxification of ROS is largely performed by Glutathione S-transferases (GSTs), therefore polymorphisms of *GSTM1*, *GSTT1* and *GSTP1* genes which decrease enzymes activity could affect SLE susceptibility. The aim of this study was to determine the effects of *GSTM1* (deletion), *GSTT1* (deletion) and *GSTP1* (Ile105Val) polymorphisms on SLE susceptibility.

Methods: Genomic DNA was extracted from blood samples of 163 SLE patients and 180 age, sex and ethnically matched controls. *GSTs* genotypes were determined by polymerase chain reaction (PCR)-multiplex procedure or polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis.

Results: *GSTT1* null genotype frequency was higher in SLE patients than controls. NO association observed between *GSTM1* null genotype or *GSTP1* Ile105Val polymorphism with SLE. Nevertheless combination of *GSTT1* null/*GSTM1* null genotypes showed 2.8-fold increase in risk of SLE. Moreover the combination of *GSTT1* null/*GSTM1* null/*GSTP1* Ile/Val and Val/Val genotypes increased the SLE risk about 8 fold.

Conclusion: Present data suggest that *GSTT1* null/*GSTM1* null/*GSTP1* Ile/Val and Val/Val genotypes might largely contribute to the pathogenesis of SLE.

Keywords: Systemic lupus erythematosus, Glutathione S-transferase, Gene, Polymorphism

Introduction

Systemic lupus erythematosus (SLE) is a multisystemic disorder with diverse incidence and prevalence between different populations. This complication is characterized by mal-regulation of the immune system, expression of impaired T cell responses, hyperactive B cells which synthesize excessive amounts of different autoantibodies and

formation of immune complexes against various nuclear antigens. SLE could affect several organs and display a complex spectrum of clinical and immunologic manifestations such as arthritis, vasculitis and nephritis (1, 2). Although the exact pathophysiology of SLE is unclear, environmental and genetic risk factors may play an important

role in susceptibility to this multi-etiological disease (2, 3).

Environmental stimulators such as ultraviolet radiation and xenobiotic compounds have critical roles in the onset and progression of SLE (4, 5). Environmental carcinogens including smoking, air contamination and occupational exposures have strong influences on individual factors (6, 7).

There are several known enzymes involved in metabolic activation and detoxification of carcinogens including polycyclic aromatic hydrocarbons (PAH) and aromatic amines. Therefore inter-individual differences in ability to activate and detoxify carcinogens might affect the risk of developing SLE (8).

Glutathione S-transferases (GSTs) are a superfamily of dimeric phase II metabolic enzymes which are divided into four major subfamilies designated as GST α (*GSTA1*), GST μ (*GSTM1*), GST θ (*GSTT1*) and GST π (*GSTP1*). These enzymes catalyze detoxification of a wide variety of potentially toxic and carcinogenic electrophiles in human environment, by conjugating to glutathione (GSH) (8, 9).

Recently, *GSTM1*, *GSTT1* and *GSTP1* have been extensively studied for their potential modulating role in individual susceptibility to environmentally induced diseases, including cancer (10).

GSTM1 and *GSTP1* gene products could detoxify polycyclic aromatic hydrocarbons (PAHs) while *GSTT1* detoxifies smaller hydrocarbons. Moreover they might play a role in scavenging reactive oxygen species (ROS) such as superoxide radicals and hydrogen peroxide (11) and play a crucial role in protecting DNA from oxidative damage (12).

GSTM1 gene, organized in a gene cluster on chromosome 1p13.3, has been shown to be polymorphic and it is absent in 35-60% of individuals (13, 14). *GSTT1* gene, located on chromosome 22q11.2, is also polymorphic and it is absent in 10-65% of different populations (15, 16). There are two common deletion polymorphisms in *GSTM1* and *GSTT1* genes, which consequently results in virtual absence of enzyme activity, particularly in subjects with both deletions (17, 18).

GSTP1 gene product plays an important role in biotransformation and bio activation of cigarette smoke carcinogens (19). Inactivated or down-regulated *GSTP1* gene could increase genomic damage when individuals were exposed to carcinogens (20-22). A313G polymorphism (Ile105Val, rs1695) in *GSTP1* gene, located in the enzyme's active site, could alter the enzymatic activity of the protein, decrease its detoxification ability for environmental mutagens and increase DNA damage. Therefore A313G polymorphism is an important risk factor for developing different diseases (23, 24).

GSTs polymorphisms have been associated with SLE and its manifestations (25, 26), whereas other studies did not observe any relation between these variants and susceptibility to SLE (27-29). These functional polymorphisms vary by race and ethnicity (30-33).

Present research was carried out in south east of Iran, to determine the effect of *GSTM1*, *GSTT1* and *GSTP1* genes polymorphisms, alone and in combination, on susceptibility of SLE.

Materials and Methods

Study population

This case-control study conducted on one hundred sixty three SLE patients (13 males and 150 females, average age 32.6 ± 8.6 years) who were referred to Rheumatology Clinic of Ali-Ebn Abitaleb Hospital in Zahedan from 2011 to 2012. The study was approved by the Ethics Committee of Zahedan University of Medical Sciences.

The control group consisted of one hundred eighty age, sex and ethnically matched volunteers (14 males and 166 females, average age 32.1 ± 11.7 years) with negative ANA test that had no systemic disease and family relation with SLE patients. SLE patients have been diagnosed with systemic lupus erythematosus according to ACR 1998 criteria (American Rheumatology Association).

Determination of Genetic Polymorphisms

Whole blood was collected in EDTA containing tubes. Genomic DNA was isolated from periph-

eral blood leukocytes by using the commercial available kit (Roche, Germany) in accordance with the manufacturer's instructions. The extracted DNA was stored at -20°C until analyzed.

Analysis of *GSTM1* and *GSTT1* polymorphisms

Genetic polymorphism analysis for the *GSTM1* and *GSTT1* genes were conducted by the multiplex PCR (34). The β -globin gene was co-amplified as an internal positive control and *GSTM1* and *GSTT1* genotypes were not scored unless the PCR product from the internal reference gene (β -globin) was evident. PCR was performed in a total volume of $25\ \mu\text{l}$ containing $200\ \text{ng}$ genomic DNA, $25\ \text{pM}$ of each primer, $2.5\ \text{mM}$ deoxyribonucleoside triphosphates (dNTPs) (Fermentas, Lithuania), $1.5\ \text{mM}$ MgCl_2 , and $1\ \text{U}$ thermostable Taq DNA polymerase (Fermentas, Lithuania) using My Cycler Thermal cycler, BIO-RAD PCR system (BIO-RAD Co., U.S.A.). The amplification conditions were initial denaturation at 94°C for $5\ \text{min}$ followed by 35 cycles of denaturation at 94°C for $30\ \text{s}$, annealing at 63°C for $30\ \text{s}$, extension at 72°C for $1\ \text{min}$, and final extension at 72°C for $10\ \text{min}$. The amplified product was visualized in an ethidium bromide stained 2% agarose gel. The absence of amplified *GSTM1* and *GSTT1* products (in the presence of control PCR product) indicated the respective null genotypes.

Analysis of *GSTP1* Ile105val polymorphism

GSTP1 Ile105val (A313G) polymorphism was genotyped using a polymerase chain reaction (PCR) amplification refractory mutation system (ARMS). Based on the flanking region of *GSTP1* Ile105val polymorphism, following primers was designed for tetra-ARMS-PCR according to the published sequence for *GSTP1* gene obtained from GenBank: Forward outer primer $5\text{'-AG-GTTACGTAGTTTGGCCCAAGGTC-3'}$, Reverse outer primer $5\text{'-CGTACTTGGCTGGTTGATGTCC-3'}$, Forward inner primer $5\text{'-GAGGACCTCCGCTGCAAATTCG-3'}$ and Reverse inner primer $5\text{'-CATAGTTGGTGTAGATGAGGGAGCT-3'}$. PCR reaction mixture ($25\ \mu\text{l}$) contained $5\ \mu\text{M}$ of each primer, $200\ \text{ng}$ of genomic DNA, $1.5\ \text{mM}$

MgCl_2 , $2.5\ \text{mM}$ each dNTPs (Fermentas, Lithuania) and $1\ \text{U}$ Taq polymerase (Fermentas, Lithuania). Amplification was performed with an initial denaturation at 94°C for $5\ \text{min}$ followed by 35 cycles at 94°C for $30\ \text{s}$, 64°C for $30\ \text{s}$, and 72°C for $1\ \text{min}$. After a final extension at 72°C for $10\ \text{minutes}$, PCR products were separated on 2% agarose gel and stained with ethidium bromide. The primer design for this polymorphism amplify a control product of 563 base pairs (bp), while the PCR products of $360\ \text{bp}$ and $260\ \text{bp}$ would identify Ile and Val variants, respectively (Fig. 1).

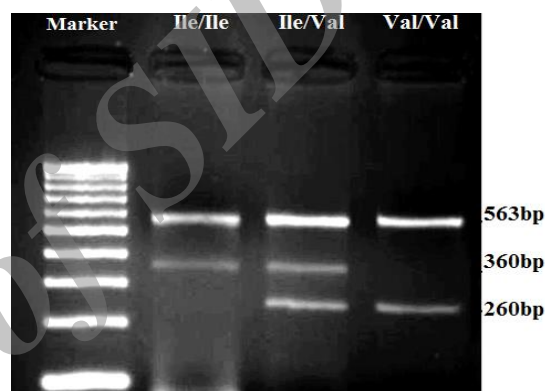


Fig. 1: Electrophoresis pattern of tetra-ARMS-PCR for detection of *GSTP1* Ile105val polymorphism on Agarose gel

All of the samples were analyzed by PCR restriction fragment length polymorphism (PCR-RFLP) using $10\ \text{U}$ *ALW 261* restriction enzyme (Fermentas, Lithuania) (35) for quality control.

Statistical analysis

All statistical analysis was performed with SPSS V.15. Demographic differences between groups were examined by χ^2 test or an independent Student's *t*-test whenever appropriate. Allele frequencies were estimated by the gene counting method. Frequencies of the alleles and genotypes were compared between patients and control groups by the χ^2 test or Fisher's exact test. The odds ratio (OR) and 95% confidence intervals (CI) were also estimated by Binary Logistic regression. The χ^2 test was used for deviation of genotype distribution from Hardy-Weinberg equilibrium. All statis-

tical tests were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Demographic characteristics of SLE patients and control group are showed in Table 1. This study included 163 SLE patients and 180 age, gender -

and ethnicity matched control subjects. Dermomucous manifestations developed in 83% of patients during the course of their disease. Arthritis was found in 87%, whereas neuropsychiatric manifestations were found in 14% of SLE patients. Lupus nephritis was advanced with raised serum creatinine in 22% of patients.

Table 1: Demographic characteristics of SLE patients and controls

Parameter	SLE n=163	Controls n=180	P-Value	χ^2
Age (yr)	32.6±8.6	32.1±11.7	0.68	0.04
Sex (male/female)	13/150	14/166	0.6	0.04
Race n (%)			0.36	0.27
Persian	82 (50)	86 (48)		
Balouch	81 (50)	94 (52)		

GSTM1 and *GSTT1* gene polymorphisms

The frequency of *GSTM1* null genotype was 53.4% and 44% in SLE patients and controls respectively, which was not statistically significant (OR, 1.4 [95% CI, 1 to 2.2]; $P=0.095$). Moreover the frequency of *GSTT1* null genotype was significantly higher in SLE patients (25.2%) compared to healthy controls (15.1%) and risk of SLE was 1.9 fold higher in individuals with null mutation of *GSTT1* after adjusting for age, gender and ethnicity (OR = 1.9, 95% CI = 1.1 to 3.2, $P = 0.02$) (Table 2).

Analysis of the combined effects of *GSTM1*, *GSTT1* null genotypes on risk of SLE after adjust-

ing for age, gender and ethnicity revealed that the null/null genotype for *GSTT1* and *GSTM1* polymorphisms increase 2.8 fold the risk of SLE compared to present/present genotype (OR, 2.8 [95% CI, 1.2 to 6.4]; $P=0.014$) (Table 2).

GSTP1 gene Ile105Val polymorphism

The frequency of Ile105Val polymorphism genotypes and alleles has shown in Table 3. No significant differences in genotypes and alleles frequency of Ile105Val polymorphism were found between SLE patients and healthy controls.

Table 2: Association of *GSTM1* and *GSTT1* polymorphisms and SLE risk

Polymorphism	SLE n=163	control n=180	P-value	Adjusted OR* (95% CI)
<i>GSTM1</i>				
Present, n (%)	76 (46.6)	100 (56)		1
Null, n (%)	87 (53.4)	79 (44)	0.095	1.4 (1 – 2.2)
<i>GSTT1</i>				
Present, n (%)	122 (74.8)	152 (84.9)		1
Null, n (%)	41 (25.2)	27 (15.1)	0.02	1.9 (1.1-3.2)
<i>GSTM1/GSTT1</i>				
Present/present, n (%)	54 (33.1)	81 (45.3)		
Null/ Null, n (%)	20 (12.3)	11 (6.1)	0.014	2.8 (1.2 - 6.4)

OR = odds ratio; CI = confidence interval./ *Adjusted OR for age, ethnicity and gender

Table 3: Association of *GSTP1* Ile105Val polymorphism and SLE risk

	SLE N=163	Control N=180	P-value	Adjusted OR (95% CI)
<i>GSTP1</i> Genotype				
Ile/Ile, n (%)	88 (54)	112 (62.2)		1
Ile/Val, n (%)	69 (42.3)	60 (33.3)	0.09	1.5 (0.9 – 2.3)
Val/Val, n (%)	6 (3.7)	8 (4.8)	0.95	1 (0.3-2.9)
Allele				
Ile	245 (75)	284 (79)		1
Val	81 (25)	76 (21)	0.3	1.2 (0.9-1.8)

OR = odds ratio; CI = confidence interval.

*Adjusted OR for age, ethnicity and gender

Combination effect of *GST*'s genotypes

In order to assess the existence of any interaction between the polymorphisms of *GSTM1*, *GSTT1* and *GSTP1* genes, we calculated the frequencies of the simultaneous presence of the putative 'high-risk' genotypes. Individuals carrying all three presumptive low-risk genotypes, *GSTM1* and *GSTT1* non-deleted (present) and *GSTP1* Ile/Ile genotypes were used as the reference group. Table 4 shows the results of the association between combined genotypes and SLE. A highly significant

difference in the combination of *GSTM1*, *GSTT1* null mutation with *GSTP1* Ile/Val, Val/Val genotypes were found between the SLE patients (8%) and controls (1.6%), with 8 fold increase in risk of SLE [OR of 8.1, (95% CI= 2.1 to 30.6, $P=0.003$)], while other combination genotypes did not show significant association with risk of SLE (Table 4). There were no significant differences in *GST*'s polymorphisms between different ethnic groups. Moreover no association was found between *GST*'s genotypes and SLE manifestations.

Table 4: The combination effect of *GST*'s polymorphisms and Risk of SLE

<i>GST</i> 's polymorphisms			Lupus N=163 n (%)	Control N=180 n (%)	P-value	Adjusted OR(95%CI)*
<i>GSTT1</i>	<i>GSTM1</i>	<i>GSTP1</i>				
Present	Present	Ile/Ile	27 (17)	50 (15)	-	1
Null	Present	Ile/Ile	12 (7.4)	9 (5)	0.08	2.5 (0.9-6.5)
Present	Null	Ile/Ile	42 (26)	44 (24.4)	0.09	1.7 (0.9-3.3)
Null	Null	Ile/Ile	7 (4.3)	8 (4.4)	0.35	1.7 (0.6-5.5)
Null	Present	Ile /Val, Val/Val	8 (5)	7 (3.9)	0.16	2.2 (0.7-6.9)
Present	Null	Ile /Val, Val/Val	26 (16)	24 (13.3)	0.05	2.1 (1-4.5)
Null	Null	Ile /Val, Val/Val	13 (8)	3 (1.6)	0.003	8.1 (2.1- 31.6)

*Adjusted OR for age, ethnicity and sex

Discussion

The present study was conducted to investigate the relation between the *GSTT1*, *GSTM1* and *GSTP1* polymorphisms and SLE susceptibility in

an Iranian population. The contribution of the *GST* supergene family to oxidative stress resistance is well established (19), therefore the absence of *GST* enzymes could increase ROS-mediated damage. Since the lack of detoxification

might be a risk factor for SLE development, analysis of *GSTs* genes status, particularly detection of *GSTM1* and *GSTT1* null mutation, and *GSTP1* polymorphisms could have prognostic and pathologic importance. *GSTT1*, *GSTM1* and *GSTP1* genes are three major phase II xenobiotic biotransforming enzymes that are known to be involved in the metabolic activation and generation of ROS. Although a few studies have been performed on the relation between *GSTs* polymorphisms and SLE, the association between these polymorphisms and SLE has not been clearly demonstrated (25-28). Furthermore a few studies showed a relation between *GSTs* polymorphisms and clinical manifestations of SLE, but not with SLE susceptibility (29-32).

In the current study, a significant difference in the *GSTT1* null genotype frequency was observed between SLE patients and control group. However no association observed between *GSTM1* null genotype and *GSTP1* Ile105Val polymorphism with SLE. Moreover combination of *GSTT1* null/*GSTM1* null genotypes showed 2.8-fold increase in SLE risk and the combination of *GSTT1* null/*GSTM1* null/*GSTP1* Ile/Val and Val/Val genotypes increased the SLE risk about 8.1-fold. Our data strongly suggest that *GSTT1* null/*GSTM1* null/*GSTP1* Ile/Val and Val/Val genotypes might substantially contribute to the pathogenesis of SLE.

Similarly Kang et al. in Korea observed no association between *GSTM1* and *GSTP1* genotypes and risk of SLE (25), however in present study, a relation between *GSTT1* null genotype and SLE was found. Moreover Kang et al. observed an association between *GSTM1* null genotype and lower frequency of hematological disorders, as well as lower frequency of discoid rash and nephritis in individuals with *GSTT1* null genotype (25). Kiohara et al. observed increased risk of SLE in smokers with combined *CYP1A1* rs4646903/*GSTM1* null polymorphisms. (OR 17.5, 95% CI 3.20-95.9) (26).

Moreover Fraser et al. in United States reported no relation between *GSTs* polymorphisms and SLE, but they observed 3 fold increase in risk of SLE in individuals with ≥ 24 months' occupation-

al sun exposure and *GSTM1* null genotype in Caucasians (27).

In contrast to our results, Zhang et.al, in China observed an association between *GSTM1* null ($P = 0.003$, OR 1.66 [95% CI 1.19-2.32]), but not *GSTT1* null ($P = 0.119$, OR 0.77 [95% CI 0.56-1.07]) genotypes and SLE. Also the analysis of genes in combination for double-null deletion of both *GSTT1* and *GSTM1* showed no significant difference ($P = 0.863$, OR 1.03 [95% CI 0.70-1.52]) (28).

In present study there was no significant difference in the frequency of the *GSTM1* null genotype between patients and controls (55% versus 47%) which is in agreement with previously published studies(25, 27). However current data was in contrast with Zhang et. al. study in Chinese population (28).

There were limited studies which examined the *GSTM1* null genotype in combination with *CYP1A1* or *TNF receptor type II (TNF-RII)* genotypes and SLE susceptibility (29). *GSTM1* null genotype was not associated with SLE risk, nevertheless combination of *CYP1A1* 3801C/*GSTM1* null polymorphisms was strongly associated with an increased risk of SLE in Japanese (OR = 4.35; 95% CI = 1.76, 10.73) (29). Moreover the authors analyzed the combined effect of *TNF-RII* 196M/*CYP1A1* 3801C/*GSTM1* null genotype and observed significant association with SLE (OR = 5.83; 95% CI = 2, 17.04) (29).

There was no previously published report about the association between combined *GSTs* genotypes and SLE. In current investigation we concluded that the combination of *GSTM1* and *GSTT1* null mutation with *GSTP1* Val/Ile, Ile/Ile genotypes increased the risk of SLE about 8 fold. However, combined studies including various genetic polymorphisms in various genes/enzymes are required to clarify genetic etiology of SLE.

There were some limitations in present study, including low sample size, environmental conditions and different ethnic groups (Fars and Balouch) existing in South East of Iran. Therefore further investigations using a larger sample size and different ethnic groups are necessary to confirm the present results.

Conclusion

GSTT1 null genotype alone and in combination with *GSTM1* and also combination of *GSTM1*, *GSTT1* null mutation with *GSTP1* Val/Ile, Ile/Ile genotype could have a significant relation with SLE and might be associated with increased risk in Iranian patients.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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