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Association of *GSTT1, GSTM1* and SNP rs1695 in *GSTP1* with the Incidence of Rheumatoid Arthritis in Southern Punjab, Pakistan: A Case-Control Study

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FI and MF designed and supervised this study. MI, HAF and RM collected blood and risk factor data from the subjects. MI, HAF and MA extracted DNA from blood samples and performed PCRs. AA and MBS analyzed the data. FI, MF and MBS edited the manuscript and finalized it. All these authors approved the final version of the manuscript.

Key words
Rheumatoid arthritis, *GSTT1, GSTM1*, rs1695, *GSTP1*

ABSTRACT

Rheumatoid arthritis (RA) is one of the most common autoimmune diseases in the world causing painful and swollen joints. RA is a progressive disease that can lead to various physical disabilities. The current study aimed to assess the association of the presence/absence of *GSTM1*, *GSTT1* and single nucleotide polymorphism [SNP] (rs1695) in *GSTP1 gene with RA*. A cross-sectional study was conducted at Institute of Zoology, Bahauddin Zakariya University Multan from May 2020 to March 2021 and included *RA* patients (cases; N = 100) and controls (N = 100) enrolled from Multan district in Pakistan. A T-ARMS PCR protocol was applied to report the genotype at studied GSTs. The association of rs1695 in *GSTP1* with RA was studied either individually or in various combinations with *GATM1* and *T1*. A significant association of absence of *GSTT1* and heterozygous genotype (AG) at rs1695 in *GSTP1* was found to be associated with RA in the present study. Subjects whose age varied between 41 and 55 years and women suffered more from RA. It is concluded that polymorphisms in GSTs may disturb the protection provided by them against oxidative stress which may influence disease progression in RA.

INTRODUCTION

Rheumatoid arthritis (RA) is reported in all parts of the world and is a chronic autoimmune disorder characterized by polyarticular inflammation, increased cytokine production and development of pannus. In advance stages of RA, erosion of underlying cartilage and bone occurs (Derek *et al*., 2002). It has been documented that during RA development, leukocytes likes neutrophils, macrophages and monocytes present in the synovial fluid of inflamed joints generate reactive

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oxygen species (ROS) which leads to damage to DNA and lipid oxidation which lead to the cartilage and bone destruction resulting in the disease state (Sheehan *et al*., 2001). Thus, genetic differences in the ability to detoxify ROS and their byproducts may influence the risk and intensity of RA (Yun *et al*., 2005). Cells has a complex defense mechanism against ROS that involves several enzymes including glutathione S-transferase (GST) which is a family of iso enzymes and catalyzes the conjugation of reduced glutathiones (GSH) to a number of electrophilic compounds which are part of the detoxification pathways (Grabar *et al*., 2009). Disturbed GST and GSH activity can disturb cellular metabolism and increases the chances for ROS disturbing normal gene expression, leading to a number of diseases including RA (Jamil *et al*., 2022).

The glutathione S-transferase mu (*GSTM1*) gene is located on chromosome 1p13.3 and this gene produces an enzyme responsible for the detoxification of metabolites of environmental carcinogens including tobacco smoke (Moghimi *et al*., 2019). Individuals that are homozygous *GSTM1*-null lack an 8-kb fragment of genomic DNA and these individuals have no glutathione transferase activity against the substrate trans-stilbene oxide in their blood and no *GSTM1*a or *GSTM1*b expression in their liver (Song *et al*., 2012). The glutathione S-transferase theta (*GSTT1*) gene has remained highly conserved through evolution (positioned at 1p13.3) and it is implicated in the detoxification of a large number of industrial products and drugs, including hydrocarbons and cytostatic drugs (Bolt and Their, 2006). The glutathione S-transferase pi (*GSTP1*) gene (positioned at 11q13) is well known for its protective role which it plays through the enzyme mediated conjugation of GSH with reactive electrophiles and it also protects the cell through its redox reversible GSH reactions which reduces the organic peroxides (Mian *et al*., 2016).

Rheumatology is a developing field in Pakistan and so far only a few hospitals have established rheumatology clinics with qualified rheumatologists. National registries also do not exist to account the various autoimmune diseases existing in this country with their treatment outcomes (Khaliq *et al*., 2020). The *GST* genotype has been associated to a number of diseases, including RA in numerous investigations (Grabar *et al*., 2009; Song *et al*., 2012), however from Pakistan, to our knowledge, there is no information available in literature regarding the association of the presence or absence of *GSTM1* and/or *GSTM1* and genotypes at rs1695 in the *GSTP1* gene with the incidence of Rheumatoid Arthritis. Hence, the current investigation aimed to address this knowledge gap among subjects enrolled from Multan and Dera Ghazi Khan Districts in Punjab, Pakistan.

MATERIALS AND METHODS

Subjects and data collection

The cross-sectional study was conducted at the Institute of Zoology from May 2020 to March 2021 and included clinically confirmed RA cases and controls with no history of RA. Following their informed consent, blood samples from the patients (N= 100) were collected at Orthopedic Unit 1 of Nishter Medical University and hospital Multan (Pakistan) and District Headquarter Hospital (DHQ) Dera Ghazi Khan. The subjects enrolled in the present investigation came from different cities of Southern Punjab, included both males and females of different age groups and had diverse cultural backgrounds. All patients were diagnosed based on their rheumatoid factor (RF) and C-reactive protein level (CRP) and anti-citrullinated peptide antibody (anti-CCP) measurements. RF and CRP values of 10 IU/ml and 0.3mg/dl, respectively, were considered normal while more than 5 U/ml of anti-CCP were considered positive case of RA (Intriago *et al*., 2019). The controls had no systemic illness, they did not suffering from RA and their age was matched to the cases. Solvin's formula was used to estimate the sample size;

$n = N / (1+N * e^2)$

In the equation above, n is the number of samples, N is the total population of the area and the margin of error is represented by e. Prior the blood sampling, the case as well as controls were interviewed by using a questionnaire to collect epidemiological data including name, gender, age, family history, marital status, smoking and the exercise habits of each subject (Jamil *et al*., 2022).

Blood collection and DNA extraction

A 3-5 ml blood sample from each subject was taken into vials containing EDTA as an anticoagulant and used for DNA extraction by using the inorganic DNA extraction protocol (Jamil *et al*., 2022).

Amplification and genotyping of GSTT1 *and* GSTM1

To document the presence or absence of *GSTT1* and *GSTM1* genes in enrolled subjects, a multiplex PCR approach was applied as previously reported (Rohr *et al*., 2008). As an internal control, as both *GSTT1* and GSTM1 can be absent in subjects, Cytochrome P450, family 1, subfamily A, polypeptide 1 (*CYP1A1*) gene (exon 7) was amplified as an internal control. The primers used for the amplification of these genes were: GSTM1 reverse 5′-GTTGGGCTCAAATATACGGTGG-3', GSTM1 Forward 5′-GAACTCCCTGAAAAGCTAAAGC-3', GSTT1reverse primer 5'-TCACGGGATCATGGCCAGCA-3', GSTT1 forward primer 5'-TTCCTTACTGGTCCTCACATCTC-3', CYP1A1 reverse 5'-CAGCTGCATTTG-

GAAGTGCTC-3' and CYP1A1 forward 5'-GAACTGC-CACTTCAGCTGTCT-3' (Rohr *et al*., 2008). A master mixture (50µl) was prepared for the PCR which contained 5 µl of PCR buffer, 2 µl of each primer (12 Pm), 3.5µl of $MgCl_2$ (25 mM), 2µl of dNTPs (2 mM), 1 µl of DNA polymerase (Thermo Scientific, USA) and 5 µl of template DNA. The thermal profile for the amplification of *GSTT1*, *GSTM1* and *CYP1A1* included an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 59 °C for 1 min and elongation at 72°C for 1 min and a final extension at 72°C for 10 min (Rohr *et al*., 2008).

Tetra ARMS PCR based amplification of rs1695 in GSTP1

A T-ARMS–PCR protocol was performed to genotype rs1695 in exon 5 of *GSTP1* (A/G; Ileu 105 Val) as previously reported by Ji and Lee (2013). The primers used in this T-ARMS PCR were outer reverse 5'-ATAA-GGGTGCAGGTTGTGTCTTGTCCCA-3', outer forward 5'-CAGGTGTCAGGTGAGCTCTGAGCACC-3', inner reverse 5'-GCTCACATAGTTGGTGTAGATGAGG-GATAC-3' and inner forward 5'-CGTGGAGGACCTC-CGCTGCAAATCCA-3'. A reaction mixture (25µl) was prepared for the amplification of rs1695 composed of 5 µl of PCR buffer, 2 µl of each primer (15 Pm), 2 µl of $MgCl_2$ (25 mM), 2 µl of dNTPs (2mM), 1µl of Taq DNA polymerase (Thermo Scientific, USA) and 3 µl of template DNA. The thermal profile for this PCR was initial denaturation at 95°C for 5 min followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 30 s, elongation at 72°C for 30 s and final extension at 72°C for 7 min (Ji and Lee, 2013).

Statistical analysis

Data were analyzed using Minitab version 17 (Mini Tab, USA). Direct counting was applied for counting genotypic and allelic frequencies. To compare the genotype and allelic frequencies between case and controls and also to report the correlation between RA and the studied risk factors, Chi-square test was applied.

RESULTS

Association GSTM1 *and* GSTT1 *with RA*

Multiplex PCR amplified 480 bp, 312 bp and 215 bp products of *GSTT1*, *CYP1A1* and *GSTM1*, respectively. Chi-square test result indicated that the *GSTM1* had no association with RA incidence (P = 0.5) (Table I). On analyzing *GSTT1* gene among enrolled cases and controls, Chi-square test revealed a significant correlation (P = 0.009). It was observed that the majority of cases were *GSTT1* null (Table I).

Table I. Genotype and allelic frequency distribution among cases and controls for *GSTM1* and *T1* and their possible association with Rheumatoid Arthritis.

Genotype	Control	Case	Chi-Square	P-value
GSTT1				
Present	8	21	6.816	0.009**
Null	92	79		
GSTM1				
Present	43	48	0.5	0.5
Null	57	52		

P > 0.05 = Non significant (**); P < 0.01 = Significant (**).

Genotypic and allelic frequency at rs1695 in GSTP1 and their association with RA

T-ARMS PCR generated a 467 bp for the outer primers. Whereas, the allele-specific primers generated a 233 bp product for homozygous wild (AA) and a 290 bp fragment for homozygous mutant (GG) genotypes. Data analysis revealed that the genotypic frequency varied significantly (Chi square test; P < 0.001) at rs1695 in *GSTP1* compared to control and cases. It was observed that wild (AA) genotype was more frequent in controls while the heterozygous (AG) genotype was more frequent in RA cases (Table II). The Chi-square test revealed that the allelic frequency also varied significantly (P < 0.001) compared to healthy control and RA patients with a more frequent wild-type (A) allele in controls and a higher frequency of mutant (G) allele in the case (Table II).

Table II. Genotype and allelic frequency distribution at single nucleotide polymorphism rs1695 in *GSTP1* gene among case and controls enrolled during present study, and their possible association with Rheumatoid Arthritis. Chi-square test was applied to compare the genotype and allelic frequency between case and controls.

Parameters	Genotypic frequency			P-value	Allelic frequency		P-value
	AA	AG	GG		A	G	
Controls	91(91%)	8(8%)	1(1%)	P < 0.0001***	190(95%)	10(5%)	P < 0.0001***
Case	25(25%)	63(63%)	12(12%)		113(56%)	87(43%)	

P < 0.001 = Highly significant (***).

GSTs interactions and their association with RA

Genotype frequency analysis indicated that certain genotypes' combination with two or more GSTs significantly increased the risk of developing RA. All significantly higher genotype combinations among controls were considered protected combinations, while those with higher frequencies among RA cases were the genetic risks to develop the RA (Tables III). Our results indicated that subjects with both *GSTMI* and *T1* present (P = 0.03), subjects having either *GSTM1* present or absent with a heterozygous (AG) genotype at *GSTP1* (P < 0.001), and subjects having either *GSTT1* present or absent with heterozygous (AG) genotype at *GSTP1* (P < 0.001) were more susceptible to RA (Tables III). The same trend was observed when the combinations of three studied SNPs were analyzed. It was observed that subjects having either both *GSTM1* and *GSTT1* present or both *GSTM1* and *GSTT1* absent but having a heterozygous (AG) genotype at *GSTP1* were significantly more susceptible to develop RA (P < 0.001) (Tables III).

Table III. Genotype and allelic frequency distribution among cases and controls for combinations of GSTs and their possible association with Rheumatoid Arthritis.

Genotype	Control	Case (%±C.I.[1])	Chi-Square	P value
GSTM1* and *GSTT1				
Both present	3	13 (81.2±0.19)	6.962	0.031*
Either Null	45	43 (48.9±0.10)		
Both Null	52	44 (45.8±0.09)		
GSTM1* and *GSTP1				
M1(+/+) and P1(AA)	39	12 (23.5±0.11)	86.630	P < 0.0001***
M1(+/+) and P1(AG)	4	31 (88.6±0.10)		
M1(+/+) and P1(GG)	1	5 (83.3±0.29)		
M1(-/-) and P1(AA)	51	13 (20.3±0.09)		
M1(-/-) and P1(AG)	4	32 (88.9±0.10)		
M1(-/-) and P1(GG)	1	7 (87.5±0.22)		
GSTT1* and *GSTP1				
T1(+/+) and P1(AA)	8	4 (33.3±0.26)	91.230	P < 0.0001***
T1(+/+) and P1(AG)	0	15 (100)		
T1(+/+) and P1(GG)	0	2 (100)		
T1(-/-) and P1(AA)	83	21 (20.2±0.07)		
T1(-/-) and P1(AG)	8	48 (85.7±0.09)		
T1(-/-) and P1(GG)	1	10 (90.9±0.17)		
GSTT1*, *GSTM1* and *GSTP1				
T1 and M1(+/+) and P1 (AA)	3	2 (40±0.42)	96.965	P < 0.0001***
T1 and M1(+/+) and P1 (AG)	0	10 (100)		
T1 and M1(+/+) and P1 (GG)	0	1 (100)		
T1(+/+), M1(-/-) and P1 (AA)	5	2 (28.6±0.33)		
T1(+/+), M1(-/-) and P1 (AG)	0	5 (100)		
T1(+/+), M1(-/-) and P1 (GG)	0	1 (100)		
T1(-/-),M1(+/+) and P1 (AA)	35	10 (22.2±0.12)		
T1(-/-), M1(+/+) and P1 (AG)	4	21 (84±0.14)		
T1(-/-), M1(+/+) and P1 (GG)	1	4 (80±0.35)		
T1(-/-), M1(-/-) and P1 (AA)	48	11 (18.6±0.09)		
T1(-/-), M1(-/-) and P1 (AG)	4	27 (87.1±0.11)		
T1(-/-), M1(-/-) and P1 (GG)	0	6 (100)		

[1]: C.I.: 95% confidence interval. P > 0.05 = non-significant; P < 0.05 (*) = Least significant; P < 0.001(***) = Highly significant. +/+ represents that two copies specific gene present. -/- represents that two copies specific gene present. *GSTP1*, Glutathione S-transferase pi; *GSTM1*, Glutathione S-transferase mu; *GSTT1, G*lutathione S-transferase theta.

Association of risk factor and RA

Chi-square test results revealed that age ($P < 0.0001$) and gender ($P < 0.0001$) were associated with the incidence of RA. Subjects aged 41 to 55 years and women were more susceptible to RA (Table IV).

Table IV. Analysis of the studied demographic factors and their association with the incidence of rheumatoid arthritis. P-value indicates the results of chi square test calculated for each parameter.

Category	Control (N = 100)	Case (N = 100)	P-value
Age (Years)			
10-25	1	14	P < 0.0001***
26-40	25	36	
41-55	60	34	
56-70	13	16	
Above 71	1	0	
Gender			
Male=1	61	34	P < 0.0001***
Female=0	39	66	

P < 0.001 = Highly significant (***).

DISCUSSION

RA is a long-term inflammatory autoimmune illness designated by a remarkable deterioration of cartilage and bone that has affected millions of people around the world (Mueller *et al*., 2021). Inflammation in RA patients leads to increased formation of ROS, which produce oxidative destruction to biological components such as DNA and lipids [4]. During the development of RA, both environment and genetics play their roles (Derek *et al*., 2002; Hashemi *et al*., 2012). GST is known to detoxify products generated in a cell due to the ROS activity (Ghelani *et al*., 2011), however no information is available in the literature regarding the role of GSTs genotypes in development of RA in the Pakistani population. Therefore, the present study was designed to report the correlation between three GSTs genotypes (*GSTT1*, *M1* and *P1*) and the incidence of RA in subjects tested from Southern Punjab, Pakistan, if any.

Although few studies have previously shown the association of *GSTM1* null genotype with RA, however, during this study, no association was observed between the presence and absence *GSTM1* with RA (Table I). This was probably due to the small number of subjects enrolled in the study and a larger number of patients would be needed to confirm whether the *GSTM1* null genotype is associated with disease susceptibility in Pakistani patients with RA. Our results are in line with those of Song *et al*. (2012) and Lundstrom *et al*. (2011) as they observed no association *GSTM1* with RA in subjects enrolled from Asia and Swedish EIRA, respectively. Contrary to our observation, Derek *et al*. (2002) observed that patients who lacked the *GSTM1* gene and who were smokers had a significantly higher incidence of RA than those who had the *GSTM1* gene and had never smoked. Hashemi *et al*. (2012) and Morinobu *et al*. (2006) had also reported a significantly higher frequency of *GSTM1* null genotype in RA patients than in controls enrolled from two different ethnic groups: Iranian and Japanese populations, respectively. The differences observed between these studies are due to the different number of subjects enrolled in these studies and to the genotypic variation of the populations as the subjects enrolled in these studies belonged to different ethnic origin.

A significant association was observed between the absence of *GSTT1* and RA during the present study (Table I). Our results are in agreement with Keenan *et al*. (2010), Grabar *et al*. (2009), Rohr *et al*. (2008) and Berhane *et al*. (1994) as they had also reported that *GSTT1* null subjects were more prone to develop RA than patients having *GSTT1* gene. The subjects enrolled in these studies haled from Caucasian, Slovenian, Korea and Serbian populations, respectively. Contrary to these observations, Hashemi *et al*. (2012) reported no association of GSTT1 genotypes with RA incidence in the Iranian population, indicating ethnicity-specific differences.

During the present study, we observed a significant association of rs1695 in *GSTP1* with RA with heterozygous (AG) genotype and G allele more common in cases (Table II). These results are interesting as SNPs in GSTP1 have not been investigated in detail as a potential causative agent for RA worldwide, despite the fact that the GSTP1 protein is widely expressed in a number of human cells, including lymphocytes and synovial fibroblasts, which have an important role to play during the RA development (Mattey *et al*., 1999). This SNP, rs1695, in *GSTP1* is also a good candidate to be screened in RA subjects because it is already established that the disturbed *GSTP1* activity makes a cell vulnerable to oxidative stress and DNA damage (Cote *et al*., 2009). This SNP has been studied several times in relation to RA and our results are contrary to those of Hashemi *et al*. (2012), Keenan *et al*. (2010), Grabar *et al*. (2009) and Morinobu *et al*. (2006) as they had reported no significant association of rs1695 in *GSTP1* with the occurrence of RA in Iranian, Caucasian and Japanese populations, respectively.

Results from studies of individual SNP are inconsistent as each individual SNP alters the function of a single gene among the many that are involved in the progression of RA. However, disease progression can

be due to the interaction of several proteins. Hence, a single SNP usually has a modest effect, however the same biological events are more greatly affected by different SNPs in different genes and how these SNP combinations interact. Keeping this observation in view, we performed SNP-SNP interaction analysis and tried to examine various possible SNP combinations from the GSTs that were screened in the present study to identify the combination of SNPs that are most likely to be associated with the risk to develop RA. Interestingly, the presence of both *GSTM1* and *GSTT1* was found to be a risk factor for the onset of RA, while, during the genotyping of single GST at a time, only the absence of *GSTT1* was found associated with RA (Table I). These observations support our hypothesis that it is the combination of genotypes with one or more SNPs that plays an important role in health and disease. Our results indicated that it is the genotype at rs1695 in *GSTP1* that plays an important role in the RA development, as regardless of the genotype at *GSTM1* and *GSTT1*, it is the heterozygous (AG) genotype at *GSTP1* that was always linked to RA, making it an interesting candidate SNP to consider for analysis in RA related studies in various populations (Table IV).

Analysis of risk factors our study indicated that subjects in the age group 41-55 years and women were more susceptible to RA (Table IV). These observations are consistent with Pakistan's earlier report, as Soomro *et al*. (2019) had reported that more women than men suffered from RA among subjects enrolled from Larkana district in Sindh and they reported that the mean age of subjects was 59 years at onset of RA. Our results are in line with Ji and Lee (2013) as they reported studying that adults, smokers and obese subjects had a higher susceptibility to the RA incidence in subjects enrolled from East Asia. Our results are in agreement with Kvein *et al*. (2006) as they reported a higher prevalence of RA women than men. They observed that the incidence was 4-5 times higher in women under 50, but above 60–70 years, the female/male ratio was only about 2. It was observed that gender also influences drug response during RA treatment as Kvein *et al*. (2006) reported that gender influenced response to methotrexate and anti-tumor necrosis factor treatment and the drug response was 30 to 50% lower in females.

The small number of samples is a limitation of the present study as a larger the sample size gives a higher power of the observations and obtained results. The genes and SNPs, we analyzed in relation to RA in this study, were limited given that there are several other genes and SNPs have been reported to be associated with RA in different populations. There is a need to explore more genes and SNPs related to RA to accurately assess the susceptibility of these genes for this disease in the Pakistani population. However, the present study is valuable as it is the pioneering study from Pakistan that reports a genotype at three GSTs with reference to RA that has never been explored in the local population.

CONCLUSION

We report here that the genotype of the studied GSTs may influence the disease process in RA. We show a significant association of the absence of *GSTT1* and heterozygous (AG) genotype at rs1695 in *GSTP1* with RA in enrolled subjects. Subjects aged 41 to 55 years and women were more susceptible to RA. We recommend similar large-scale studies in the Pakistani population to establish the role of *GSTM1* in the onset of RA.

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DICLARATIONS

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IRB approval

Ethical Research Committee of the Institute of Pure and Applied Biology (Pakistan) approved all experimental procedures and protocols applied in this study through letter number Zool/Ethics/41/2020.

Ethics approval

Ethical Research Committee of the Institute of Pure and Applied Biology (Pakistan) approved all experimental procedures and protocols applied in this study through letter number Zool/Ethics/41/2020.

Consent to participate

Informed consent was obtained from all subjects before including them in this study.

Data availability statement

All data generated during this study are presented in the manuscript.

Permission to reproduce material from other sources

Not applicable.

Statement of conflicts of interest

The authors have declared no conflict of interest.

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