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Role of Glutathione S Transferase Polymorphism in the Pathogenesis of Cardiovascular Diseases: A Case Control Study

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ABSTRACT

Cardiovascular diseases (CVDs) are major health problems all over the world. Oxidative stress contributes an important pathological role in the development of CVDs. To counter this oxidative stress, the most important natural antioxidant defense mechanisms include endogenous glutathione concentration, superoxide dismutase, catalase, and glutathione S-transferase (GST). GST neutralizes reactive oxygen species to regulate physical homeostasis in the body. The target of this study was to evaluate the molecular role of GST genotypic polymorphism involved in the development of CVD. For this case-control study, a total of 504 participants including 261 CVD patients and 243 healthy individuals were enrolled after taking informed consent. The analysis of the three allelic variants GSTM1, GSTT1, and GSTP1 was carried out through PCR-based amplification. Amplification of GSTM1 and GSTT1 was performed using the specific primers designed by Primer-3 software. GSTT1 and GSTMI genotypes were determined by comparing the sizes of amplified PCR product of genotypes with β Globulin gene, used as internal standard and 100-bp DNA ladder. GSTP1 genotype was determined using the PCR-restriction fragment length polymorphism. Analysis of data was carried out using SPSS software Version 22.0. Statistical significance of $p< 0.05$ was considered as valuable results. Results demonstrated that Null and GSTP1b$_{(105)}$ genotypes were more frequent in CVD patients than controls (23.0 vs 8.6 and 69.0 vs 44.4) with strong statistical association of Null=OR: 0.317, CI: 0.126-0.797 and GSTP1b$_{(105)}$ OR: 0.360, CI: 0.192 – 0.677 respectively. GSTM1 and GSTT1 were less frequent in CVD patients (46.0% vs 74.1% and 49.4% vs 74.1%) with significant statistical association of GSTM1= OR: 3.367, CI: 1.75-6.44 vs GSTT1=OR: 2.292, CI: 1.52-5.60 respectively. These findings concluded that Null and GSTP1b$_{(105)}$ genotypes have a significant association with CVD in the Pakistani population.

INTRODUCTION

Cardiovascular Diseases (CVDs) are the leading cause of death worldwide and are a major health problem globally (Amini *et al*., 2021). According to an estimation, CVD has caused 17.8 million deaths in 2017 worldwide and millions of disability (Roth *et al*., 2018). Further, worldwide CVD mortality is projected to be 23.4 million, involving 35% of all deaths in 2030 (Jilani *et al*., 2021). Statistically in Pakistan, 15.36 % of deaths are caused by CVDs, and about 0.2 million lives suffered from coronary heart diseases (CHD) since 2011 (Zeb *et al*., 2016). Various modifiable (lifestyle such as diet, physical inactivity, and stress), and non-modifiable (oxidative stress, poor antioxidant system, and genetic predisposition) risk factors have critical roles in the development of various heart diseases (Qasim, 2012). Dyslipidemia, the key feature of cardiovascular diseases, arises due to elevated serum lipoproteins levels (Syed *et al*., 2010).

High levels of cholesterol, triglycerides, LDL, and low level of anti-atherogenic HDL are the major risk

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factors for various types of chronic heart disease (Kontush and Chapman, 2006). Oxidative stress (OS), originating as a result of irregularities between free radicals production and antioxidant defense, destroy proteins, nucleic acids, and lipids. OS also leads to cellular dysfunctions which result in various pathological conditions including cancer, diabetes, and atherosclerosis (Zhao *et al*., 2022). Experimental and clinical pieces of evidence showed that OS plays a significant role in the pathology of CVD. Moreover, OS is also involved in dyslipidemia and other various pathological disorders which lead to the development CVD. OS is generated as a consequence of the interaction of ongoing metabolism and a variety of environmental factors (Natarajan *et al*., 2010). The well-known most potent natural non-enzymatic and enzymatic antioxidant defense mechanisms included endogenous glutathione (GSH) concentration, superoxide dismutases, catalases, and glutathione S-transferases (GST). GST neutralizes the reactive oxygen species to regulate physical homeostasis in the body (Tiwari *et al*., 2013).

GSH participates in several physiological functions e.g, it keeps the sulfhydryl group of proteins in the reduced state, detoxification of foreign hazardous compounds, transportation of amino acids, assist in enzymatic degradation of endogenous peroxide and also acts as a co-enzyme for many enzymatic reactions. It has also been demonstrated that GSH has a vital role in detoxifying the cells from free oxygen radicals to prevent damage from oxidative stress (Yan *et al*., 2020). GST is a family of stress-responsive detoxification enzymes with the ability to react to active compounds generated by reactive oxygen species (Aldini *et al*., 2013). GSTs isoenzymes possess tissues specific expressions and represent an important multigene family of isoenzymes prevalently expressed in almost all higher animals. It has been demonstrated that these isoenzymes help in the conjugation of GSH to a diversity of ionic molecules, and establish the role of the GST family as cell housekeepers, always active for detoxification of cellular toxic molecules (Rahman et al., 2012). GSTs allele/null genotype unable to cope the body against toxic free radicals causes tissues injury (Shimizu *et al*., 2004).

Several studies have shown that patients with chronic diseases, for example, heart disease, malignancies, diabetes, and even arthritis exhibit lower plasma levels of GSH than healthy individuals, indicating that GSH provides a protective role against such chronic conditions (Forman *et al*., 2009). Various studies have described the association of plasma or RBCs level of GSH to CVD (Bajic *et al*., 2019). A deletion in the GST gene has caused the polymorphisms with two variants GSTT1 and GSTM1. The individuals with the total absence of these two enzymes, due to deletions are called Null genotypes. Many studies produced great concordance (> 95%) among the genotypic and phenotypic ratios (Phulukdaree *et al*., 2012). In other studies, high frequency of genotypes M1 and P1 has been demonstrated in coronary artery disease (CAD) patients with smoking (Singh *et al*., 2011). This research aims to assess the genetic role of GST polymorphism in the development and progression of CVD. Many studies suggest that GSTP1 Ile105Val polymorphism could have strong associations with the development of coronary heart diseases (Singh *et al*., 2011; Shimizu *et al*., 2004). However, the results in the literature remained elusive, which might be due to the limitation of individual studies with a relatively small sample size. Therefore, we also aimed to conduct a case study to rule out the overall effects of the GSTP1 Ile105Val genetic polymorphism as a risk of CHD.

MATERIALS AND METHODS

Study sample collection

A case-control study was conducted at Sheikh-Zayed Hospital, Lahore, Pakistan. The study was conducted after approval from the Ethical Review Board of Federal PGM Institute of Sheikh-Zayed Medical Complex Lahore. After taking informed consent of total of 504 participants, 261 CVD patients and 243 controls (Healthy Individuals) were enrolled. Patients suffering from different cardiovascular disorders e.g. coronary artery disease (CAD), acute coronary syndrome (ACS), including myocardial infarction of ST-elevation and non-ST-elevation were included.

A questionnaire was used to record the personal record of participants containing demographic data e.g. age, weight, height along with the history of smoking and other diseases. Blood samples were collected under sterile conditions from all participants at Shaikh Zayed hospital, the 6cc blood was obtained from the cubital vein by using venipuncture out of which 3cc drained in tubes with 3.2% sodium citrate containing vial for plasma extraction, and the remaining 3cc blood was stored in EDTA blood vials for DNA extraction.

Analysis of genetic polymorphism

GSTM1, GSTT1, and GSTP1 genes amplification were performed using the specific primers for each isoform designed by "Primer 3 software. A mixture of 25 µl PCR master mix (15 pmol of each oligo, 800 µM of dNTPs, 2.5 mM MgCl2, 1x PCR buffer, 0.5U of Taq DNA-polymerase and 100 ng genomic DNA template) was used for PCR reaction in each PCR tube. PCR products were electrophoresed in 2% agarose gel. GSTT1

and GSTMI genotypes were determined by comparing the sizes of amplified PCR product of genotypes with β Globulin gene, used as internal standard and 100-bp DNA ladder. And GSTP1 genotype was determined by using the technique of PCR-RFLP. Following primers were applied for the determination of genetic polymorphism (Table I).

For amplification of GSTP1 following PCR conditions were applied, the initial denaturation of 96°C for 5 min, 30 cycles of DNA denaturation at 96°C for 30 sec, primers annealing at 55°C for 30 sec and amplification at 72°C for 30 sec, followed by a single cycle of final DNA amplification of 72°C for 5 min. A 176 bp PCR product was amplified and subjected to RFLP to analyze the polymorphic restriction sites. For this purpose, 0.5 µl (5 U) restriction enzyme *Bsm*AI (Fermentas) and 4.5 µl restriction reaction buffer were mixed in 15 µl PCR product in 25 µl reaction tube and left for overnight in shaking incubator at 37°C for digestion. Restriction amplicons were electrophoresed on 3% agarose gel and visualized under UV light. PCR amplicons containing homozygous allele G105 were subjected to complete digestion and produced two fragments of 91bp and 85bp, respectively.

Statistical analysis

The collected data was evaluated using the SPSS v22.0 software (Statistical Package for Social Sciences). Data were expressed as the mean ± SD (continuous variables) or by frequency and the percentage (categorical variables). The Independent Samples t-test was used to compare the means of continuous significant variables of independent groups in normal distribution data. A chi-squared statistical test was used to assess and compare the genotype frequencies between CVD and healthy individuals as separate groups. To evaluate the effect of genotype on the cardiovascular risk an odds ratio (OR) with a 95% confidence interval was used. The association between GST genotypes and different biochemical parameters was retrieved, by using Pearson's or Spearman's correlation coefficient depending upon the linearity and normality of data. Statistical significance of data was considered at $p < 0.05$.

Table I. List of primers used for genetic polymorphism.

Genes/Alleles	Oligonulceotides (5`→3`)		Annealing
GSTT1	Sense	TTCCTTACTGGTCCTCACATCTC	58°C and 72°C
	Antisense	TCACCGGATCATGGCCAGCA	
GSTM1	Sense	GAACTCCCTGAAAAGCTAAAGC	58°C and 72°C
	Antisense	GTTGGGCTCAAATATACGGTGG	
GSTP1	Sense	ACCCCAGGGCTCTATGGGAA	55°C
	Antisense	TGAGGGCACAAGAAGCCCCT	

Table II. Demographic parameters of 261 CAD patients and 243 healthy control groups.

	CAD patients No's (%)	Healthy individuals (n=243) No's (%)	χ^2 value T- test (p-value)
Gender			
Male	225 (86.2%)	60 (24.7%)	64.60 (<0.001***)
Female	36 (13.8%)	183 (75.3%)	
Age (Years)			
Mean age	53.63±13.34	49.38±14.48	1.98 (0.049*)
Age ≤ 40	36.12±4.14	31.09±6.9	5.92 (0.261)
Age > 40	58.03±11.48	55.78±10.35	2.31 (0.026*)
Smoking	96 (36.8%)	N/A	
Non-smoking	165 (63.2%)	N/A	

* p<0.05, ** p<0.01, ***p<0.001: *significant, **strong significant, *** Very strong significant.

RESULTS

The statistical analysis of demographic variables regarding gender, age, and smoking of CVD patients and healthy controls produced significant probability value (p-value) ranging from $p<0.05$ to $p<0.001$ as shown in Table II. In cases 225 (86.2%) were male and 36 (13.8%) were female. The overall mean age was 53.63±13.34, in Age ≤ 40 was 36.12±4.14 and in Age > 40 (years) was 58.03±11.48 respectively. In Controls mean age was 49.38±14.48, in Age ≤ 40 was 31.09±6.9 and in Age > 40 (years) was 55.78±10.35 respectively. There was a prominent association between gender and groups (P-value = 0.001). It was observed that the mean age of both groups exhibit a significant difference (P-value = 0.049).

PCR amplification of GST variants e.g. M1 and T1 (Fig. 1) showed that Null genotype does not produced any band for GSTT1 and GSTM1 genes, but only bands for internal control were generated. Whereas healthy control samples produced bands for GST1 and GSTM1 genes, and CAD diseased patient's samples produced either GSTT1 or GSTM1 bands along with bands of β Globulin gene as internal control.

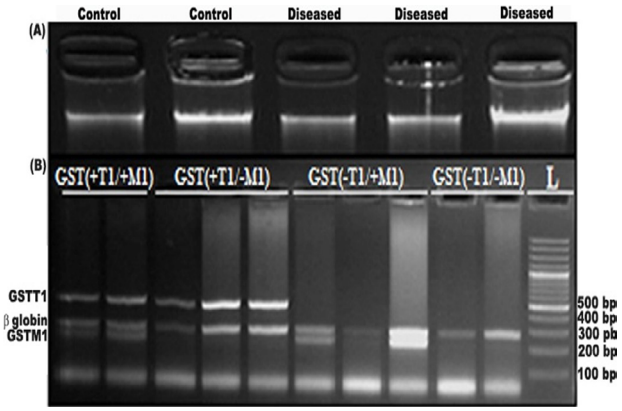

Fig. 1. (A) DNA extraction of control and disease and (B) agarose gel electrophoresis (2%) of GST polymorphism PCR, showing GSTM1 and GSTT1 alleles of healthy control and CAD patients. Extreme right lane contain 100 bp DNA marker, and extreme two left lane contain amplicons of control individual then followed by two lanes of CAD patients and β globin DNA fragment of 300 bp act as an internal standard.

The restriction (*Bsm*AI) digestion of GSTP1 homozygous Ile/Ile genetic variant produced two DNA fragments of 484 and 9 base pairs, the fragment of 9 base pairs is constant and is considered as an internal standard during the restriction digestion. The heterozygous Ile/Val variant generated four fragments of different lengths of 484, 259, 225, and 9 base pairs whereas, the mutant homozygous Val/Val genotype gives three fragments of 225, 259, and 9 base pairs, respectively (Fig. 2).

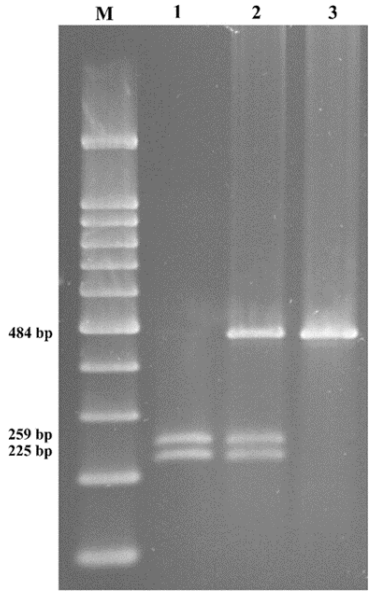

Fig. 2. Agarose gel electrophoresis (2%) of PCR-RFLP of the GSTP1 variant Ile105Val (A to G transition). M lane is 100 bp DNA ladder, Lane 1 contain variant GSTP1 (Val/Val), Lane 2 contain variant GSTP1 (Ile/Val) and Lane 3 contain GSTP1 (Ile/Ile). Band of Fragment of 9 base pairs being very small in size not shown in picture.

Frequencies of GST genotypes (M1, T1, and P1) among MI patients and healthy participants are mentioned in the form of a graph (Fig. 3), where GSTM1 is present in 120(46.0%) cases and 180(74.1%) in controls. GSTT1 is present in 129(49.4%) cases and 180(74.1%) in controls. GSTP1 ILE105Allele (a) was present in 198(75.9%) cases and 174(71.6%) in controls. Null genotype is present in 60(23.0%) cases and 21(8.6%) in controls.

The statistical analysis (Table III) revealed that patients with GSTM1 have 3.367 (OR: 3.367, CI: 1.75-6.44) fold more risk of having CVD. The patients with GSTT1 genotype exhibit 2.92 (OR: 2.292, CI: 1.52-5.60) fold more chances of developing CVD. The patients with Null genotype have 0.317 (OR: 0.317, CI: 0.126-0.797) times more chances for CVD. The patients who have GSTP1 ILE105Allele (a) was present have 0.802 (OR: 0.802, CI: 0.403-1.598) times have more chances of having CVD. GSTP1 ILE105Allele (b) was present in 60(69.0%) cases and 36(44.4%) in controls. The patients who have GSTP1 ILE105Allele (b) was present have 0.360 (OR: 0.360, CI: 0.192–0.677) times have more chances of having CVD.

Table III. Relationship of genotype and allele frequency in patient CAD and control group.

GST genotyping based variables		CAD group (n=261) N (%)	Control group (n=243) N (%)	χ^2 value	OR (95%CI)	p-value
GSTM1	No (-)	141 (54.0%)	63 (25.9%)	13.74	3.367(1.75-6.44)	<.001***
	Yes (+)	120 (46.0%)	180 (74.1%)			
GSTT1	No (-)	132 (50.6%)	63 (25.9%)	10.74	2.92 (1.52-5.60)	.001**
	Yes (+)	129 (49.4%)	180 (74.1%)			
Null	No (-)	201 (77.0%)	222 (91.4%)	6.40	0.317 (0.126-0.797)	.011*
	Yes (+)	60 (23.0%)	21 (8.6%)			
GSTP1	No (-)	63 (24.1%)	69 (28.4%)	0.393	0.802 (0.403-1.598)	.531
	Yes (+)	198 (75.9%)	174 (71.6%)			
GSTP1 ILE105 Allele (a)	No (-)	63 (24.1%)	69 (58%)	0.393	0.802 (0.403-1.598)	.531
	Yes (+)	198 (75.9%)	174 (71.6%)			
GSTP1 Val105 Allele (b)	No (-)	81 (30.0%)	135 (55.6%)	10.299	0.360 (.192-.677)	.001**
	Yes (+)	180 (69.0%)	108 (44.4%)			
ILE105/Val105 (a/b)	a/a	75 (28.7%)	135 (55.6%)	21.595	-	-
	a/b	123 (47.1%)	36 (14.8%)		.635(.296-1.36)	.243
	b/b	63 (24.1%)	72 (29.6%)		3.905(1.636-9.32)	.002**

* p<0.05, ** p<0.01, ***p<0.001: *significant, **strong significant, *** Very strong significant.

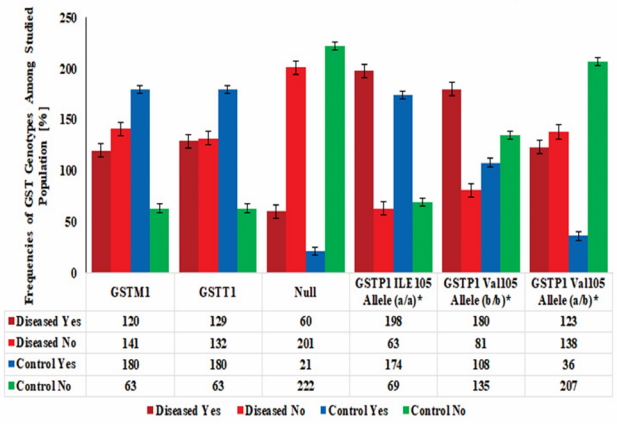

Fig. 3. Frequencies of GST genotypes (M1, T1 and P1) among MI patients with reference to healthy individuals.

DISCUSSION

Oxidative stress is a major cause of changes in hemodynamics, leading to the formation of thrombosis and atherosclerosis which ultimately results in endothelial cellular damage in vasculature. This kind of cyclic inflammation in arterial wall lesions and atherosclerotic plaques plays a major role to increase the level of C-reactive protein in CAD patients. The mild inflammation due to the activation of leucocytes in the atherosclerotic region has a significant role in the production of reactive oxygen species (ROS) to overcome or limit the spread of exotic or endogenous toxic materials, this strategy at the same time also exposes the essential biomolecules to the oxidative stress (Zhao *et al*., 2022; Palmer *et al*., 2003).

This study demonstrates the linkage of wild-type genotypes GSTP1A105/A105 and GSTM10/0 with CVD. The presence of allele GSTP1 G105 in healthy individuals indicates the importance of this genetic variant in increasing the potential of antioxidant defense mechanisms. GST functions as a base catalyst, accelerating the rate of GSH conjugation to hydrophobic substrates molecules by de-protonation of GS by an active tyrosine. Mutation of codon 105 in the GST gene causes the Ile to Val amino acid substitution. The GST105 mutation has been reported previously in some studies, to cause the co-ordinates deflection of atomic H-site in side-chains, thus changing the GST catalytic activity and enhancing susceptibility of individuals to develop smoking-related CAD (Zhang *et al*., 2018; Singh *et al*., 2011; Hulsman and Holvoet, 2010). Earlier literature also demonstrated that GST105 has sevenfold increased conjugation and catalytic abilities for aromatic epoxides (Hu *et al*., 1997).

Many Asian and European countries e.g. India, Bangladesh, Saudi Arabia, Turkey, and Italy have reported that Null genotype (individuals absent in both GSTT1 and

GSTM1) have two to eight-fold increased risk of CAD in their populations (Khanum *et al*, 2020; Cora *et al*., 2013). Whereas, a study from Taiwan reported no significant association between CAD and Null genotype (Yeh *et al*., 2013). However, in this study, the frequency of Null genotype was recorded (OR= 0.317, CL: 0.126-0.797) which is quite less than previously reported from China (Zhou et al., 2010).

These different results from different countries might be due to the interaction of different environmental factors to different ethnic races with diverse genetic makeup. It is a known fact that GSTP1 has a significant role in maintaining the cellular redox state due to its glutathionylation and antioxidant activities (Tew *et al*., 2011). It has been observed that 198 individuals from the CAD group have been found positive for GSTPI, statistically which is very high in this study (OR=0.802 and CL: 0.403-1.598). GSTP1 is also required for the activation of peroxiredoxin protein VI (Prdx6), which detoxifies the lipid peroxides especially in biological membranes (Manevich *et al*., 2013). Even the role of GSTPI in synchronizing the activation of endothelial lining cells during atherosclerosis has been studied well (Mowbray *et al*., 2008). Data also suggest that differential expression of GSTPI also interferes with the Prdx6 activities indicating the GSTPI genotypic individuals will have a prominent antioxidant response (Manevich *et al*., 2013). A study demonstrated that increased levels of GSTP1 expression in CAD patients, were strongly associated with declined GSTP1: JNK (JNK: c-Jun N-terminal kinase) interaction then resulted in activation of the JNK-MAPK (mitogen-activated protein kinase) signaling cascade, which is important for cardiomyocyte apoptosis (Andrukhova *et al*., 2014).

Studies have reported a common polymorphism in GSTPI at position 105 with Ile/Val in CHD patients with altered antioxidant activity (Phulukdaree *et al*., 2012). A study has demonstrated that the variant of the GSTP1-Val genotype exhibits an elevated level of TNF*α,* indicating the pathological importance of GSTP1 polymorphism (Simeunovic *et al*., 2019). This study demonstrates that the Pakistani population has a high frequency of GSTPI-Val genotype (OR= 0.360, CL: 0.192-0.677) which might contribute to declined antioxidant activity in HF patients and may also disturb the plasma concentration of MDA, TNF*α* in CAD patients (Rababa'h *et al*., 2018; Pocok *et al*., 2013).

CAD is a group of destructive disorders caused by genetic and environmental factors, which interact differently in different ethnic groups. Despite the number of investigations, the exact mechanism of interactions of these factors is still unknown (Khanam *et al*., 2020). Several genes have been reported to be associated with CAD including myocardial infarction (Cicoira *et al*., 2001; Çine *et al*., 2002). It is suggested that GST isoforms have a significant role in decreasing antioxidant activity leading to atherosclerotic plaque formation (Singh *et al*., 2011; Bhat *et al*., 2016). Two isoforms (M1 and T1) of GST are reported to be directly involved in CAD and MI (Abu-Amero et al., 2006). In this study, we have explored the relationships of these two isoforms (GSTM1 and GSTT1) in Pakistan.

In this study, the frequencies of GSTM1 in CAD and control groups were markedly different. The risk of occurring of CAD due to GSTM1 is 3.37 times (OR=3.367; 95% CI= 1.75-6.44; p< 0.001) higher in patients than null genotype (OR=0.317; 95% CI= 0.126-0.797; p=0.011). Moreover, the association of GSTT1 (OR=2.92; 95% CI= 1.52-5.44; p=0.001) is 2.92 fold higher in patients than the control group and null genotype. All these findings show a very close association to a study conducted in Bangladesh, where the GSTM1 genotypic allele frequencies were prominently different between the two groups, and the M1 was more frequent in myocardial infarction (MI) than the control group. The risk of occurring CAD was 2.5-fold (OR= 2.5; 95% CI= 1.4–4.3; p< 0.01) higher in patients in comparison to null genotypes (Khanam *et al*., 2020). Our findings regarding null genotype (OR=0.317; 95% CI= 0.126-0.797; p=0.011) are supported by a very similar study conducted on the North Indian population which suggest that GSTT1 null genotype could provide protection against CAD, on the other hand, GSTM1 could be involved in development and pathogenesis of CAD in the population of Punjab (Bhat *et al*., 2016).

CONCLUSION

It is concluded that Null and GSTP1 $b_{(105)}$ are significantly associated with CVD. However, this study was conducted on samples collected from a single hospital which was a limitation so these results could not be considered for all regions of Pakistan. So an extensive study with samples collected from all populated regions of the country would certainly produce more conclusive findings, which would help in the early diagnosis of CAD using GST molecular genotyping.

Statement of conflict of interest

The authors have declared no conflict of interest.

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