

Association of *GSTP1*, *GSTT1* and *GSTM1* Gene Variants with Coronary Artery Disease in Iranian Population: A Case–Control Study

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Background: Coronary artery disease (CAD) is a multifactorial disease that may be caused by the interaction between environmental and genetic risk factors. Glutathione S-transferases (GSTs) are known to participate in detoxification and metabolism of a wide range of xenobiotic compounds and oxidative stress products. Considering the interaction between environmental and genetic factors in CAD, we investigated the genetic polymorphisms of *GSTM1*, *GSTT1*, and *GSTP1* in the Iranian population.

Patients and Methods: Two hundred and forty-four CAD cases and 281 healthy controls were studied. The genotype of *GSTM1*, *GSTT1*, and *GSTP1* genes was determined by multiplex polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism (PCR-RFLP) techniques. Multivariable logistic regression analysis was used to calculate the odds ratios (ORs) and 95% confidence intervals (CI). Multifactor dimensionality reduction (MDR) analysis was also carried out to analyze the gene–gene and gene–environment interaction.

Results: The genotype and allele distribution of the three variations were not significantly different between CAD patients and controls ($p > 0.05$). The subgroup analysis revealed no significant gene–gene interactions or gene–gene combination effects linked to CAD susceptibility. However, MDR analysis selected the *GSTM1*, *GSTT1* pairwise and three genes combination models associated with the susceptibility to CAD. In addition, its result revealed that smoking in combination with *GSTM1* (two-way) and *GSTT1*, *GSTP1* (three-way) genes might increase the risk of CAD. Furthermore, a significant interaction between *GSTT1*-null polymorphism and dyslipidemia was found in multivariable logistic regression analyses in the gene–environmental interactions on CAD risk.

Conclusion: Our results suggest that the *GSTM1*, *GSTT1* and *GSTP1* genetic variations are not directly associated with the susceptibility to CAD in Iranian patients. Due to MDR results, there might be a non-linear association between interactions of two or three genes and smoking with CAD. There is also an association between CAD risk factors and GST variations, which requires supplementary confirmation with larger sample sizes.

Keywords: *GSTM1*, *GSTT1*, *GSTP1*, detoxification system, polymorphisms, coronary artery disease

Introduction

Coronary artery disease (CAD) is the most common type of heart disease worldwide, which can lead to a heart attack or death.¹ A high prevalence of CAD in the Iranian adult population has indicated.² CAD is a multifactorial disease affected by both acquired and inherited factors.³ Modifiable risk factors could be ameliorated

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through lifestyle changes and medication management. However, other risk factors are non-modifiable, such as genetic predisposition and sex.⁴ It is hypothesized that the development of CAD or atherosclerosis is affected by the interaction between predisposing genes and environmental factors.⁵ To improve strategies for prevention, early diagnosis, and therapy, the genetic factors involved in CAD development need to be understood.³

There are two main phases associated with detoxification in the eukaryotic cells. In Phase I, cytochrome P450 superfamily of enzymes (CYP450) have a fundamental role in bio transforming xenobiotics, steroid hormones, and pharmaceuticals to highly reactive compounds by adding active groups, including hydroxyl, carboxyl, or an amino group. These procedures through oxidation, reduction, and/or hydrolysis reactions can create reactive electrophilic species, which cause oxidative damage in cellular pathways.⁶ Phase II detoxification encompasses a procedure, in which an endogenous hydrophilic substance is conjugated to the reactive site of a product from phase I, turning it to a hydrophilic compound increasing its excretion in bile and/or urine. Several enzymes, such as glucuronyl transferases, sulfotransferases, glutathione transferases, amino acid transferases, N-acetyl transferases and N- and O-methyltransferases are involved in this phase.⁷

Glutathione S-transferase (GST) is one of phase II enzymes, which catalyzes conjugation reactions between glutathione and a wide range of electrophilic substrates. It also detoxifies a wide range of substrates, such as the compounds found in cigarette and environmental contamination that may increase the risk of CAD.^{8,9} It is also involved in the reactive oxygen species (ROS) reduction and protects the cell against ROS damage.¹⁰ The vascular layer including endothelium, smooth muscle and adventitia produce ROS. These molecules act as a signaling molecule under normal physiological condition; however, increased amount of them can cause cell necrosis, apoptosis and dysfunction through oxidizing macromolecules, such as proteins, lipids, and DNA.¹¹ Oxidization of low-density lipoproteins (LDL) is the primary step in atherosclerosis progression, as the main cause of CAD. The oxidized LDL (OxLDL) is involved in several biological procedures, which can be responsible for atherosclerosis in CAD. It increases the activation and proliferation of monocyte and macrophage in the arterial wall through chemotactic activity.¹² OxLDL promotes the production of collagen from smooth muscle cells (SMCs), resulting in the formation of the fibrous cap in atherosclerotic plaque and the

growth of the lesion.^{13,14} It also has a cytotoxic effect and increases apoptosis in vascular cells.^{15,16} Also, OxLDL increases the aggregation and adhesion of platelet by reducing the synthesis of nitric oxide and increasing the production of prostaglandins and related precursors.^{17,18} Consequently, any changes in the expression of GSTs, responsible for the reduction of ROS, can increase the susceptibility to CAD.

The genetic variants influence the expression and functional activities of the GST proteins. Among the cytosolic GST enzymes, the variations of mu, theta, and pi classes have intensively investigated in various studies.^{19,20} The glutathione S-transferase mu 1 (*GSTM1*), glutathione S-transferase theta 1 (*GSTT1*), and glutathione S-transferase pi 1 (*GSTP1*) genes are polymorphic and some allelic variants cause enzyme deficiency.⁹

GSTP1 gene is located on chromosome 11q13 and is expressed in normal epithelial cells, such as cardiovascular system.²¹ An A to G transition in codon 105 of *GSTP1* enzyme leads to the substitution of isoleucine (Ile) to valine (Val) amino acids (Rs 1625).^{22,23} This alteration affects the enzyme activity compared with the wild-type. Amino acid 105 lies near the active site of the enzyme, by which influences the *GSTP1* catalytic activity.^{24,25}

The *GSTT1* and *GSTM1* encoding genes are organized in gene clusters on chromosomes 22q11 and 1p13.3, respectively.^{26–28} The common deletion polymorphism of *GSTM1* and *GSTT1* genes consequently results in the absence of functional enzyme.^{29,30} Deleting variants or null variants in *GSTT1* and *GSTM1* genes may arise by homologous recombination of the left- and right-repeated sequences, which results in a 54-kb and 16-kb deletion including the entire two genes.^{31,32} Regarding the importance of these polymorphic genes, we conducted this study to understand whether the polymorphisms of these three genes or their combinations have any effect on disease progression or susceptibility. Furthermore, another objective of this study was to determine the possible high-level gene–gene and gene–environment interaction between *GSTT1*, *GSTM1*, and *GSTP1* genes and environmental factors using multifactor dimensionality reduction (MDR) to increase the risk of CAD.

Patients and Methods

Study Population

Two hundred and forty-four unrelated patients with CAD and 281 age- and sex-matched unrelated healthy control

subjects were enrolled in this case-control study. The study protocol was approved by the Ethics Committee of Islamic Azad University, Yazd Branch (Ethics code: IR. IAU.REC1396.23). All subjects were given information about the study before their enrollment, and the written informed consent was obtained from patients and healthy individuals. This study was conducted in accordance with the Declaration of Helsinki. Cases were selected randomly from the patients who were referred to the Rajaei Cardiovascular, Medical Research Center, Tehran, Iran from 2011 to 2013. CAD was detected by coronary angiography for patients and defined as stenosis more than 70% in at least one of the major coronary arteries. Patients with concomitant inflammatory or malignant disease were excluded. Control subjects were collected randomly from healthy people who volunteered for the study and examined by a cardiologist. Subjects with concerning signs or symptoms were subjected to coronary angiography to ensure about their health conditions. Subjects with no history of cardiac pain and other CAD risk factors were selected as controls.

Definition of Cardiovascular Risk Factors

The well-known independent risk factors for CAD are hypertension, diabetes, smoking, and dyslipidemia. Hypertension was diagnosed in patients based on a systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg or the need for blood pressure-lowering medicine.³³ Having at least two measurements of fasting blood glucose ≥ 126 mg/dl was defined as diabetes.³⁴ Smoking was defined as smoking constantly or over repeated periods at least six months. Dyslipidemia was defined as having high levels of blood lipids (eg triglycerides >200 mg/dl, total cholesterol levels ≥ 240 mg/dl, HDL cholesterol ≤ 40 mg/dl, LDL cholesterol ≥ 130 mg/dl) or use of lipid-lowering drugs.³⁵ The Body Mass Index (BMI) was calculated as weight/height² in all subjects.³⁴

Genotyping

Five mL of peripheral blood samples were collected in Conical Centrifuge Tubes containing EDTA and stored at 4°C. Genomic DNA was isolated using the GenEx™ blood genomic DNA purification kit (GeneAll, Korea) according to the experimental protocol. DNA quality and quantity were assessed by the NanoDrop™ 2000 Spectrophotometer.

GSTP1 Genotyping

The *GSTP1* (Ile105Val) genetic variant was identified using the polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis. A 493-bp fragment including exon 5 substitution was amplified with a set of primers: forward primer: 5'-TCTCATCCTTCCACGCATC-3' and reverse primer: 5'-TGCTGGAGGTCTCTGTCCTTG-3'. The PCR conditions were 95°C for 5 minutes, 35 cycles of 94°C for 45 seconds, 63°C for 40 seconds and 72°C for 40 seconds, followed by 72°C for 10 minutes. The PCR products were digested with *Alw26I* (Thermo Scientific™, Norway) at 37°C for 16 h in a total volume of 10 μ L. The digestion products were analyzed on a 2% agarose gel prepared in 0.5X TBE buffer. The *GSTP1* genotypes were determined as follows: The AA genotype (Ile/Ile) yields two fragments of 484 and 9 bp; the 9 bp is an invariant polymorphism, which used as an internal standard for digestion process; AG genotype (Ile/Val) gives four fragments with 484, 225, 259, and 9 bp and GG mutant genotype (Val/Val) produces three fragments with 225 and 259 bp and 9 bp (Figure 1). After genotyping all samples with PCR-RFLP, some of them were retested by sequencing the PCR product by Macrogen, Korea; no discrepancies were found.

GSTM1 and GSTT1 Genotyping

Multiplex PCR was used to detect the *GSTM1* and *GSTT1* genes in a total volume of 25 μ L buffered solution. The reaction mixture was heated at 95°C for 5 min, followed by 35 cycles of amplification as follows: a denaturing step at 94°C for 30 s, an annealing step at 63°C for 1 min, and an extension step at 72°C for 1 min. The final extension was at 72°C for 10 min. PCR samples were analyzed on a 2% agarose gel prepared in 0.5X TBE buffer runs at 110 V for 70 min at room temperature. The absence of a 219-bp band for *GSTM1* or a 558-bp band for *GSTT1*, with the presence of a 268-bp β -globin (as control fragment) band, was recorded as null genotype (Figure 2). This method did not permit the detection of heterozygous carriers of *GSTM1* or *GSTT1* deletions, but it identified the null genotypes conclusively. The following primers were used: designed *GSTT1* primers: forward: GGTCTCACA TCTCCTTAGC; *GSTT1* reverse: AGTCTTAGGCAAGC CATTCC; *GSTM1* forward: GAACTCCCTGAAAAGC TAAAGC; *GSTM1* reverse: GTTGGGCTCAAATATACG GTGG;³⁶ β -globin forward: CAACTTCATCCACGTTCC; β -globin reverse: GAAGAGCCAAGGACAGGTAC.³⁷

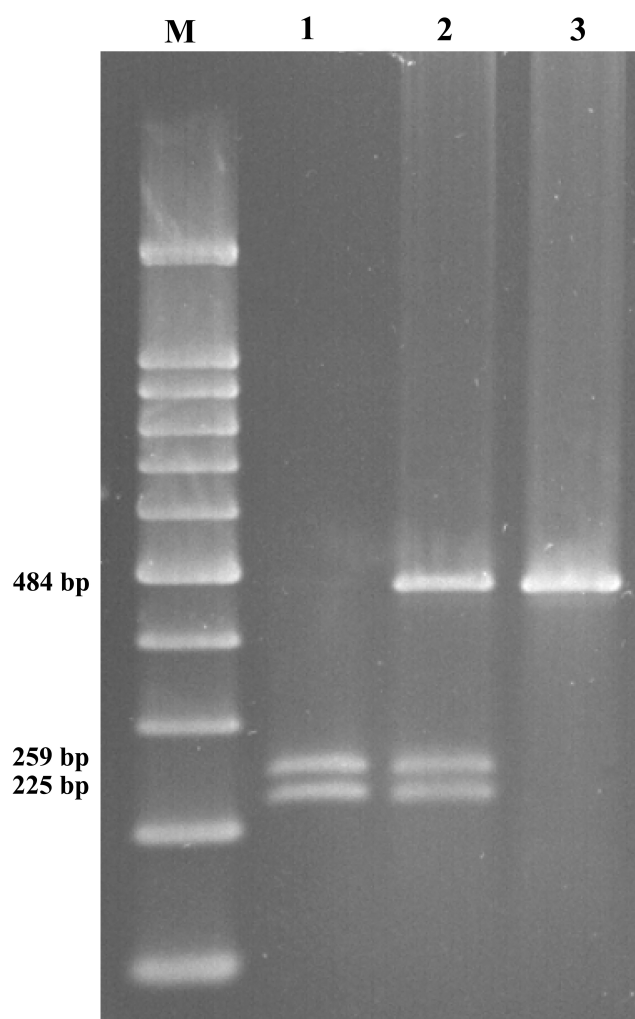


Figure 1 Detection of the Ile105Val (A to G transition) of GSTP1 by RFLP on 2% agarose gel. (M) 100 bp DNA ladder (CinnaGen co, Iran); lane 1: GSTP1 (Val/Val), lane 2: GSTP1 (Ile/Val); lane 3: GSTP1 (Ile/Ile).

Statistical Analysis

Statistical analysis was carried out using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA). Allele and genotype frequencies in cases and controls were compared using a chi-square (χ^2) test, and odds ratios (ORs) and their 95% confidence intervals (CIs) were calculated. Age, the ratio of low-density lipoprotein (LDL) to high-density lipoprotein (HDL), triglycerides and BMI were evaluated by Student's *t*-test. The risk factors for CAD and the association between CAD and GSTs polymorphisms were evaluated by multiple logistic regression analysis. Tests for Hardy–Weinberg equilibrium was also conducted by the χ^2 test. A *p*-value <0.05 was defined as statistically significant.

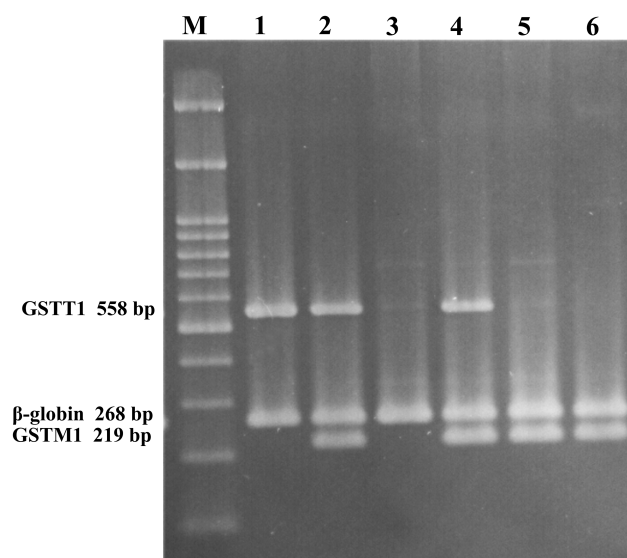


Figure 2 Multiplex PCR products electrophoresed on 2% agarose gel. (M) 100 bp DNA ladder (CinnaGen co, Iran); lane 1: GSTM1 null & GSTT1 present; lanes 2 and 4: GSTM1 & GSTT1 present; lane 3: GSTM1 & GSTT1 null; lanes 5 and 6: GSTM1 present & GSTT1 null.

MDR Analysis

Gene–gene interaction and gene–environment interaction analysis between *GSTT1*, *GSTM1*, and *GSTP1* genes and smoking, as an environmental factor, were executed using the MDR software package (version 3.0.2).³⁸ MDR, as a non-parametric approach, and data mining approach can characterize the gene–gene and gene–environmental non-linear interaction in a small group and overcome size limitation.^{38,39} In this analysis, the optimal prediction model was found due to maximum testing balance accuracy (TBA) and cross-validation consistency (CVC). During the analysis, 1000-fold permutation testing and χ^2 test at 0.05 significance level were used to test the model with the highest TBA and CVC.

Results

Demographic Information

This study was conducted on 244 patients with CAD and 281 healthy controls. The lifestyle and clinical parameters of the patient and control subjects are summarized in Table 1. No significant difference was found between the case and control groups in age (56.56 ± 9.27 vs 55.46 ± 10.95 years, respectively). Subjects with CAD had a significantly higher frequency of hypertension than controls (45.9% vs 28.4%, respectively, *p*=0.002), as well as diabetes mellitus (53.7% vs 19.2%, respectively, *p*=0.000),

Table 1 Clinical Parameters of the Patients and Controls

Variable	CAD Patients (N=244)	Control Group (N=281)	p-value
Age (Years)	56.56±9.27	55.46±10.95	0.332
BMI (Kg/m ²)	27.07±3.76	27.53±4.58	0.389
LDL/HDL (Mean)	4.17±1.39	2.20±0.99	0.000
Gender (Male), n (%)	174 (71.3)	164 (58.3)	0.015
Smoking status, n (%)	113 (46.3)	53 (18.8)	0.000
Hypertension, n (%)	112 (45.9)	80 (28.4)	0.002
Diabetes mellitus, n (%)	131 (53.7)	54 (19.2)	0.000
Dyslipidemia, n (%)	187 (76.6)	89 (31.6)	0.000
Number of involved vessels, n (%)	One 44 (18.0)	–	–
	Two 69 (28.3)	–	–
	Three 131 (53.7)	–	–

Abbreviations: BMI, body mass index; LDL, low-density lipoproteins; HDL, high-density lipoproteins; CAD, coronary artery disease.

history of cigarette smoking (46.3% vs 18.8%, respectively, $p=0.000$), and dyslipidemia (76.6% vs 31.6%, respectively, $p=0.000$). Male subjects showed a higher rate of CAD (cases: 71.3% vs controls: 58.3%).

CAD patients were classified into three groups based on the CAD severity according to the coronary angiographic results. Of these, 44 (18%), 69 (28.3%), and 131 (53.7%) CAD cases had one-, two-, and three-vessel disease, respectively.

Genotype Frequencies and Their Associations with CAD

The distribution of *GSTP1* genotypes between the two groups was not significantly different ($p=0.921$). The frequency of AA, AG, and GG genotypes among the studied group was 52.9%, 40.9%, and 6.2% in patients; and 50.8%, 42.8%, and 6.4% in the control group, respectively

(Table 2). The chi-square test revealed that genotype distributions between patients and controls were not significantly different. The allelic distribution of the rs1625 genotype in the cases and controls showed no deviation from the Hardy–Weinberg equilibrium ($p>0.05$).

The proportion of the *GSTM1*-null genotype was not significantly different between the patients (47.5%) and control (50.9%) groups ($p=0.566$). This variant did not increase the risk of CAD development. The frequency of the *GSTT1*-null genotype was 26.2% and 19.9% in the patient and control groups, respectively ($p=0.165$). No significant association was found between *GSTT1* mutation and susceptibility to CAD. Additionally, the distribution of *GSTM1* and *GSTT1* phenotypes and *GSTP1* genotypes in three subgroups of CAD severity is summarized in Table 3.

The Combined Effect of GST Polymorphisms and Risk of CAD

To further assess the existence of an interaction between the studied GST genes, the combination of their polymorphisms was investigated. The frequencies of these combinations are summarized in Tables 4 and 5. Those who carried all three wild-types (low-risk) genotypes [*GSTM1* and *GSTT1* (present) and *GSTP1* (AA) genotypes] were defined as the reference group (Table 4). No statistically significant association was observed with each genotype combination and the risk of CAD ($p=0.192$).

Interactions Between GST Genes and CAD Risk Factors

Supplementary subgroup analyses were performed to determine the interactions between GST genotypes and

Table 2 GST Genotypes and the Risk of Coronary Artery Disease

Gene	Genotype	CAD Patients (%)	Control Group (%)	OR CI (95%)	p-value
<i>GSTM1</i>	Positive	128 (52.5)	138 (49.1)	1.000	–
	Null	116 (47.5)	143 (50.9)	0.86 (0.62–1.23)	0.566
<i>GSTT1</i>	Positive	180 (73.8)	225 (80.1)	1.000	–
	Null	64 (26.2)	56 (19.9)	1.43 (0.95–2.15)	0.165
<i>GSTP1</i> (rs1695)	AA	129 (52.9)	143 (50.8)	1.000	–
	AG	100 (40.9)	120 (42.8)	0.902 (0.361–2.250)	0.825
	GG	15 (6.2)	18 (6.4)	0.917 (0.586–1.434)	0.704
	AA (dominant)	129 (52.9)	143 (50.8)	1.000	–
	AG+GG	115 (47.1)	138 (49.2)	0.92 (0.66–1.33)	0.69
	Allele A	358 (0.73)	406 (0.72)	1.000	–
	Allele G	130 (0.27)	153 (0.28)	0.96 (0.73–1.27)	0.70

Abbreviations: GST, glutathione S-transferase; CI, confidence interval; OR, odds ratio; CAD, coronary artery disease.

Table 3 Distribution of the GST Genotypes Stratified by the Severity of Coronary Artery Disease

Genotype	Number of Involved Vessels				p-value
	0 (n=281)	1 (n=44)	2 (n=69)	3 (n=131)	
<i>GSTM1</i>					0.145
Present	138 (49.1)	31 (70.4)	30 (43.5)	67 (51.1)	
Null	143 (50.9)	13 (29.6)	39 (56.5)	64 (48.9)	
<i>GSTT1</i>					0.152
Present	225 (80.0)	33 (75.0)	58 (84.0)	90 (68.7)	
Null	56 (20.0)	11 (25.0)	11 (16.0)	41 (31.3)	
<i>GSTP1</i>					0.421
AA	143 (50.9)	26 (59.1)	41 (59.4)	62 (47.3)	
AG	120 (42.7)	16 (36.4)	28 (40.5)	56 (42.7)	
GG	18 (6.4)	2 (4.5)	0	13 (10.0)	

Abbreviation: GST, glutathione S-transferase.

Table 4 Combined Effects of *GSTM1*, *GSTT1*, and *GSTP1* Polymorphisms in the Study Subjects

<i>GSTM1</i>	<i>GSTT1</i>	<i>GSTP1</i>	Cases	Controls	p-value	OR ^a (95% CI)
Present	Present	AA	56 (22.9)	53 (18.9)	–	1.000
Null	Present	AA	46 (18.8)	55 (19.6)	0.472	0.79 (0.46, 1.36)
Present	Null	AA	12 (4.9)	21 (7.4)	0.203	0.54 (0.24, 1.21)
Null	Null	AA	16 (6.7)	14 (5.0)	0.796	1.08 (0.48, 2.43)
Present	Present	AG+GG	44 (18.0)	52 (18.5)	0.512	0.8 (0.46, 1.39)
Null	Present	AG+GG	34 (13.9)	65 (23.1)	0.055	0.5 (0.28, 0.87)
Present	Null	AG+GG	16 (6.6)	12 (4.3)	0.636	1.26 (0.28, 0.87)
Null	Null	AG+GG	20 (8.2)	9 (3.2)	0.193	2.1 (0.88, 5.03)

Note: ^aAdjusted OR: adjusted in multivariate logistic regression models including age and sex.

Abbreviations: GST, glutathione S-transferase; CI, confidence interval; OR, odds ratio.

CAD risk factors (such as hypertension, smoking, dyslipidemia, male gender, and diabetes mellitus) and the risk of CAD development (Table 6). Based on the analysis of the

interactions between *GSTM1*, *GSTT1* phenotypes or *GSTP1* genotypes and CAD risk factors in CAD pathogenesis, a significant interaction between *GSTT1* deletion

Table 5 Combination of Double GST Polymorphisms

Genotype	Cases	Controls	p-value	OR ^a (95% CI)
MI present/TI present	100 (40.9)	105 (37.5)	–	1.000
MI null/TI present	80 (32.8)	120 (42.8)	0.162	0.7 (0.47, 1.04)
MI present/TI null	28 (11.5)	33 (11.8)	0.744	0.89 (0.5, 1.58)
MI null/TI null	36 (14.8)	22 (7.9)	0.168	1.72 (0.95, 3.12)
MI present/PI AA	67 (27.5)	74 (26.3)	–	1.000
MI null/PI AA	60 (24.8)	65 (23.1)	0.928	1.02 (0.63, 1.65)
MI present/PI AG+GG	62 (25.5)	69 (24.4)	0.966	1.013 (0.557–1.841)
MI null/PI AG+GG	54 (22.1)	74 (26.2)	0.482	0.81 (0.5, 1.31)
TI present/PI AA	102 (41.8)	108 (38.4)	–	1.000
TI null/PI AA	79 (32.2)	117 (41.6)	0.183	0.71 (0.48, 1.06)
TI present/PI AG+GG	28 (11.3)	35 (12.5)	0.675	0.85 (0.48, 1.49)
TI null/PI AG+GG	36 (14.7)	21 (7.5)	0.117	1.82 (0.99, 3.32)

Note: ^aAdjusted OR: adjusted in multivariate logistic regression models including age and sex.

Abbreviations: CI, confidence interval; OR, odds ratio.

Table 6 Interaction of the GST Genotypes and Coronary Artery Disease (CAD) Risk Factors on CAD Development

SNP/Genotype	Number	Smoking (%)	Hypertension (%)	Dyslipidemia (%)	Male Gender (%)	Diabetes (%)
<i>GSTP1</i> (rs1695)						
Patients	244	113	112	187	174	131
AA	129	60 (53.2)	57 (51.8)	97 (51.9)	90 (51.7)	70 (53.5)
AG	100	46 (40.7)	50 (44.6)	75 (40.1)	76 (43.7)	51 (38.9)
GG	15	7 (6.1)	5 (4.4)	15 (8.0)	8 (4.6)	10 (7.6)
Control	281	59	80	89	164	54
AA	143	19 (32.2)	38 (47.5)	49 (55.0)	83 (50.4)	36 (66.7)
AG	120	27 (45.7)	38 (47.5)	37 (41.6)	68 (41.3)	12 (22.2)
GG	18	13 (22.9)	4 (5.0)	3 (3.4)	13 (8.3)	6 (11.1)
p-value		0.055	0.877	0.622	0.702	0.234
<i>GSTM1</i> (deletion)						
Patients	244	113	112	187	174	131
Positive	128	61 (54.0)	53 (47.3)	97 (51.9)	87 (50.0)	74 (56.5)
Null	116	52 (46.0)	59 (52.7)	90 (48.1)	87 (50.0)	57 (43.5)
Control	281	59	80	89	164	54
Positive	138	37 (62.8)	50 (62.5)	50 (56.1)	83 (50.6)	33 (61.1)
Null	143	22 (37.2)	30 (37.5)	39 (43.8)	81 (49.4)	21 (38.9)
p-value		0.500	0.101	0.629	0.946	0.711
<i>GSTT1</i> (deletion)						
Patients	244	113	112	187	174	131
Positive	180	85 (75.2)	84 (75.0)	133 (71.1)	128 (73.6)	97 (74.1)
Null	64	28 (24.8)	28 (25.0)	54 (28.9)	46 (26.4)	34 (25.9)
Control	281	59	80	89	164	54
Positive	225	44 (74.6)	66 (82.5)	81 (91.0)	128 (78.0)	39 (72.2)
Null	56	15 (25.4)	14 (17.5)	8 (9.0)	36 (22.0)	15 (27.8)
p-value		1.000	0.336	0.002	0.497	0.966

polymorphism and dyslipidemia on CAD development was observed ($p=0.002$). No association was observed between and within the other study subgroups.

Gene–Gene and Gene–Environment Interactions by MDR Analysis

The current study also investigated the interaction between *GSTT1*, *GSTM1*, *GSTP1* genes and smoking as an environmental factor using MDR analysis in controls and CAD patients. The best possible interaction between three genes is listed in Table 7. The *GSTT1* gene (null and present genotypes) with the cross-validation of 9/10 had the highest testing-balanced accuracy (51.63%) among the three genes. Among the two-way gene interaction models, the best model was the interaction of *GSTM* and *GSTT* genes with a testing-balanced of 47.72% and permutation testing p -value of 0.0254. However, the testing-balanced accuracy of this model is lower compared with the three-way gene interaction model. The three-way genes interaction model showed cross-validation of 10/10 and permutation testing

p -value of 0.0087. These results showed that the two-way (*GSTM*, *GSTT*) and three-way interaction of three genes might have a non-linear association with the susceptibility to CAD. Figure 3 summarizes the dimensional reduction of the three-way gene interaction of *GSTT1*, *GSTM1*, and *GSTP1* genes (12 genotypes) showing the high- and low-risk combination of genotypes associated with CAD, as well as the distribution of cases and controls for each combination.

A summary of the best models for two-way and three-way gene-environment interactions between the three genes and smoking are listed in Table 8. These three models have a cross-validation consistency of 100% and significant permutation testing p -value ($P<0.0001$). The best models for two-way and three-way gene-environment interactions were *GSTM*-smoking and *GSTT*-*GSTP*-smoking, respectively.

Discussion

ROS are the initiator of oxidative stress involved in the pathogenesis of atherosclerosis through several important

Table 7 Summary of Multifactor Dimensionality Reduction Gene–Gene Interaction Result

Best Model	Training Bal. Acc. (%)	Testing Bal. Acc. (%)	CV Consistency	P-value
GSTT	53.16	51.63	9/10	0.0864
GSTM, GSTT	55.23	47.72	6/10	0.0254
GSTM, GSTT, GSTP	56.03	48.73	10/10	0.0087

Abbreviations: CV, cross-validation; Bal, balanced; Acc, accuracy.

enzyme systems.⁴⁰ Risk factors for atherosclerosis and CAD increase the production of ROS by endothelial cells. Although certain enzymes are acting against ROS, such as superoxide dismutase and glutathione peroxidase, these molecules can oxidize lipid, DNA, protein, and carbohydrate, leading to degradation of them and increase their toxicity and mutagenicity. Other enzymes, such as aldehyde dehydrogenase, alcohol dehydrogenase, Aldo-Keto Reductase, and GST neutralize the excessive ROS and the byproducts of oxidative stress. Among these enzymes, GST enzymes catalyze the conjugation of glutathione to a wide variety of electrophile compounds and make them more soluble and less active. Studies also revealed that materials generating free radicals and H₂O₂ can induce GST in mammalian cells. Therefore, the level of GST expression can be a determining factor for evaluating the response of cells to xenobiotic and chemical compounds, and considered as a potential biomarker for ROS-related diseases.^{41–43}

Table 8 Summary of Multifactor Dimensionality Reduction Gene–Environment Interaction Results

Best Model	Training Bal. Acc. (%)	Testing Bal. Acc. (%)	CV Consistency	P-value
Smoking	63.73	63.73	10/10	<0.0001
GSTM, Smoking	63.73	63.73	10/10	<0.0001
GSTT, GSTP, Smoking	64.81	63.70	10/10	<0.0001

Abbreviations: CV, cross-validation; Bal, balanced; Acc, accuracy.

Many studies have evaluated GST polymorphisms in CAD; however, few studies have addressed the role of *GSTM1*, *GSTT1* and *GSTP1* gene polymorphisms in this disease simultaneously.^{9,44} This study aimed at assessing the association between polymorphisms of GST genes and CAD in the Iranian population. The *GSTP1* A1578G transition leads to isoleucine substitution to valine, which affects the enzyme activity. The proportion of Val allele in the control group was 28%. No significant differences were observed in the frequencies of the *GSTP1* genotypes between the patient and control groups. Our result is comparable to the allele frequencies in Caucasians (33.1%) and also to a population from the west of Iran (31.9%).^{44,45} Few studies evaluated the association of *GSTP1* polymorphism and CAD,^{9,44} and our findings are consistent with the results obtained in the west of Iranian and Taiwanese populations. The *GSTM1*-null polymorphism is caused by homozygous deletion. The frequency of

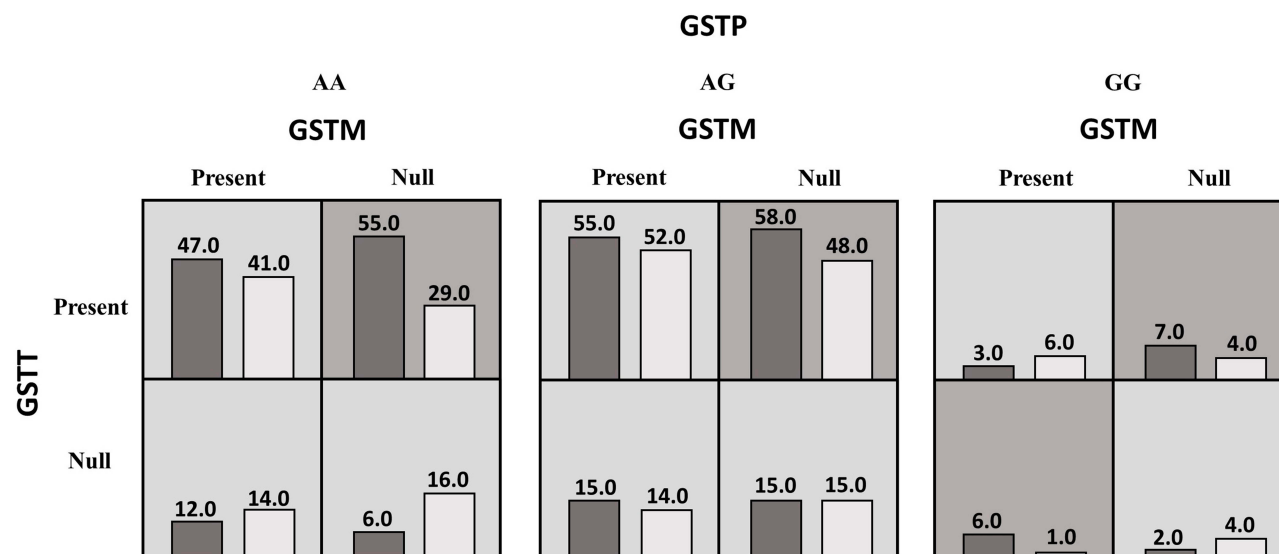


Figure 3 A summary of the best gene–gene interaction analysis by multifactor dimensionality reduction for 12 genotypes (*GSTP*, *GSTM*, and *GSTT* genes) associated with coronary artery disease. The dark shading box represents high-risk combinations and the light shading box shows low-risk combinations. In each box, the left and right columns represent the percentage of the cases and controls, respectively.

the *GSTM1* null genotype in our control subjects was 50.9%, similar to frequencies of this genotype in the Caucasian, Asian, and Iranian control populations (53.1, 52.9, and 50.9%, respectively).^{46,47} The prevalence of *GSTM1*-null genotype was 52.8% in a similar study in the west of Iran.⁴⁴ The frequency of the *GSTT1*-null genotype in our control participants was 19.9%, which was similar to that reported in the Caucasian and Iranian control population.^{46,47} The Asian control population showed a higher prevalence of 47% of *GSTT1*-null genotype.⁴⁶ The frequency of the *GSTT1*-null genotype in a control population from the west of Iran was 15.7%.⁴⁴ In our study, no significant differences were found in the frequencies of *GSTM1*- and *GSTT1*-null mutations between the case and control groups as observed in the Taiwanese and Brazilian populations.^{9,48} In contrast, some studies reported the association between *GSTM1*- and *GSTT1*-null genotypes and CAD development in the Italian, Saudi Arabian, North Indian, Chinese, and especially Iranian (west of Iran) populations.^{44,49-52} A study in the North India found a protective role of the *GSTT1*-null genotype against CAD.⁵³ Moreover, a study in young South African Indians documented that *GSTM1*-null genotype is associated with a 2.6-fold higher risk of CAD development.⁵⁴

Although there was no direct association between the genes and susceptibility to CAD based on traditional statistical analysis, high-level MDR analysis revealed that there might be an association between the interactions of these genes and susceptibility to CAD in our study. Determining the gene–gene and gene–environment interactions are fundamental in epidemiological studies to identify the etiology of different diseases and predict the risk factors for disease prevention.⁵⁵

Earlier studies investigating the interactions between GST genes and cigarette smoking on CAD disease showed a significant association between GST polymorphisms and CAD risk in smokers.^{5,56} No direct association was found among the smoker subgroup; however, MDR demonstrated that there might be an association between *GSTM* and smoking (the best two-way model) and *GSTT*, *GSTP*, and smoking (the best three-way model) with the risk of developing CAD in our population. No previously published studies have investigated the interaction between GST genotypes and other CAD risk factors in CAD development. Our results showed that the interaction between *GSTT1*-null genotype and dyslipidemia significantly affected CAD development ($p=0.002$). The other interaction between

GST genotypes and risk factors for CAD was not significantly different between the two studied groups.

Ethnic differences and varied environmental, lifestyle, cultural, socioeconomics and nutritional factors might explain inconsistent results from different studies.^{57,58} In terms of ethnicity, it might be determined by self-reported data, which increases its complication. In some population, individuals are inclined to conceal their main ethnicity and use other ethnicity due to the political or cultural sensitivity. Marriage between different ethnic groups can also add more variety to the results of different relevant studies.⁵⁹ On the other hand, the genetic background and expression of compensatory functions can cover the effect of a gene polymorphism or mutation.²⁵

We observed that the well-known CAD risk factors, like male gender, cigarette smoking, hypertension, dyslipidemia, and diabetes were significantly associated with CAD development. Our data is consistent with the results of previous studies.^{9,44,54,60}

Prior studies have demonstrated conflicting results regarding the combination effect of GSTs genotypes on CAD. Here, we investigated the effects of a combination of two and three genotypes on CAD. The results showed that different combinations of genotypes did not affect CAD. The incidence of a combination of *GSTM1*- and *GSTT1*- null genotypes in our control individuals was 8%, which is similar to the previous reports of the frequency of the double nulls between Iranian and Caucasian populations, and a population from the west of Iran (11.8, 10.4 and 10.2%).⁴⁴ However, in an Asian control population, both *GSTM1*- and *GSTT1*-null genotypes were higher (24.6%).⁴⁶

Conclusion

In conclusion, our study revealed no association between the *GSTM1*, *GSTT1*, and *GSTP1* genetic variations and the susceptibility to CAD in Iranian patients. However, MDR analysis revealed a two-way interaction between *GSTM* and *GSTT* and a three-way combination between *GSTT* and *GSTP* of the genes associated with the susceptibility to CAD. In addition, our results revealed that smoking in combination with *GSTM1* (two-way) and *GSTT* and *GSTP* (three-way) might increase the risk of CAD. There was an association between *GSTT1* deletion polymorphism and dyslipidemia, as one of the CAD risk factors on CAD development.

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Disclosure

The authors report no conflicts of interest in this work.

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