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ORIGINAL RESEARCH

Association Of GSTM1, GSTT1 And GSTP1 Polymorphisms With Breast Cancer Among Jordanian Women

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Purpose: Genetic predisposition to disease has become one of the most investigated risk factors in recent years, and breast cancer (BC) is no exception. In this study, we investigated specific genetic variants of three candidate genes belonging to the glutathione-S-transferase superfamily that have been implicated in increased risk of cancers.

Materials and methods: This case-control study comprised 241 Jordanian women who were diagnosed with BC in addition to 219 matched controls. Gel electrophoresis of PCR products was used to visualize and genotype both the *GSTM1* and *GSTT1* genes, while PCR-RFLP was employed to genotype the rs1695 of the *GSTP1* gene.

Results: Our findings did not reveal any correlation between the investigated polymorphisms of GST genes and BC risk among Jordanian women. Otherwise, the combination of *GSTM1* entire gene deletion and (GG) genotype of *GSTP1* polymorphism (rs1695) was significantly associated with BC with p-value <0.05 (i.e. p-value was not significant after correcting for multiple comparison).

Conclusion: We suggest that the interaction between *GSTM1* polymorphism and rs1695 of *GSTP1* may influence BC development and progression among Jordanian women. More epidemiological studies are needed to provide a baseline for the underlying role of GSTs polymorphisms in tumorigenesis.

Keywords: breast, cancer, genetic variation, GST genes, polymorphisms

Introduction

Breast cancer (BC) occurs when the multiplication of specific cells in the breast becomes abnormal and uncontrollable, leading to tumor formation.[1] It accounts for 16% of invasive cancers in women worldwide and is responsible for 18.2% of all cancer deaths.[2] Despite the high mortality rate, BC incidence and survival rates vary between developed and developing countries, as evidenced by the fact that North American survival rates exceed 80% compared to those that fall below 40% in low-income countries.[3] As well, BC is the most common female cancer in Jordan, accounting for 37.3% of all cancers in Jordanian women.[4]

A range of different factors contribute to increasing the risk of developing BC including an individual's lifestyle, environment, and genetic makeup.[5] Nonetheless, the bulk of BC research has been directed towards the role of inherited factors, mainly genetic polymorphisms in critical genes, in the development of this particular form of cancer.[6]

Glutathione S-transferases (GSTs) are a family of essential Phase II detoxification enzymes that protect against oxidative stress by catalyzing the conjugation of

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glutathione and xenobiotic compounds.[7] The soluble dimeric is one of the GSTs superfamilies that found mainly in the cytosol and comprise eight classes d Alpha, Kappa, Mu, Pi, Sigma, Theta, Zeta and Omega. Individuals defect the expression of GST-M1, GST-T1 and GST-P1 enzymes are more susceptible to different types of cancers such as breast, colorectal and lung cancers.[8] Three common polymorphic genes of this family (*GSTM1, GSTT1*, and rs 1695 of *GSTP1*) have been widely investigated in the context of BC research.[9] It has previously been reported that whole gene deletion of both the *GSTM1* and *GSTT1* genes in addition to the rs1695 of *GSTP1* gene could be responsible for BC progression.[10] rs1695 is a single nucleotide polymorphism (SNP) at codon 105 in the *GSTP1* gene that is responsible for the substitution of isoleucine amino acid with valine. The degree of involvement of the aforementioned GST genetic variants with BC varies among different ethnic groups.[11] In this regard, a study by Helzlsouer et al revealed that the deletion of both *GSTM1* and *GSTT1* genes as well as the (AG) genotype of *GSTP1 (*rs1965) might be associated with BC risk among White Americans.[12] In contrast, another study conducted on the Thai population indicated no significant influence of the *GSTM1* and *GSTT1* deletion genotypes and the rs1695 of *GSTP1* on BC progression.[13]

This study focus on three polymorphic well-identified genes *GSTM1* (deletion), *GSTT1* (deletion) and *GSTP1* (rs1695) in which polymorphisms were extensively investigated with respect to cancer in molecular epidemiologic research. The variability in GST function is attributed to the polymorphisms in these GST. Therefore, characterizing the frequencies of these polymorphisms within Jordanian population from Arab descent and compare it to other population may help to understand the exact role of GST in cancer development and progression.

Materials And Methods

Study Population

This case-control study included 241 patients diagnosed with BC in addition to 219 unrelated healthy females with no family history of BC disease and was approved by the Human Ethics Committee at Jordan University of Science and Technology. Samples from Jordanian BC patients were collected from the Jordanian Royal Medical Services (JRMS) hospital. In addition, 219 samples from randomly selected healthy Jordanian women were recruited from the blood bank at JRMS. Both the patients and the controls were age- and gender-matched and came from the same ethnic background (Arab).

GSTM1, GSTT1, And GSTP1 (rs1695) Genotyping

For each sample, the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA) was used to extract genomic DNA from 5 mL of blood according to the manufacturer's instruction. DNA quantity (ng/μL) and purity (A260/280) were verified using the NanoDrop™ spectrophotometer. For both the *GSTM1* and *GSTT1* genes, genotypes were assessed by detecting the presence or absence of each gene.

Traditional PCR was carried out to genotype each gene using specific sets of primers (Table 1).[14] PCR protocol was conducted in the following manner: 12.5 μL of ready-made master mix plus 10 μL of deionized water

Table 1 Primer Sequences And Predicted Product Sizes Of PCR Amplified Products Of The *GST* Genetic Variants

Polymorphic Loci	Primer Name And Oligonucleotide Primer Sequences	Amplicon Size (bp)
GSTT1 Gene	GSTT1_GENE_F: 5′-TCTTTTGCATAGAGACCATGACCAG-3 GSTT1_GENE_R: 5′-CTCCCTACTCCAGTAACTCCCGACT-3′	969
GSTT1 Deletion	GSTT1_DEL_F: 5′-GAAGCCCAAGAATGGGTGTGTGTG-3′ GSTT1_DEL_R: 5′-TGTCCCCATGGCCTCCAACATT-3	3106
GSTM1 Gene	GSTM1_GENE_F: 5′-CAAATTCTGGATTGTAGCAGATCATGC-3′ GSTM1_GENE_R: 5′-CACAGCTCCTGATTATGACAGAAGCC-3′	625
GSTM1 Deletion	GSTM1_DEL_F:5-′AAGACAGAGGAAGGGTGCATTTGATA-3′ GSTM1_DEL_R:5′-ACAGACATTCATTCCCAAAGCGACCA-3′	4748
GSTP1(rs1695)	GSTP1_SNP_F: 5′-TCCTTCCACGCACATCCTCT-3′ GSTP1_SNP_R: 5′-AGCCCCTTTCTTTGTTCAGC-3′	436

Note: GSTP (rs1695) responsible for the substitution of Isoleucine amino acid with valine.

were added to a 25 µL reaction tube, followed by 2 µL of each forward and reverse primer in addition to 50 ng of the purified DNA.

The amplified conditions for *GSTM1* presence, *GSTT1* presence, *GSTT1* deletion and the (rs1695) of *GSTP1* (A/G) were carried out as previously described.[15] On the other hand, *GSTM1* deletion was detected by a PCR program involving an initial denaturation of 3 mins, 35 cycles of denaturation (30 s of at 94°C), annealing (1 min at 64.5°C), and extension (1:30 mins at 68°C), and a final extension for 10 mins at 72°C. PCR products were separated using 1.5% agarose gel and then visualized under UV light. SNP detection within the *GSTP1* gene was done by using PCR followed by restriction fragment length polymorphism (RFLP).[16]

Statistical Analysis

The Hardy–Weinberg equilibrium equation was used to calculate the genotypic and allelic frequencies, while Pearson's chi-squared and ANOVA tests were used to perform the genetic association analysis. In terms of P values, the level of significance was taken as $P<0.05$. The Statistical Package for the Social Sciences (SPSS) version 21.0 was used to perform all analyses.

Correction For Multiple Testing

Method of Li and Ji (2005) was used to estimate the effective number of genetic variants (N_{em}),[17] which employs a modification of an earlier approach by Nyholt (2004).[18] Modified Bonferroni procedure was applied to determine a target alpha level ($0.05/N_{em}$) that would maintain an overall significance level of 0.05 or less.

Results

Sample Characteristics

This study involved 241 breast cancer female patients selected from Jordanian population in addition to 219 healthy subjects. Both the cases and controls were randomly chosen and adjusted to be matched with regard to age, sex, and ethnic origin and were all 100% native Arab ancestry (genetically homogenous). The study cohorts were analyzed and summarized in previously published study.[4] However, the demographical clinical and pathological data were available for 230 patients. Briefly, patients' ages ranged from 24 to 95 with the average (±SD) of 53.9 ± 12.777 years, while the average age of controls was 50.4 ± 12.607 years and ranged from 24 to 90 years.

Clinico-Pathologic Features Of Breast Cancer (BC) Patients

Table 2 shows several features of BC including clinical and pathological. The majority (67.89%) of participants were older than 45 years old at first diagnosis with BC. In addition, most of the patients had gotten pregnant and breastfed from an early age (less than 20 years old) (83%), while 72.49% of the cases experienced menarche at an age older than 13 years old. Furthermore, pathological parameters including histopathological characteristics, progesterone (PR) and estrogen receptors (ER) status, lymph node involvement, axillary lymph nodes metastatic, and tumor size were considered in this study. We found that 76.7% of the cases expressed the ER receptor while 48.9% of them were PR positive. Moreover, 90% of patients were diagnosed with low tumor grade compared to 10% who had high grade of tumor. The tumor size of patient was extracted from their medical records, we estimated that 74% of BC patients had tumor size of more than 2 cm. Our results revealed that 51.3% of the patients had axillary lymph nodes free of tumor, whereas 48.67% of the BC patients were diagnosed with metastatic carcinoma in the axillary lymph nodes.

DNA Genotyping And Gel Electrophoresis

The gel electrophoresis technique was used to detect the genotype for the *GSTM1, GSTT1*, and *GSTP1* genetic variants. *GSTM1* genotyping was based on the detection of a 625 bp band of one or both gene copies while the 4748 bp band indicated the absence of one or both gene copies. Figure 1 exhibits the three genotypes for the *GSTM1* gene. As for *GSTM1*, the *GSTT1* gene was genotyped in the same manner. Figure 2 demonstrates the three genotypes for the *GSTT1* gene.

The amplicon size of the *GSTP1* gene before digestion was 436 bp. A PCR-RFLP assay was used to detect the SNP within the *GSTP1* gene via the BSMA1 restriction digestion enzyme. Incubating the PCR product with this enzyme gave three different genotypes displayed by gel electrophoresis: (A/A), (A/G), and (G/G). Figure 3 illustrates these genotypes and the corresponding band sizes for each one.

Table 2 Clinical And Pathological Features Of BC Patients (n = 241)

Clinical Parameters	Freq (%)	Pathological Parameters	Freq (%)
Age at BC diagnosis		Estrogen receptor (ER) status	
<45	32.11	Positive	76.74
≥45	67.89	Negative	23.26
Age at first menstruation		Progesterone receptor (PR) status	
<13	27.51	Positive	48.90
≥13	72.49	Negative	51.10
Age at first pregnancy		Tumor differentiation	
<20	83.02	G*1: Low.D	33.48
≥20	39.62	G2+G3: mid and high D	66.52
Breast feeding		Lymph node involvement	
Yes	65.49	Yes	81.78
No	34.51	No	18.22
Allergy		Axillary lymph nodes	
Yes	27.51	Free of tumor	51.33
No	72.49	Show metastatic carcinoma	48.67
Co-morbidity		Tumor stage	
No	46.88	PT1–PT2	90.34
Yes[a]	53.13	PT3–PT4	9.66
Smoking		Histological classification	
Yes	30.09	In situ carcinoma	18.01
No	69.91	Invasive carcinoma	81.99
Family history			
Yes	32.02		
No	67.98		
Menstrual age			
≤50	45.16		
>50	54.84		
Body Mass Index (BMI)			
≤25	25.44		
>25	74.56		

Note: [a]Co-morbid with hypertension, coronary artery disease, asthma, and/or diabetes.

Frequency Distribution And Genetic Association Of GSTM1, GSTT1 And GSTP1 Polymorphism With Breast Cancer (BC)

Genotypic and allelic frequencies were statistically analyzed. Table 3 shows the genotypic and allelic distributions of the *GSTM1, GSTT1*, and *GSTP1* (rs1695) polymorphisms among Jordanian BC patients and controls. Our results indicated that the frequency distributions of the double deletion of *GSTM1* and *GSTT1* genes among Jordanians of Arab descent were 53.4% and 26.9%, respectively, while it was 8.2% for the rs1695 mutant genotype (G/G) within the *GSTP1* gene. Allelic distribution for the *GSTM1, GSTT1*, and rs1695 of *GSTP1* polymorphisms also revealed no statistically significant difference between patients and controls.

Remarkably, the heterozygous genotype frequency (presence/deletion) for the *GSTT1* gene was more frequent than the each of homozygous presence and homozygous deletion genotypes among cases and controls. Furthermore, our finding revealed that the distribution of *GSTM1* double deletion genotype was slightly higher among BC patients (57.7%) than it among controls (53.4%). However, we did not find any correlation between each of *GSTM, GSTT1* and rs1695 of *GSTP1*

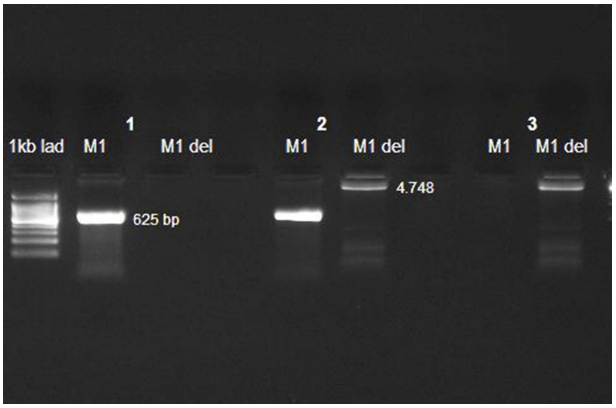

Figure 1 *GSTM1* genotypes detected by agarose gel electrophoresis. Case one represents a homozygous presence indicated by a 625 bp band in the *GSTM1* lane but no band in the *GSTM1* deletion lane. The double deletion is represented by one band (4.748 kpb size) in the*GSTM1* deletion lane (case 3). Case two shows the heterozygous genotype (presence/deletion).

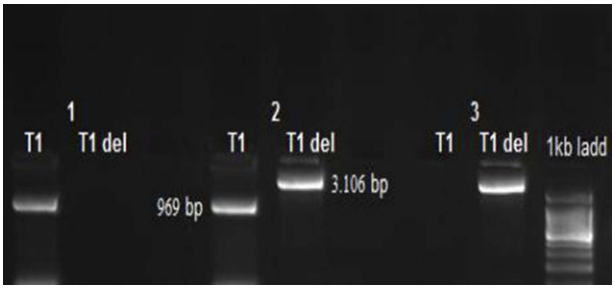

Figure 2 *GSTT1* genotypes detected by agarose gel electrophoresis. Case one represents the homozygous presence indicated by a 969 bp band in the *GSTM1* presence lane but no band in the*GSTT1* deletion lane. The double deletion is represented by one band (3.106 kbp size) in the *GSTM1* deletion lane (case 3). Case two shows the heterozygous genotype (presence/deletion).

and BC risk (P = 0.226, 0.590 and 0.659), respectively (Table 3).

Moreover, we investigated the influence of the combination genotypes between the *GSTM1, GSTT1*, and rs1695 of *GSTP1* on BC risk. As shown in Table 4, we estimated a correlation between a (combined *GSTM1* homozygous presence with *GSTP1* variant genotypes) and BC risk (P = 0.32). We also detected a statistically significant association of combined *GSTM1* heterozygous (presence/deletion) and *GSTP1* (rs1695) genotypes with BC risk (P = 0.021). However, we propose that the combination between *GSTT1* genotypes and *GSTP1* (rs1695) was not involved in BC development or progression (Table 4).

Furthermore, we inspected the genetic association of the investigated GSTs with BC using different genetic models. Table 5 illustrates the different categories of the test and also indicates the chi-squared values. In this study,

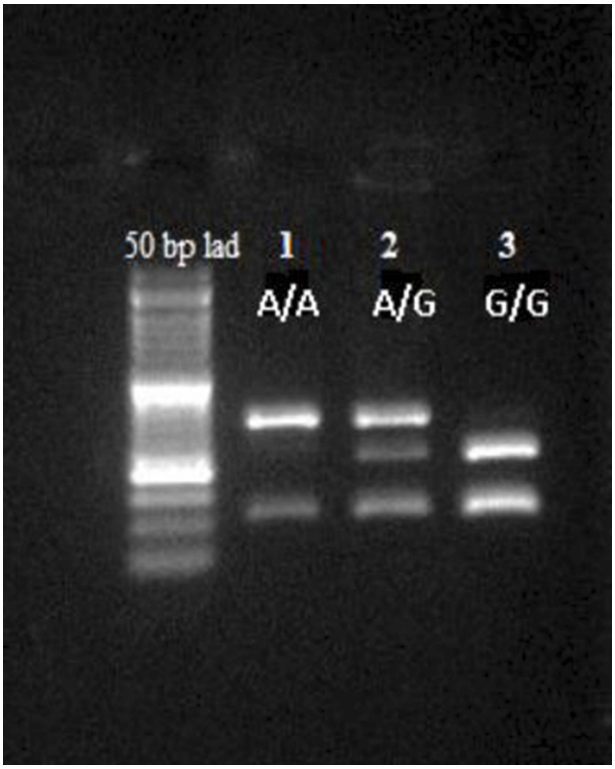

Figure 3 *GSTP1* genotypes detected by agarose gel electrophoresis. Lane 1 represents the wild-type genotype (A/A) with two bands at 329 and 107 bp. Lane 2 illustrates 3 bands at 329, 222, and 107 bp and represents the heterozygous genotype (A/G). Lane 3 depicts the homozygous mutant genotype (G/G) with two bands at 222 and 107 bp.

there was no significant difference between patients and controls for each tested category.

Discussion

BC is a disease that influenced by both genetic and environmental factors. BC susceptibility genes are responsible for the development of 20% to 25% of all BC cases.[19] GSTs, which take part in the cell's detoxification process, comprise three common genes (*GSTM1, GSTT1* and *GSTP1*) that are suggested to be involved in BC progression.[20] BC is a spectrum of many subtypes with distinct biological characteristic. To understand BC treatment personalization, more accomplished analysis and evaluation of the molecular characteristics of the disease in the individual patient are required. However, the interaction between the genetic variants and BC should be more specific because of the stratified etiology of BC.

Studies have shown that specific genetic variants of these three genes (double deletion for both the *GSTM1* and *GSTT1* genes neither the rs1695 within the *GSTP1* gene) are involved in BC risk.[11,12,21] In contrast, other studies have reported that the aforementioned variants

Table 3 Genotypic And Allelic Distributions Of The *GSTM1, GSTT1*, And *GSTP1* (rs1695) Polymorphisms In Jordanian BC Patients (n = 241) And Controls (n = 219)

Gene/Marker	Allele/Genotype	Breast Cancer (%)	Control (%)	χ^2	p-Value*
GSTM1	Presence	27.8	32.9	2.804	0.094
	Deletion	72.2	67.1		
	Presence/presence	13.3	19.2	2.966	0.226
	Presence/deletion	29	27.4		
	Deletion/deletion	57.7	53.4		
GSTT1	Presence	46.9	46.8	0.974	0.974
	Deletion	53.1	53.2		
	Presence/presence	18.3	20.6	1.052	0.590
	Presence/deletion	57.3	52.5		
	Deletion/deletion	24.4	26.9		
GSTP1 (rs1695)	A	71.8	73.5	0.346	0.556
	G	28.2	26.5		
	A/A	51.4	55.3	0.834	0.659
	A/G	40.7	36.5		
	G/G	7.9	8.2		

Note: *p value < 0.005 is considered significant using (χ^2) Chi-square test after the multiple correction analysis.

are not associated with an increased risk of BC.[22–24] Table 6 reviews the most important studies regarding the relationship between BC and the candidate gene variants in different ethnic groups.

The present study's cohort involved 241 female patients and 219 unrelated healthy female controls. It was revealed that 53.4% of the healthy Jordanian population does not have any copy of the *GSTM1* gene, a figure which is close to another finding by previous study within the same population.[15] Meanwhile, 57.7% of all patients did not express both *GSTM1* copies, which is comparable to findings by Yang et al (2004) in Shanghai (55.8%) but different from findings in a Thai population (35.0%).[13] On the other hand, the frequency of the *GSTT1* double deletion polymorphism among BC patients was 24.4%, which is incompatible to what was found in the Brazilian (58.8%),[1] Thai (41.9%),[13] and Californian (82%) populations.[10] In this study, we proposed that both *GSTM1 and GSTT1* genes were not significantly related to BC risk among Jordanian women.

On the other hand, the frequency of the *GSTP1* (AG) genotype was also estimated as a part of the present study. Interestingly, we found that the mutant genotype (GG) among controls (8.2%) is slightly higher than it is in patients (7.9%). Nevertheless, there was no statistical association between the *GSTP1* (GG) genotype and increased BC susceptibility, which is in concordance with the frequencies in the Thai population but in contrast to those in the Turkish,[25] Chinese,[26] and Washingtonian populations.[12] In this work, we deduce that the rs1695 of *GSTP1* gene was not involved in BC risk among Jordanian females.

Additionally, the association of the combined *GSTM1* and *GSTT1* polymorphisms with rs1695 of *GSTP1* genotypes with BC risk was conducted. In this regard, there are only few studies that inspect the interaction between GST genetic variants within different genes. While the *GSTM1* polymorphism alone was not related to BC, our results demonstrated that the combined *GSTM1* and *GSTP1 (rs1695)* genotypes might influence BC risk among Jordanian females, which is contrary to reports for the Chinese population.[26] A possible explanation for the divergence between findings among different studies inconsistent results could be that other members of the GST family or other enzymes involved in similar chemical detoxification compensate for the deletion of a functional GSTM1 enzyme. In addition, this gene has not face a

Table 4 Distributions of the Combination Genotypes of GSTM1, GSTT1 and Rs1695 of GSTP1 Polymorphisms among Jordanian BC Patients (n = 241) and Controls (n = 219)

Gene Markers	Genotypes Combination	Breast Cancer (%)	Controls (%)	χ^2	p-value*
Combination genotypes between *GSTM1*& (rs1695) of *GSTP1*	Presence/presence A/A A/G G/G	5.4 7.1 0.8	13.2 4.6 1.4	6.884	0.032
	Presence/deletion A/A A/G G/G	17 10 2.1	10.5 13.7 3.2	7.726	0.021
	Deletion/deletion A/A A/G G/G	28.6 24.1 4.9	31.1 19.2 3.2	2.01	0.367
Combination genotype between *GSTT1*& (rs1695) of *GSTP1*	Presence/presence A/A A/G G/G	10.4 6.2 1.7	13.2 6.4 0.9	0.986	0.611
	Presence/deletion A/A A/G G/G	30.7 22.4 4.1	27.4 20.5 4.6	0.192	0.908
	Deletion/deletion A/A A/G G/G	11.2 11.6 1.7	14.6 9.6 2.7	1.824	0.402

Note: *p value < 0.005 is considered significant using Chi-squared test after the multiple correction analysis.

Table 5 Genetic Association Analysis Of *GSTM1, GSTT1*, And *GSTP1* (1695) In BC Patients And Controls Using Different Genetic Models

Gene	Category test	Odd ratio	95% CI	χ^2*	P-Value*
GSTM1	Presence/deletion vs presence/presence	0.65	0.37–1.16	2.12	>0.05
	Deletion/deletion vs presence/deletion	0.98	0.64–1.5	0.01	>0.05
	Deletion/deletion vs presence/presence	0.64	0.38–1.08	2.81	>0.05
GSTT1	Presence/deletion vs presence/presence	0.81	0.5–1.32	0.69	>0.05
	Deletion/deletion vs presence/deletion	1.2	0.77–1.86	0.67	>0.05
	Deletion/deletion vs presence/presence	0.98	0.56–1.7	0.01	>0.05
GSTP (rs1695)	A/G vs A/A	1.2	0.81–1.76	0.82	>0.05
	G/G vs A/G	0.86	0.42–1.75	0.17	>0.05
	G/G vs A/A	1.03	0.52–2.06	0.01	>0.05

Notes: *For significant association χ^2 should be >3.84 with P<0.05. CI indicates confidence interval.

Table 6 Genetic Association Studies Of Breast Cancer (BC) Among Different Populations

Population	Gene Symbol	Polymorphism	No. Case/ Control	Association	Reference
Thai	*GSTM1* *GSTT* *GSTP1* (rs1695)	Deletion Deletion A/G	43/56	No No No	13
Brazilian	*GSTM1* *GSTM1*	Deletion Deletion	105/278 49/49	No Yes	20 11
French	*GSTM1*	Deletion	92/–	No	21
Korean	*GSTM1* *GSTT1*	Deletion Deletion	176/118	Yes yes	19
American (Washington)	*GSTM1* *GSTT* *GSTP1*(rs1695)	Deletion Deletion A/G or G/G	115/115	Yes Yes Yes	12
Iranian	*GSTM1*	Deletion	59/59	No	22
Chinese (Shanghai)	*GSTM1* *GSTT1* *GSTP1*(rs1695) Combined *GSTM1* and *GSTP1* (rs1695) Combined *GSTT1* and *GSTP1* (rs1695)	Deletion Deletion A/G GSTM1 deletion and (rs1695) of *GSTP1* *GSTT1*deletion and (rs1695) of *GSTP1*	1034/–	No No Yes No No	23
Turkish	*GSTM1* *GSTT* *GSTP1*(rs1695)	Deletion Deletion A/G or G/G	264/233	No No Yes	24

strong environmental selection pressure during evaluation.[27,28] However, the genetic interaction between the *GSTT1* and *GSTP1* polymorphisms was not related to BC risk among Jordanian women. Comprehensive epidemiological studies regarding the genetic variations of GSTs among populations and ethnicity are key for implementing the individualization of treatment depending on individual genetic background.

Much significance can be derived from these results, as they help shift the focus of cancer therapy from blanket treatments to more efficient protocols tailored to an individual's genetic makeup. It can be concluded from the results of the present study that there is no relationship between the genetic variants among the studied GST genes (*GSTM1, GSTT1*, and rs1695 of *GSTP1*) and BC risk. However, we proposed that the combination between *GSTM1* and *GSTP1* polymorphism may be implicated in BC development and progression among Jordanian females of Arab descent. Bearing in mind the accelerated rates of BC incidence around the world, more studies should focus on the nature of this disease in developing countries especially so as to adequately address the impact of the genetic etiology of BC.

Ethical Statement

Ethical approval was obtained from the Intuitional Review Board (IRB) at Jordan University of Science and Technology. Written informed consent was obtained from all participants in this study. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Disclosure

The authors report no conflicts of interest in this work.

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