RESEARCH ARTICLE

Editorial Process: Submission:08/09/2024 Acceptance:03/11/2025

Genetic Polymorphism of *GSTM1, GSTT1, GSTP1* Genes and Breast Cancer Risk in Rural Maharashtra: Insights from a Case- Control Study

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Abstract

Background: Breast cancer (BC) is a complex, multifactorial disease where genetic factors are one of the key determinants playing an important role in carcinogenesis process. The discrepancies in the reports all around the world in relation with the association of polymorphisms of glutathione S- transferase (GST) genes with BC risk encouraged us to assess the correlation of polymorphism in GST gene isoforms with BC susceptibility in the rural population of Maharashtra. **Methods:** The association of *GSTM1* and *GSTT1* gene polymorphisms with BC risk was studied by polymerase chain reaction (PCR) method using 400 clinically confirmed BC cases and equal number of healthy controls. The *GSTP1* Ile/Val of exon 5 and Ala/Val of exon 6 polymorphism was determined by PCR followed by restriction fragment length polymorphism (PCR-RFLP). The logistic regression model was used to study the association of polymorphism with BC risk which was confirmed by Odds ratio (OR) with 95% confidence interval. **Results:** The frequency distribution of *GSTT1* showed contributory increase of BC risk in association with null genotypes (OR = 2.45; 95%CI = 1.73–3.48, p<0.0001) where, *GSTT1* null (-/-) genotypes increased risk of BC by 2.45 folds in the studied population. The results of genetic association analysis of *GSTP1* showed that heterozygous Ala/Val genotype of *GSTP1* was associated with decreased risk of BC (OR=0.26, 95% CI: 0.18-0.35; p<0.0001, $\chi^2 = 71.48$) in the studied population. **Conclusion:** Our results indicated that *GSTT1* null genotype was significantly associated and *GSTP1* heterozygous variant genotype was negatively associated with BC risk in women of rural Maharashtra.

Keywords: Breast Cancer- *GSTM1*- *GSTT1*- *GSTP1*- PCR-RFLP

Asian Pac J Cancer Prev, **26 (3)**, 829-837

Introduction

Breast cancer (BC) is the world's most prevalent cancer among women which has surpassed all other cancers as a leading cause of global cancer incidence in 2020 with an estimated 2261, 419 (24.5%) new cases of all age females and 684, 994 deaths accounting 15.5% of all cancers cases [1]. As per Global Cancer Observatory data, BC accounted largest cause of cancer deaths in India where 13.5 % (178, 361) of new cases and 10.6% (90,408) deaths were reported in 2020 [2]. It is a challenge to reduce BC burden in India as compared to Western countries because of early onset age, late disease presentation stage and delayed and inadequate management [3]. The etiology of BC is complex which is resulted from interactions of genetic and environmental risk factors where genetic factors play a key role in determining host susceptibility towards developing BC [4-5]. It has been hypothesized that genetic polymorphism in genes involved in DNA repair pathway and carcinogen metabolism increase the risk of BC in susceptible population. Glutathione S-Transferases (GSTs) are a family of phase II metabolizing enzymes that play a crucial role in detoxification of wide range of endogenous reactive oxygen species as well as exogenous toxic and carcinogenic electrophillic compounds. *GSTM1*, *GSTT1*, and *GSTP1* are members of the GST family that play a vital role in preserving genomic integrity by regulating the activation of related enzymes and other protein molecules involved in the cellular DNA repair pathway.

The genetic polymorphisms are the genetic variations occurred in two or more alleles of any gene occur in different populations which may influence the susceptibility of individuals towards diseases. The gene

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polymorphisms are grouped into different types based on single base pair change which lead to single nucleotide polymorphism (SNP), copy number variations (CNVs) leading to deletion or duplication of large fragment of DNA or variable number of tandem repeats (VNTR) with repetition of short repeats of tandem. The GSTs exhibit polymorphism, and variations in these enzymes can lead to dysfunction, resulting in a decreased ability to detoxify a wide range of carcinogens and reactive oxygen species. Genetic polymorphisms in *GSTM1*, *GSTT1*, and *GSTP1* may serve as potential risk modifiers by increasing an individual's susceptibility to carcinogenesis through reduced metabolism of pro-carcinogens and carcinogens. Polymorphisms in *GSTM1* and *GSTT1* may lead to gene deletion which causes absence of metabolic enzyme activity in individuals with *GSTM1* and *GSTT1* null genotypes. *GSTP1* polymorphism with single nucleotide substitutions in exon 5 with Ile105Val and exon 6 with Ala114Val amino acid substitution are also known. Thus, individuals with polymorphic GSTs with reduced or no enzymatic activity might be at higher risk of developing cancer due to reduced detoxification of carcinogenic compounds.

Earlier, epidemiological studies have reported that polymorphism in GST genes were associated with risk of several cancers including lung [6-9], bladder [10-11], liver [12] , gastric [13-14], cervix [15-16] and head and neck cancer [17]. However, other studies refused to agree with any association of *GSTM1*, *GSTT1* or *GSTP1* with variety of malignancies such as pancreas [18], lung [19], gastric [20], cervix [21] and prostate cancer [22-24]. Several other studies investigated relationship between *GSTM1*, *GSTT1* null genotypes and *GSTP1* polymorphism and their association with BC risk [25-28], but others reported contradictory results with no association of either *GSTM1 GSTT1* or *GSTP1* gene polymorphism with BC risk [29-30]. Similar studies conducted in India have shown an association between *GSTM1* and *GSTT1* null polymorphisms and an increased risk of BC in North Indian Population [31-32]; however other research found no such association among South Indian women [33].

The cited literature highlights that numerous studies across various populations have demonstrated the role of GST gene polymorphisms in influencing an individual's susceptibility to carcinogenesis, however; other studies have presented conflicting findings, either within the same populations or in different ones. Therefore, establishing a significant influence of GST gene polymorphisms on breast carcinogenesis remains challenging, as opinions on this matter are still inconclusive. The impact of genetic variations in *GSTM1*, *GSTT1*, and *GSTP1* on BC development in Maharashtrian women has not yet been explored and remains unknown. We hypothesized that polymorphisms in GST genes might be linked to an increased risk of breast cancer. Therefore, in this study, we aimed to investigate the association between individual and combined genotypes of *GSTM1*, *GSTT1*, and *GSTP1* gene polymorphisms and BC risk in rural women from South-Western Maharashtra.

Materials and Methods

Study design

The present hospital based case-control study comprised n=400 histopathologically confirmed BC cases and equal number of healthy unrelated controls. The inclusion criterion for the cases was presence of histopathologically diagnosed BC and no previous chemotherapy or radiotherapy treatment. Patients receiving preoperative chemotherapy or radiotherapy were excluded from the study. The inclusion criteria for the controls was absence of prior history of cancer and no history of hysterectomy or mastectomy. The healthy controls were recruited from women donors who accompanied patients seeking treatment or volunteers attending to the hospital for blood donation. All the patients ranged in age from 23-85 years (Mean ± SD) (52.43 ±12.40) were recruited from the Krishna Institute of Medical Sciences during year 2017-2020. The sample size was determined by the formula n= [(p1 x q1) + (p2 x p2)] X (Z1-α/2) +Z1-β)2/ (p1-p2)2; Where p1- presence of allele1, q1- absence of allele1, p2- presence of allele 2, q2- absence of allele 2, α- probability of detecting false results, β- power. After obtaining written informed consent all eligible cases and controls were individually interviewed using a structured questionnaire to collect demographic and other clinical data. The data pertaining to histopathological diagnosis and clinical staging were collected from hospital records. This study was reviewed and approved by the Institute Ethics Committee of Krishna Institute of Medical Sciences.

Study methods

Genomic DNA extraction and Genotyping assays

Five milliliter (mL) of intravenous blood from patients and controls was collected in ethylenediaminetetraacetic acid (EDTA) containing vacutainer. Genomic DNA extraction was carried out from the peripheral blood sample using HiPurA blood genomic DNA miniprep purification kit ((MB504; HiMedia Laboratories) following the manufacturer's instructions and used for polymorphism studies.

The genotyping of *GSTM1* and *GSTT1* were performed by polymerase chain reaction (PCR). The PCR amplification of *GSTM1* and *GSTT1* were carried out separately in 20 microliter (µL) reaction mixtures containing 1X PCR buffer 0.2 mM each dNTP, 10 picomole (pmol) of each primers (IDT technologies) , 1U Taq DNA polymerase (GeNei, Merck Bioscience) and 100 nanogram (ng) of purified genomic DNA. The primers selected to amplify the *GSTM1*; forward primer (FP): 5′- CAA ATT CTG GAT TGT AGC AGA TCA TGC-3′, reverse primer (RP): 5′-CAC AGC TCC TGA TTA TGA CAG AAG CC -3′ and *GSTT1*; FP: 5'- TTC CTT ACT GGT CCT CAC ATC TC-3', RP: 5'- TCA CCG GAT CAT GGC CAG CA-3'. The PCR conditions for amplification of 625 bp fragment of *GSTM1*: Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 30 seconds (sec) , 56°C- 30 sec, 72°C- 30 sec and final extension at 72°C for 10 min. The conditions for *GSTT1* of 480 bp: Initial denaturation at 95°C for 5 min followed by

30 cycles of 95°C- 30 sec, 60°C- 30 sec, 72°C- 30 sec and final extension at 72°C- 10 min. After performing PCR program for each reaction with a Master Cycler Gradient PCR (Eppendorf), the PCR products were checked by agarose gel electrophoresis in Tris-Acetate-EDTA (TAE) buffer thereafter stained with ethidium bromide (10 mg/mL) and visualized under UV-transilluminator and photographed in gel documentation system (BioRad Laboratories). The nonfunctional allele homozygous null for *GSTM1* and *GSTT1* was evidenced by the absence of gene fragment, and presence of gene was indicated by amplification gene fragment in the PCR. The *GSTP1* Ile/Val of exon 5 and Ala/Val of exon 6 polymorphism was determined by PCR followed by restriction fragment length polymorphism (PCR-RFLP). The exon 5 and 6 of *GSTP1* were amplified by using specific primers FP: 5'-AGC CAC CTG AGG GGT AAG-3', RP: 5'-GGG AGC AAG CAG AGG AGA AT-3 and FP: 5'-GTA GTT TGC CCA AGG TCA AG-3' & RP: 5'-CAG GTT GTA GTC AGC GAA GGA G-3' respectively. The PCR cycling conditions for amplification of 433 bp fragment of *GSTP1* Ile105Val: Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 20 seconds (sec) , 55°C- 20 sec, 72°C- 20 sec and final extension at 72°C for 10 min) and 420 bp of *GSTP1* Ala114Val : Initial denaturation at 95°C for 5 minutes (min) followed by 30 cycles of 95°C- 30 seconds (sec) , 57°C- 20 sec, 72°C- 30 sec and final extension at 72°C for 10 min) respectively. The PCR amplicon of 433bp of exon 5 was subjected to restriction digestion using BsmAI enzyme (New England Biolabs) at 37°C for 1 hour. Following restriction digestion the products were separated on 3% agarose (GeNei, Merck Biosciences) gel. Complete digestion of *GSTP1* exon 5 with BsmAI was characterized by wild type (Ile/Ile) genotype with two bands 328 bp & 105 bp; heterozugous (Ile/Val) genotype with 4 bands 328 bp, 222 bp, 106 bp & 105 bp and variant (Val/Val) genotype with 222 bp,106 bp & 105bp). Similarly restriction digestion of *GSTP1* exon 6 with AciI was characterized by Ala/Ala genotype with three bands 246 bp, 116 bp & 58 bp; Ala/Val genotype with 4 bands 362 bp, 246 bp, 116 bp & 58 bp and Val/Val genotype with 362 bp & 58 bp.

Statistical Analysis

The chi-square test was used to test the deviations from Hardy-Weinberg equilibrium in the genotype frequencies of the polymorphism in controls along with the differences in demographic variables between cases and controls which are summarized as Mean ± SD. The association between the *GSTM1*, *GSTP1* and *GSTT1* genotypes and risk of developing BC were studied by odds ratio (OR). Logistic regression model was used to calculate the OR and 95% confidence intervals (CI) with adjustment of variables to determine the BC risk associated with genotypes. All P values were two-sided and differences were considered statistically significant for $p \leq 0.0001$. All statistical analyses were performed with SPSS (Version 11.0).

Results

Demographic characteristics of breast cancer cases and healthy controls

The case-control study was conducted comprising 400 BC cases and 400 age matched controls where, mean ± SD age of cases was 52.43 ±12.40 (Median age: 50; age range: 23-85) and that of control was 42.37 ±13.90 (Median age: 40; age range: 24-81) with no much difference in age distribution between cases and controls (p =0.01). In the present study, significant occurrence of BC development (80.5%) was observed in rural women at the age 40 years and above. When we checked the tobacco habit status, 54.75 % of the cases were tobacco users and 45.20% were non-users, whereas in the control group 28.25 % were tobacco users and 71.25% were non-users thus, we observed significant relation with BC (OR 3.07; 95%CI, 2.29-4.12; p<0.0001) in women of rural population. There was no significant difference between cases and controls in their diet (OR 1.57; 95%CI, 1.14-2.17; p=0.05) and economic status (OR 0.74; 95%CI, 0.55-1.01; p=0.06). Of these 400 BC cases, 299 (74.75%) were diagnosed with invasive ductal carcinoma, 27 (6.75%) had medulary

Table 1. The Genotype Frequency Distribution of *GSTM1, GSTT1* and *GSTP1* Genes and Their Association with Breast Cancer Risk in Breast Cancer Cases and Healthy Controls

Gene	Genotype	Cases N=400 n (%)	Control N=400 n (%)	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
GSTM1	Present	255 (63.75)	285 (71.20)	1 (Reference)		1 (Reference)	
	Null	145 (36.25)	115 (28.80)	1.40 (1.04-1.89)	0.023	1.61 (1.16-2.23)	0.004
GSTT1	Present	259 (64.75)	320 (80.00)	1 (Reference)		1 (Reference)	
	Null	141 (35.25)	80 (20.00)	2.17 (1.58-2.99)	<0.0001*	2.45 (1.73-3.48)	<0.0001*
GSTP1	Ile/Ile	239 (59.75)	253 (63.20)	1 (Reference)		1 (Reference)	
Exon-5	Ile/Val	131 (32.75)	131 (32.80)	1.05 (0.78-1.42)	0.709	1.11 (0.80-1.53	0.534
A>G	Val/Val	30 (7.50)	16 (4.00)	1.98 (1.05-3.73)	0.033	2.13 (1.05-4.35)	0.03
	Ile/Val+ Val/Val	161 (40.25)	147 (36.80)	1.15 (0.87-1.54)	0.309	1.23 (0.91-1.67)	0.185
GSTP1	Ala/ Ala	326 (81.50)	214 (53.50)	1 (Reference)		1 (Reference)	
Exon-6	Ala /Val	49 (12.25)	169 (42.25)	0.19 (0.13-0.27)	0.033	0.17 (0.11-0.25)	0.001
C>T	Val/Val	25 (6.25)	17 4.25)	0.96 (0.51-1.83)	0.914	0.91 (0.47-1.75)	0.776
	Ala /Val+ Val/Val	74 (18.50)	186 (46.50)	0.26 (0.18-0.35)	<0.0001*	0.24 (0.17-0.33)	<0.0001*

OR, Odds ratio; CI, Confidence interval; *, Indicates significance (p<0.001); p value determined based on χ^2, 1.0 (Reference)

carcinoma, 12 (3%) had mucinous and invasive apocrine carcinoma and 15 (3.75 %) had lobular carcinoma. Most of the BC patients 205 (51.25%) were in >III stage histological grade and 195 (48.75%) were in stage I and stage II. When hormone receptor status was considered, out of 400 cases, 218 (54.50) were positive for estrogen receptor (ER), 197 (49.25) were progesterone receptor (PR) positive and 57 (14.25) were human epidermal growth factor receptor 2 (Her2) positive and 343 (85.75) were negative. Out of these, 134 (33.50%) showed triple negative status for these prognostic markers.

Genotype frequency distribution of GSTM1, GSTT1 and GSTP1 genes and risk of breast cancer

The genotype frequency distribution of *GSTM1*, *GSTT1* and *GSTP1* in BC cases and age matched controls was determined using logistic regression analysis in order to find out their association with BC. The prevalence of *GSTM1* null genotype was 36.25% and 28.80% in cases and controls respectively; whereas the frequency of GSTT null genotype was 35.25% in cases and 20% in controls. The genotype distributions of *GSTM1* and *GSTT1* null genotypes were in Hardy-Weinberg equilibrium. The distribution of homozygous *GSTM1*, *GSTT1* and their null genotype frequency among BC cases and healthy controls did not deviate from the Hardy-Weinberg equilibrium as shown in Table 1. The frequency distribution of *GSTM1* and *GSTT1* showed contributory increase of BC risk in association with null genotype (*GSTM1*: OR = 1.40; 95%CI = 1.04–1.89, p = 0.02, *GSTT1*, OR = 2.45; 95%CI = 1.73–3.48, p<0.0001) as compared to the subjects with *GSTM1* and *GSTT1* gene. In spite of the frequency of *GSTM1* null genotype was high in BC patients than healthy controls but it was not statistically significant. It was found that, the *GSTT1* null genotype frequency was significantly higher in BC cases than the controls which signifies significant relation of *GSTT1* null genotypes with risk of BC (p<0.0001) where, *GSTT1* null (-/-) genotypes increased risk of BC by 2.45 folds in the studied population. When we studied genotypic distribution of Ile105 Val and Ala114Val of *GSTP1*, we noted that *GSTP1* (Ile105 Val) showed allelic frequency of 59.75, 7.50,

Table 2. Combined Genotypes of *GSTM1, GSTT1* and *GSTP1* and Relative Risk of Breast Cancer

Gene & Genotype		Cases N=400 n (%)	Control N=400 n (%)	Crude OR	95% CI	p value
GSTM1	*GSTP1* Ex-5					
+/+	Ile/Ile	155 (38.75)	183 (45.75)	1 (Reference)		
+/+	Ile/Val	82 (20.50)	93 (23.25)	1.04	0.72-1.50	0.829
+/+	Val/Val	17 (4.25)	9 (2.25)	2.23	0.96-5.14	0.068
-/-	Ile/Ile	84 (21.00)	70 (17.50)	1.41	0.96-2.07	0.074
-/-	Ile/Val	48 (12.00)	38 (9.50)	1.49	0.92-2.40	0.1
-/-	Val/Val	14 (3.50)	7 (1.75)	2.36	0.92-5.99	0.07
GSTM1	*GSTP1* Ex-6					
+/+	Ala/Ala	204 (51.00)	164 (41.00)	1 (Reference)		
+/+	Ala/Val	34 (8.50)	109 (27.25)	0.25	0.16-0.38	<0.0001*
+/+	Val/Val	17 (4.25)	14 (3.50)	0.97	0.46-2.03	0.948
-/-	Ala/Ala	121 (30.25)	51 (12.75)	1.9	1.29-2.80	0.001
-/-	Ala/Val	15 (3.75)	57 (14.25)	0.21	0.11-0.38	<0.0001*
-/-	Val/Val	9 (2.25)	5 (1.25)	1.44	0.47-4.40	0.515
GSTT1	*GSTP1* Ex-5					
+/+	Ile/Ile	156 (39.00)	206 (51.50)	1 (Reference)		
+/+	Ile/Val	88 (22.00)	104 (26.00)	1.11	0.78-1.58	0.536
+/+	Val/Val	16 (4.00)	13 (3.25)	1.62	0.75-3.47	0.21
-/-	Ile/Ile	83 (20.75)	49 (12.25)	2.23	1.48-3.36	0.0001*
-/-	Ile/Val	42 (10.50)	25 (6.25)	2.21	1.29-3.79	0.003
-/-	Val/Val	15 (3.75)	3 (0.75)	6.6	1.87-23.20	0.003
GSTT1	*GSTP1* Ex-6					
+/+	Ala/Ala	214 (53.50)	176 (44.00)	1 (Reference)		
+/+	Ala/Val	31 (7.75)	129 (32.25)	0.19	0.12-0.30	<0.0001*
+/+	Val/Val	15 (3.75)	15 (3.75)	0.82	0.39-1.72	0.606
-/-	Ala/Ala	111 (27.75)	38 (9.50)	2.4	1.58-3.65	<0.0001*
-/-	Ala/Val	19 (4.75)	40 (10.00)	0.39	0.21-0.69	0.001
-/-	Val/Val	10 (2.50)	2 (0.50)	4.11	0.88-19.01	0.07

OR, Odds ratio; CI, Confidence interval; *, Indicates significance (p<0.001); p, value determined based on $\chi 2$, 1.0 (Reference)

Table 3. Distribution of Double and Triple Combinations of *GSTM1, GSTT1* and *GSTP1* Genotypes and Their association with Breast Cancer

Gene	Genotype	Cases N=400 n (%)	Controls N=400 n (%)	Odds' Ratio (95% CI)	P value
	Double combinations				
GSTM1 and *GSTT1*	Both present (+/+)	152 (38.00)	228 (57.00)	1 (Reference)	
	M1 null −/+	106 (26.50)	92 (23.00)	1.72 (1.22-2.44)	0.002
	T1 null (+/−)	103 (25.75)	57 (14.25)	2.71 (1.84-3.97)	<0.0001*
	Both null (−/−)	39 (9.75)	23 (5.75)	2.54 (1.46-4.42)	0.001
GSTM1 and *GSTP1* (Ex5)	M1 (+/+), P1 (Ile/Ile)	155 (38.75)	184 (46.00)	1 (Reference)	
	M1 (+/+), P1 (Ile/Val+ Val/Val)	100 (25.00)	102 (25.50)	1.16 (0.82-1.64)	0.394
	M1 (−/−),P1 (Ile/Ile)	84 (21.00)	69 (17.25)	1.44 (0.98-2.12)	0.059
	M1 (−/−), P1 (Ile/Val+ Val/Val)	61 (15.25)	45 (11.25)	1.06 (1.06-2.49	0.034
GSTM1 and *GSTP1* (Ex 6)	M1 (+/+), P1 (Ala/Ala)	205 (51.25)	165 (41.25)	1 (Reference)	
	M1 (+/+), P1 (Ala/Val+ Val/Val)	50 (12.50)	122 (30.50)	0.32 (0.22-0.48)	<0.0001*
	M1 (−/−),P1 (Ala/Ala)	121 (30.25)	49 (12.25)	1.98 (1.34-2.93)	0.0006
	M1 (−/−), P1 (Ala/Val+ Val/Val)	24 (6.00)	64 (10.00)	0.30 (0.18-0.50)	<0.0001*
GSTT1 and *GSTP1* (Ex5)	T1 (+/+), P1 (Ile/Ile)	155 (38.75)	205 (51.25)	1 (Reference)	
	T1 (+/+), P1 (Ile/Val+ Val/Val)	104 (26.00)	115 (28.75)	1.19 (0.85-1.67)	0.298
	T1 (−/−),P1 (Ile/Ile)	83 (20.75)	48 (12.00)	2.28 (1.51-3.45)	0.0001*
	T1 (−/−), P1 (Ile/Val+ Val/Val)	58 (14.50)	32 (8.00)	2.39 (1.48-3.87)	0.0004
GSTT1 and *GSTP1* (Ex 6)	T1 (+/+), P1 (Ala/Ala)	215 (53.75)	176 (44.00)	1 (Reference)	
	T1 (+/+), P1 (Ala/Val+ Val/Val)	47 (11.75)	144 (36.00)	0.26 (0.18-0.39)	<0.0001*
	T1 (−/−),P1 (Ala/Val)	111 (27.75)	39 (9.75)	2.32 (1.53-3.53)	0.0001*
	T1 (−/−), P1 (Ala/Val+ Val/Val)	27 (6.75)	41 (10.25)	0.53 (0.31-0.911)	0.021
	Triple Combinations				
GSTM1, GSTT1 and *GSTP1* Ex-5	M1 (+/+), T1 (+/+), P1(Ile/Ile)	96 (24.00)	147 (36.75)	1 (Reference)	
	M1 (+/+), T1 (+/+), P1(Ile/Val+Val/Val)	57 (14.25)	81 (20.25)	1.07 (0.70-1.64)	0.73
	M1 (-/-), T1 (+/+), P1(Ile/Ile)	59 (14.75)	59 (14.75)	1.53 (0.98-2.38)	0.059
	M1 (-/-), T1 (+/+), P1(Ile/Val+Val/Val)	47 (11.75)	34 (8.50)	2.11 (1.27-3.52)	0.004
	M1 (+/+), T1 (-/-),P1(Ile/Ile)	59 (14.75)	36 (9.00)	2.50 (1.54-4.08)	0.0002
	M1 (+/+), T1 (-/-), P1(Ile/Val+Val/Val)	43 (10.75)	21 (5.25)	3.13 (1.75-5.60)	0.0001*
	M1 (-/-), T1 (-/-),P1(Ile/Ile)	25 (6.25)	11 (2.75)	3.48 (1.63-7.39)	0.001
	M1 (-/-), T1 (-/-), P1(Ile/Val+Val/Val)	14 (3.50)	11 (2.75)	1.94 (0.84-4.47)	0.115

(+/+), Present; (-/-), Null; * Indicates significance ($p \leq 0.005$), p value determined based on χ^2, 1.0 (Reference)

Table 3. Continued

Gene	Genotype	Cases N=400 n (%)	Controls N=400 n (%)	Odds' Ratio (95% CI)	P value
GSTM1, GSTT1 and *GSTP1* Ex-6	M1 (+/+), T1 (+/+), P1(Ala/Ala)	128 (32.00)	132 (33.00)	1 (Reference)	
	M1 (+/+), T1 (+/+), P1(Ala/Val+Val/Val)	26 (6.50)	96 (24.00)	0.27 (0.16-0.45)	<0.0001*
	M1 (-/-), T1 (+/+),P1(Ala/Ala)	87 (21.75)	44 (11.00)	2.03 (1.31-3.15)	0.001
	M1 (-/-), T1 (+/+),P1(Ala/Val+Val/Val)	19 (4.75)	48 (12.00)	0.40 (0.22-0.73)	0.002
	M1 (+/+), T1 (-/-),P1(Ala/Ala)	76 (19.00)	32 (8.00)	2.44 (1.51-3.95)	0.0002
	M1 (+/+), T1 (-/-),P1(Ala/Val+Val/Val)	25 (6.25)	25 (6.25)	1.03 (0.56-1.88)	0.92
	M1 (-/-), T1 (-/-),P1(Ala/Ala)	34 (8.50)	13 (3.25)	2.69 (1.36-5.34)	0.004
	M1 (-/-), T1 (-/-),P1(Ala/Val+Val/Val)	5 (1.25)	6 (1.50)	0.85 (0.25-2.88)	0.806

(+/+), Present; (-/-), Null; * Indicates significance (p≤0.005), p value determined based on χ^2, 1.0 (Reference)

32.35 for homozygous wild type (Ile/Ile), homozygous variant (Val/Val) and heterozygous Ile/Val for BC cases and 63.20%, 4%, 32.80% respectively for the controls. The Ala114Val genotype distribution of *GSTP1* showed allelic frequency of wild type 114Ala was 81.50%, variant 114Val was 6.25% and heterozygous Ala/Val was 12.25% for BC cases and that of controls was 53.50%, 4.25% and 42.25% respectively. In order to find out the association of Ile/Val and Val/Val genotypes of exon 5, Ala/Val and Val/Val genotypes of exon 6 of *GSTP1*, we observed that neither of Val/Val (OR = 1.98; 95%CI = 1.05–3.73, P = 0.03, X2 = 1.03) of exon 5 of *GSTP1* nor Val/Val (OR=0.96, 95% CI: 0.51-1.83; p=0.914) genotypes of exon 6 of *GSTP1* showed functional association with BC risk. The heterozygous Ala/Val genotype of *GSTP1* was associated with decreased risk of BC (OR=0.26, 95% CI: 0.18-0.35; p<0.0001, X2 = 71.48). The results of distribution of *GSTP1* (Ile/Val and Ala/Val) genotypes in controls and BC cases are shown in Table 1.

Furthermore, the combination of *GSTM1* null genotype and *GSTP1* (Ala/Val) heterozygous genotypes showed negative association with BC risk (OR=0.21, 95% CI: 0.11-0.38; p<0.0001), similarly the presence of *GSTM1* gene and *GSTP1* variant (Ala/Val) combination showed negative association (OR=0.25, 95% CI: 0.16-0.38; p<0.0001). The combination of *GSTP1* (Ile/Ile) along with *GSTT1* null genotypes revealed two fold increased risk of BC which was statistically significant (OR=2.23, 95% CI: 1.48-3.36; p=0.0001); similarly the *GSTT1* null genotype in combination with *GSTP1* (Ala/Ala) genotype showed two fold elevated risk of BC in the studied population (OR=2.40, 95% CI: 1.58-3.65; p<0.0001), whereas the subjects with *GSTT1* genotype and heterozygous (Ala/Val) genotypes showed negative association with BC (OR=0.19, 95% CI: 0.12-0.30; p<0.0001). The results of combined *GSTM1*, *GSTT1* and *GSTP1* genotypes with relative risk of BC are illustrated in Table 2. Additionally, the double combination effects of *GSTM1*, *GSTT1* and *GSTP1* genotypes were studied, where combined *GSTM1* and *GSTT1* null genotypes showed BC risk as compared to those with both the genes (OR = 1.72; 95%CI = 1.22–2.44, p= 0.002), but with no significance. The study subjects with *GSTT1* null genotype and the *GSTM1* genotype had significant association with increased risk of BC (OR = 2.71; 95%CI = 1.84–3.97, p< 0.0001) as compared to both *GSTM1* and *GSTT1* null genotypes (OR = 2.54; 95%CI = 1.46–4.42, p= 0.001). Double combination of either *GSTM1* null genotype and *GSTP1* (Ile/Val) heterozygous or variant genotypes do not deviate both in cases and controls. On examining the combined effects of *GSTM1* and *GSTP1* (Ala/Val) genotypes, we observed negative association of both *GSTM1* gene and *GSTP1* (Ala/Val) heterozygous genotype (OR = 0.32; 95%CI = 0.22–0.48, p< 0.0001) as well as *GSTM1* null genotype and *GSTP1* (C/T) heterozygous genotype (OR = 0.30; 95%CI = 0.18–0.50, p< 0.0001) with BC development in studied women population. However, when combination of *GSTT1* genotypes was compared with *GSTP1* Ile/Val and Ala/Val genotypes the risk of BC was more prominent in case of *GSTT1* null genotype with wild type Ile/Ile genotype of *GSTP1* at exon 5 (OR

= 2.28; 95%CI = 1.51–3.45, p< 0.0001), and wild type Ala/Ala genotype of *GSTP1* at exon 6 (OR = 2.32; 95%CI = 1.53–3.53, p=0.0001). In case of triple combinations, *GSTM1* (+/+), *GSTT1* (-/-) and *GSTP1* (Ile/Val + Val/Val) showed significant association (OR = 3.13; 95%CI = 1.75–5.60, p=0.0001) with three fold increased risk of BC. However, genotypic combinations of *GSTM1* (+/+), *GSTT1* (+/+) and *GSTP1* (Ala/Val +Val/Val) showed negative relation with developing risk of BC (OR = 0.27; 95%CI = 0.16–0.45, p<0.0001) in studied population. The distribution of double and triple combinations of *GSTM1*, *GSTT1* and *GSTP1* genotypes and their association with BC are represented in Table 3.

Discussion

Breast cancer is a complex and multifactorial disease thought to result from a combination of genetic and environmental factors. Among these genetic factors, genes involved in carcinogen detoxification, such as GSTs, may play a role in the development of breast cancer. In this hospital based case-control study, we investigated the genetic polymorphisms of GSTs and their association of individual or combined genotypes of homozygous null genotypes of *GSTM1* and *GSTT1* with *GSTP1* gene polymorphisms with breast cancer risk among rural women in western Maharashtra. To the best of our knowledge, this is the first study to demonstrate the association of polymorphisms in GSTs genes with BC risk where such large scale studies were not carried out earlier in relation to BC risk from the rural parts of Maharashtra. *GSTM1* and *GSTT1* Null genotypes and their association with variety of cancers are illustrated by numerous studies, but with limited literature on their role with BC risk. Our results observed non significant association of *GSTM1* null genotype with BC risk which are in agreement with several recent studies [29, 34], but in contrast with other Indians [32] and Western populations [30, 35]. There are conflicting views regarding the role of the *GSTT1* null genotype in breast carcinogenesis, with some researchers indicating its association with breast cancer risk in the Asian population [25, 32, 36] whereas others have reported negative findings and do not support the role of *GSTT1* null genotype in breast cancer development in other populations [29-30, 37]. In present study we observed considerable association of *GSTT1* null genotype with BC risk where *GSTT1* (-/-) genotype increased with 2.45 fold (OR = 2.45; 95%CI = 1.73–3.48, p<0.0001) in the studied population which are in accordance with other Indian studies [32] and contrast to other north Indian studies [31] and other Iranian [37] and Mexican [29] studies. In contrast to other Asian women, *GSTP1* genotypes did not show significant association with BC [38], but individuals with combinations of Ala/Val and Val/Val genotypes of exon 6 showed significant negative association with BC risk (<0.0001) in studied population which was in accordance with other Asian BC population [25] Similarly, we also looked into the correlation between the combined genotypes of *GSTM1* (-/-) and *GSTP1* heterozygous (Ala/Val) which showed negative association (OR = 0.21; 95%CI = 0.11–0.38, p<0.0001). We also detected statistically significant association of *GSTT1* (-/-) and *GSTP1* heterozygous (Ala/Val) genotype combinations which showed 2.40 fold increased risk of BC in the studied population. However we detected no significant association of combined *GSTM1* null genotype and *GSTP1* homozygous variant (Val/Val) genotype of exon 5 (OR = 2.36; 95%CI = 0.92–5.99, p=0.070) or variant (Val/Val) genotype of exon 6 (OR = 1.44; 95%CI = 0.47–4.40, p=0.51) which were in accordance with other studies reported in Jordanian women [26] The discrepancies in the literature information of genetic association of GST isoforms mainly *GSTM1*, *GSTT1* and *GSTP1*and susceptibility towards the development of BC allowed us to explore the influence of functional polymorphism in GSTs in relation to BC risk in the susceptible women from the rural population of South-Western Maharashtra. In this study *GSTT1* null genotype showed significant association with BC risk however, no significant positive association of *GSTM1* null genotype or *GSTP1* homozygous variant Val/Val genotypes were conferred the BC in studied population.

In conclusion ours was the first analysis of GST gene polymorphisms and BC risk in rural women population of South-Western Maharashtra. The investigation confirmed the significant association of polymorphism of *GSTT1* (-/-) and negative association of heterozygous Ala/Val genotype of *GSTP1* at exon 6 with BC risk in the studied population of India. The results of this analysis required to be confirmed with large sized cohort studies in order to obtain more precise information and better understanding of the role of the polymorphisms in GSTs in BC susceptibility.

Author Contribution Statement

Concept: KDD, SJB, AKG, RAG, Design: KDD, SJB, AKG, Experimental Studies: PPD, NJJ, ALM Clinical studies: AKG, RAG, Data analysis: PPD, KDD, Statistical analysis: PPD, KDD, Manuscript preparation: KDD, SJB, AKG, RAG. All authors read and approved the final manuscript

Acknowledgements

Funding statement

Authors are thankful to Krishna Vishwa Vidyapeeth (Deemed to be University) for financial assistance to the research project

Approval

The study protocol was approved by protocol committee of Krishna Vishwa Vidyapeeth (Deemed to be University)

Declaration of Conflict of interest

The authors declare that they have no competing financial or any other conflict of interests that could have appeared to influence the work reported in this paper.

Ethics Committee Approval

The study protocol was approved by Institutional Ethics Committee of Krishna Vishwa Vidyapeeth

(Deemed to be University), Karad.

Abbreviations

BC: Breast cancer
GST: Glutathione S-transferase
DNA: Deoxyribose Nucleic acid
EDTA: Ethylenediaminetetraacetate
ER: Estrogen receptor
PR: Progesterone receptor
Her2: Human epidermal growth factor receptor 2
PCR-RFLP: Polymerase chain reaction: Restriction Fragment Length Polymorphism
SNP: Single nucleotide polymorphism
CNV: Copy number variation
VNTR: Variable number of tandem repeats
OR: Odds Ratio
CI: Confidence Interval

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