RESEARCH ARTICLE

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Investigating the Role of Glutathione S- Transferase Genes, Histopathological and Molecular Subtypes, Gene-Gene Interaction and Its Susceptibility to Breast Carcinoma in Ethnic North- Indian Population

Priyanka Gautam[1], Zainab Feroz[1], Sonia Tiwari[2], Sivakumar Vijayaraghavalu[3], Girish C Shukla[4], Munish Kumar[1]*

Abstract

Background: Breast Cancer (BC) is a genetically and clinically heterogeneous disease including complex interactions between gene-gene and gene-environment components. This study aimed, to explore whether the Glutathione S- transferase (*GSTs*) gene polymorphism has role in BC susceptibility. We further evaluated the frequency of four subtypes of BC based on molecular classification followed by microscopic histological analysis to study the grades of invasive ductal carcinoma (IDC). **Materials and Method:** Polymorphism in *GST* genes in North-Indian BC patients was assessed by multiplex-PCR and PCR-RFLP methods. 105 BC patients and 145 healthy controls were enrolled for this study. Data was analyzed by calculating the odds ratio (OR) and 95% CI from logistic regression analyses. **Results:** Our findings revealed that *GSTM1* null genotype (OR = 2.231; 95% CI = 1.332–3.737; p-value= 0.002) is significantly associated to BC risk in ethnic North- Indian population. However, the risk for BC susceptibility in North–Indians does not appear to be associated with *GSTT1* null genotype. The *GSTP1* (Val/Val) genotype (OR=1.545; CI=0.663-3.605; p-value= 0.314) was also found to be susceptible for BC risk. Combination of three high risk *GST* genotypes association exhibiting gene-gene interaction further confirmed the increased risk to BC in this region. **Conclusions:** The results of present study indicated that polymorphism in *GSTM1* and rs1695 of *GSTP1* genes may influence BC development among North-Indian women. Thus, the screening of *GSTM1* and *GSTP1* gene should be recommended for the earlier investigation for BC as a precautionary measure.

Keywords: Breast Cancer- Genetic Polymorphism- Ductal carcinoma- *GST*

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Introduction

Breast cancer (BC) remains a major health care problem throughout the world, affecting more than 1.15 million women annually (Siegel RL et al., 2017). Studies performed in China, India and Russia revealed that BC is the second most widespread cancer in females after Lung cancer (Goss et al., 2014). This disease is accountable for more than 23% of all cancer cases and 14% of cases in women representing the highest incidence among all cancers and topmost cause of cancer-associated death (6.8%) in women (Barrios 2022). Timely diagnosis of markers of BC can raise the chances of positive outcomes of the disease, leading to enhanced survival rates and improved quality of life.

BC is considered to be a multifactorial disorder. Epidemiological studies have shown that genetic factors together with environmental factors (such as carcinogens, xeno-estrogens and chemical mutagens) are involved in BC pathogenesis. Some of the most powerful carcinogens involved in mammary gland carcinogenesis are polycyclic aromatic hydrocarbons present in tobacco smoke, benzo(a)pyrene, poly-chlorinated biphenyls(PCBs), and heterocyclic aromatic amines present in the diet (Lee et al., 2019). The existence of genetic variations in the nucleotide sequences of metabolic genes concerned in DNA repair, transcription control, chromatin remodeling and cell cycle regulation amplify the risk of different types of cancers in some individuals (Dunning et al., 1999). Heredity plays a vital role in BC progression, misregulation and inherited

*[1]Department of Biochemistry, University of Allahabad, Prayagraj, India. [2]Department of Radiation Oncology, Kamala Nehru Memorial Hospital, Prayagraj, India. [3]Department of Life Science (Zoology), Manipur Central University (A Central University), Imphal, India. [4]Department of Biological Sciences and Center for Gene Regulation in Health and Disease, Cleveland State University, Cleveland, OH, 44115,USA. *For Correspondence: munishkp@gmail.com*

mutations in some specific highly penetrant genes such as BRCA1, BRCA2, CHEK 2, STK11, CDH1, TP53 or PTEN leads to the accumulation of genetic alterations, with an average cumulative lifetime risk of developing BC is about 80% (Naeem et al., 2019, Yang et al., 2019). Non-hereditary causes of BC which may play an interacting part with predisposing genes are race and ethnicity, advanced age, personal medical or family history of BC and lifestyle factors namely, (alcohol intake, smoking, high fat intake, low-fibre diet, and physical inactivity) (Osei-Afriyie et al., 2021). Having long-term exposure to estrogen via early menarche (< 12) , delayed menopause (>55), late age at first birth (≥30), nulliparity and nonstop use of oral hormonal contraceptives are some of the reproductive factors that enhance the chances of BC (Feng et al., 2018; Anderson et al., 2014).

BC is a heterogeneous disease featuring different histological, molecular and clinical phenotypes. Breast tumors are classified into four main subtypes depending on their status of hormone receptors: estrogen receptor (ER), progesterone receptor (PR) and expression of HER2; luminal A, luminal B, triple-negative/basal-like and HER2. One of the most frequently observed histological tumour type is invasive ductal carcinoma (IDC), followed by invasive lobular carcinoma (ILC) (Loibl et al., 2021).

Cells perform essential functions in a pro-oxidant condition that counter excess formation of free radicals and other reactive species which stem from both mitochondrial metabolism and exogenous processes. The system of biotransformation and elimination of these reactive species is classified into phases I and II. The phase I system includes components of the P450 cytochrome enzyme system and phase II constitutes the glutathione S-transferases (*GSTs*) enzymes (Pacholak et al., 2021). The Phase II detoxifying enzymes are stimulated under conditions of oxidative stress and catalyse the detoxifcation of xenobiotics, including chemotherapeutic drugs involved in BC treatment by glutathione conjugation that are obtained from the process of oxidation carried out by the phase I enzymes resulting in the formation of less water-soluble conjugates (Jancova et al., 2010;Lee et al. 2020). Moreover, the *GSTs* also participate in numerous cellular procedures, for example, they are involved in cell proliferations, stress response, apoptosis and drug resistance (Oliveira et al., 2010). Because oxidative DNA damage and mutations guide the process of tumor formation, numerous presumed functional genetic polymorphisms in *GSTs* were investigated in this study for plausible association with BC.

The *GST* gene family comprises of overwhelmingly important genes, which are considered to be significant in various disease manifestations (Chatterjee & Gupta 2018). The *GST* gene family in human is highly polymorphic and their frequency varies in population to population. *GSTM1* and *GSTT1* genes are deleted in approximately 40-45 % of the Caucasian population, respectively with a significant loss of enzyme function (Ranjbar et al., 2018). Polymorphism in GST genes can aggravate the aggregation of reactive metabolites in the body, enhancing the probability of interaction with biomolecules in the cells which triggers the oncogenesis process (Datkhile et al., 2019;Henkler et al., 2012). Previous reports on polymorphisms in *GSTT1* and *GSTM1* genes demonstrated the presence of 49.4% of *GSTM1* (null) and 28.6% of *GSTT1*(null) in Italy, 28% of *GSTM1*(null) and 46.7% of *GSTT1*(null) in Cameroun, 48.8% of *GSTM1*(null) and 37.8% of *GSTT1*(null) in Ethiopia, 55.8% of *GSTM1*(null) and 27.6% of *GSTT1*(null) in Spain (Kiendrebeogo et al., 2019).

GSTP1 acts as a tumor suppressor enzyme. It has a distinguished genetic variation entitled rs1695 single nucleotide polymorphism (SNP) (Farmohammadi et al., 2020). *GSTP1* has a polymorphic site, where an adenosine (A) to guanosine (G) transition leads to (Ile)→(Val) substitution at codon 105 in exon 5 (Yadav et al., 2020). Individuals with the mutated Val genotype have considerably lowered enzyme activity towards its substrate (Millar et al. 1999). Thus, it is practical to contemplate that xenobiotic metabolizing enzymes (XMEs) with reduced enzymatic activity may be associated with a prominent risk of developing cancer.

Functional polymorphisms in *GSTM1*, *GSTT1* and *GSTP1* genes have been scanned in numerous studies, looking for new markers for susceptibility to BC development (Van Emburgh et al., 2008; Sohail et al., 2013). It is estimated that about 45-50% Caucasian and about 30% of South Indians lacked the *GSTM1* gene due to inherited homozygous deletion of both alleles (Naveen et al., 2004). Several scientific studies have exhibited the association of polymorphisms in *GSTT1* and *GSTM1* with elevated BC risk in Asians, particularly in Chinese populations (Tang et al., 2015; Kalacas et al., 2019). The *GSTM1* null genotype was found to be significantly associated with BC risk in post-menopausal women (Ambrosone et al., 1995; Mitrunen et al., 2001).

Majority of the available data found no or fewer association, but these discrepancies in the findings could be due to the differences in study populations, risk factor distributions, and potential confounders. These factors might result in false-negative conclusions thus; there is a need to study polymorphism of breast-cancer-susceptibility genes in different populations.

Materials and Methods

Study population

The study population comprised of 105 histo-pathologically confirmed BC patients, treated at Kamala Nehru Memorial Cancer Hospital, Prayagraj, Uttar Pradesh, India between March 2018 till July 2020. Age matched control subjects (n=145) were randomly chosen from the general population. Both cases and controls came from the similar ethnic background to counteract the impact of ethnicity. Inclusion and exclusion criteria for BC patients and controls are as follows: Inclusion criteria for cases: (a) Women in the age group range of 18 to 65 with histo-pathologically confirmed BC were included in this study (b) Patients diagnosed with any other malignancies were excluded. Inclusion criteria for controls: (a) No personal history of cancer (b) No relatives with breast or ovarian cancer. The following data was collected from medical records: age at diagnosis,

family history of BC, menopausal status, marital status, ethnicity, smoking status and alcohol use and tumor stage. From the histopathologic report, following data were collected: the expression of hormone receptors (estrogen and progesterone), HER-2 overexpression, subtypes, and histopathological grade.

Consent to Participate and Ethical Statement

All the volunteers included this study signed a written informed consent form before sample collection. Our research proposal was reviewed and approved by the Population Resource and Research centre, Prayagraj, Institute Ethical Committee (IERB Reference: 18/9.39).

Molecular analyses of GSTM1, GSTT1 and GSTP1 gene polymorphisms

Blood sampling and DNA Isolation

Around (2ml) blood was collected from all the study volunteers in EDTA vials and genomic DNA was extracted from blood leukocytes by using DNA blood mini isolation kit following the manufacturers' protocol (Qaigen GmbH, Hilden, Germany). NanoDrop™ spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA) was used to quantitatively assess and determine DNA purity at 260/280 nm wavelengths and stored at -20° C prior to analysis.

Genotyping Protocol

The screening for *GSTT1* and *GSTM1* null genotypes was accomplished simultaneously by using multiplex-PCR procedure proposed by (Abdel-Rahman et al. 1996). Cytochrome P450 1A1 gene (CYP1A1) was used as internal control of the reaction for confirming successful PCR amplifcation. Primer sequences utilized for *GSTT1* and *GSTM1* genotypes detection are mentioned in Table 1. PCR amplification was carried out in a 25 µl multiplex reaction mixture containing about 1X PCR master mixes, 100-150 ng of template DNA and 10 pmol of each primers of *GSTM1* and *GSTT1*. The thermocycling procedure was done in a thermal cycler (Eppendorf AG, Hamburg, Germany). PCR amplification started with 5min of initial denaturation at 94°C, followed by 35 cycles of denaturation at 94° C for 30 sec, annealing at 64° C for 1 min and extension at 72 °C. A final extension was performed at 72 °C for 10 min. The amplified PCR products for the genotyping of polymorphisms were then separated on 1.5% agarose gel with ethidium bromide (EtBr) and documented in Geldoc XR+ system (Biorad System, Canada). Fragments of 215 and 480 bp were observed, respectively, in *GSTM1* and *GSTT1* active individuals, and the CYP1A1 fragment was 312 bp in size. The electrophoretic profile of the polymorphisms *GSTM1* and *GSTT1* is represented in Figure 1.

GSTP1 (Ile 105→Val) gene polymorphism was determined by PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) as previously illustrated by (Harries et al., 1997). Primer sequences used for amplification of *GSTP1* variants are mentioned in Table 1. PCR cycling conditions were as follows: early denaturation at 95° C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 sec, primer annealing at 59° C for 45 sec and a final extension at 72° C for 5 min. The amplified product of 176bp was checked by electrophoreses on 1.5% agarose gel. The 176bp PCR products (12 ml) were then digested with 5 units BsmA1 restriction enzyme (Fermentas, Germany) at 37 °C for 16hrs. The detection of the different alleles was performed by horizontal EtBr 10% native polyacrylamide gel electrophoresis. Persons with homozygous wild type allele (Ile/Ile) exhibited a single band of 176 bp, while those with homozygous mutant allele (Val/Val) exhibited two bands of 91bp and 85 bp. Individuals with the heterozygous variant allele (Ile/Val) exhibit three bands (176 bp corresponding to Ile; and 91 and 85 bp corresponding to Val).

Histopathological study of BC tissue

Histological type and grade was evaluated by microscopic examination of Hematoxylin and Eosin stained tissue sections. The slides obtained from the hospital were examined under the light microscope at 10 X and 40 X magnifications to observe the histological grades of Invasive Ductal Carcinoma of breast.

Data analysis

Data analysis was carried out using Statistical software package, SPSS 16.00 (SPSS In; Chicago, IL, USA). Genetic association analysis between BC and *GST* gene polymorphism was evaluated by calculating the odds ratio (OR) and 95% CI from logistic regression analyses (P-values <0.05 significant).

Results

The current case–control study was conducted on 105 BC patients (age: 45.1±9.26 years), and 145 healthy controls (age: 40.4±9.75 years), unrelated to the patients.

Table 2 depicts clinicopathological and demographic characteristics of BC patients and controls enrolled for this study. As per the clinicopathological characteristics of participants, 25.7% of the BC patients were diagnosed in Early Stage (T1+T2) and 74.2% of the patients were

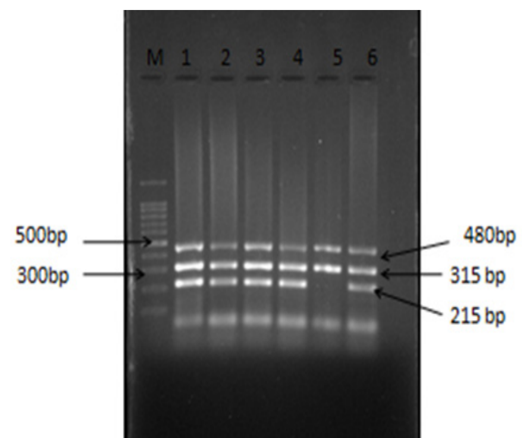

Figure 1. Electrophoretic Profile for the Polymorphisms of *GSTT1* and *GSTM1* Genes, Showing Bands at 480 bp and 215bp Respectively. *CYP1A1* (315bp) serving as internal control. Lane ''M''represents 100bp marker (Ladder), lane 1,2,3,4 and 6 shows the presence of both *GSTT1* and *GSTM1*. Lane 5 shows absence of *GSTM1*.

Table 1. PCR Primers and Electrophoretic Separation Pattern for *GSTM1, GSTT1* and *GSTP1*

Polymorphism	Primer sequence	Restriction enzyme	PCR Product
GSTM1	F -5'GAACTCCCTGAAAAGCTAAAGC3'	_	215bp
	R-5'GTTGGGCTCAAATATACGGTGG3'		
GSTT1	F- 5'TTCCTTACTGGTCCTCACATCTC3'	_	480bp
	R- 5'TCACCGGATCATGGCCAGCA3'		
CYP1A1	F- 5'GAACTGCCACTTCAGCTGTCT3'	_	312bp
	R- 5'CAGCTGCATTTGGAAGTGCTC3'		
GSTP1 (rs 1695)	F- 5'ACCCCAGGGCTCTATGGGAA3'	BsmA1	*Ile/Ile :176bp*
	R- 5'TGAGGGCACAAGAAGCCCCT3'		*Ile/Val:176bp,91bp,85bp*
			Val/Val : 91bp, 85bp

diagnosed in Late Stage (T3+T4). With regards to the tumor size, 54.3% of patients had tumors ranging between 1.5 and 3.0 centimeters (cm) in size while 35.2% of patients had tumors greater than 3.0 cm. The result showed that lymph node metastasis occurred in 35.2% of patients.

In the present study of North-Indian region , the highest number of cases of IDC (81.9%) were found followed by (8.5%) cases of ILC, (1.9%) cases of

Table 2. Clinicopathological and Demographic Characteristics of BC Patients (n=105) and Controls Subjects (n=145).

Characteristics	Cases n (%)	Control n (%)	p-value	OR (95% CI)
Age (Range)	18-70yrs	18-65yrs		
Mean± SD	45.1±9.26	40.4±9.75		
Menopausal status				
Pre-menopause	43 (40.9)	54 (37.2)	1	1
Post-menopause	62 (59%)	91 (62.7)	0.552	1.169 (0.699-1.955)
Marital status				
Unmarried	30 (28.5)	43 (29.6)	1	1
Married	65 (61.9)	87 (60)	0.813	1.071 (0.608-1.887)
Divorced	10 (9.5)	15 (10.3)	0.924	0.956 (0.378-2.412)
Ethnicity				
Rural	70 (66.6)			
Urban	35 (33.3)			
Family History				
No	74 (70.4)			
Yes	31 (29.5)			
Smoking history				
No	58 (55.2)			
Yes	47 (44.7)			
Tumor stage				
Early (T1+T2)	27 (25.7)			
Late (T3+T4)	78 (74.2)			
Tumor size				
<1.5 cm	11 (10.4)			
1.5-3.0 cm	57 (54.3)			
>3.0 cm	37 (35.2)			
Histopathological grade				
I	26 (24.7)			
II	57 (54.2)			
III	22 (21)			
Lymph node metastasis				
Absent	68 (64.7)			
Present	37 (35.2)			

*All p-values <0.05 were considered statistically significant.

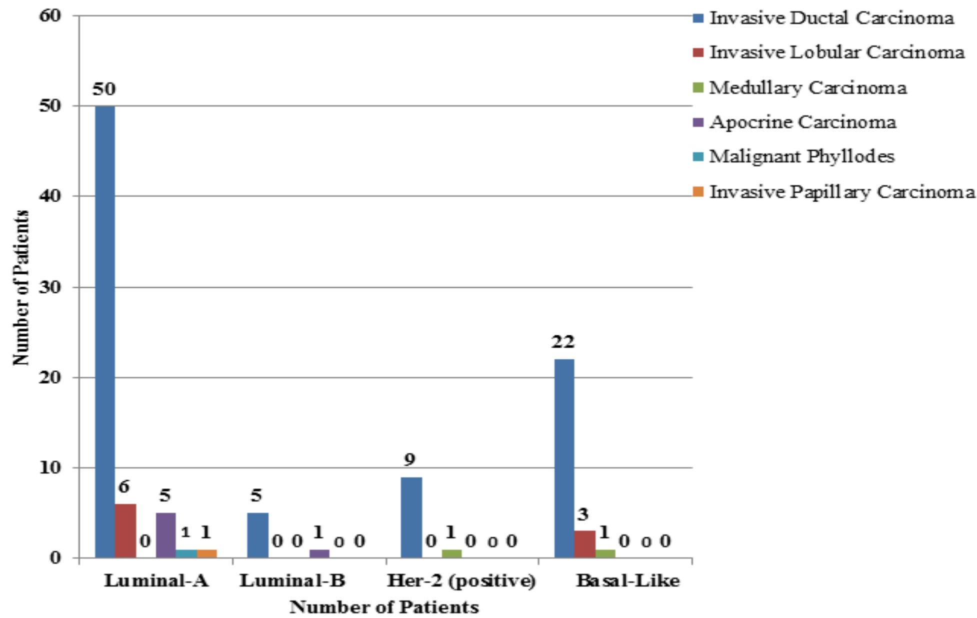

Figure 2. Comparison between Molecular Subtypes and Hhistopathological Types of Breast Cancer

Table 3. Histopathological Subtypes Diagnosed in Patients (n=105) with Breast Carcinoma.

Histopathological subtypes	No. of cases n (%)
InvasiveDuctal carcinoma (IDC)	86 (81.9)
InvasiveLobular carcinoma (ILC)	9 (8.5)
Medullary carcinoma	2 (1.9)
Apocrine carcinoma	6 (5.7)
Malignant phyllodes	1 (0.9)
Invasive papillary carcinoma	1 (0.9)
Total	105

medullary carcinoma, (5.7%) cases of apocrine carcinoma, (0.9%) case of malignant phyllodes and (0.9%) of invasive papillary as shown in (Table 3). Out of the 86 cases of IDC, 50 (58.13%) cases were of Luminal A type, 5 (5.81%) cases were of Luminal B type, 9 (10.46%) cases were of HER2 over expression type and 22 cases (25.58%) were basal like. Of the 9 ILC cases, 6 (66.66%) cases were of Luminal A type and 3 (33.33%) cases were basal like. No cases of ILC in the Luminal B type or the HER2 over expression type was found. Of the 6 cases of apocrine carcinoma, 5 (83.33%) cases were of luminal A type and 1 (16.66%) was luminal B. Of the 2 cases of medullary carcinoma, 1 case (50%) was of HER2 positive type and 1 (50%) case was of Basal-like. The single cases of malignant phyllodes and invasive papillary carcinoma were found to be of Luminal A type (Figure 2).

Figure 3 shows the histological grades of IDC. In this study we found that maximum number of tumors were moderately differentiated grade II showing less tubule formation, accounting 57 (54.2 %) of total cases, followed by 26 (24.7%) tumors with well differentiated grade I consisting of small angulated glands with fairly uniform nuclei and 22 (20.9%) tumors with poorly

Table 4.Frequency Distribution of the *GSTM1* and *GSTT1* Genotypes in BC Patients and Healthy Controls

Genotype frequency	*GSTM1*(Null)	*GSTM1*(Non-Null)	p-value	OR (95% CI)
Control (%)	64 (44.13)	81 (55.86)	1	1
Case (%)	67 (63.80)	38 (36.19)	0.002	2.231(1.332-3.737)
	GSTT1 (Null)	*GSTT1* (Non-null)		
Control (%)	45 (31.03)	100 (68.96)	1	1
Cases (%)	40 (38.09)	65 (61.90)	0.245	1.368 (0.806-2.319)

*All p-values <0.05 were considered statistically significant.

Table 5. Genotype Frequency of *GSTP1* with Odds Ratio

Genotype Frequency	Control n (%)	Cases n (%)	p-value	OR (95% CI)
GSTP1 (Ile/Ile)	54 (37.2)	54 (51.4)	1	1
GSTP1 (Ile/ Val)	80 (55.17)	34 (32.4)	0.002	0.425 (0.245-0.737)
GSTP1 (Val/Val)	11 (7.6)	17 (16.2)	0.314	1.545 (0.663-3.605)

*All p-values <0.05 were considered statistically significant.

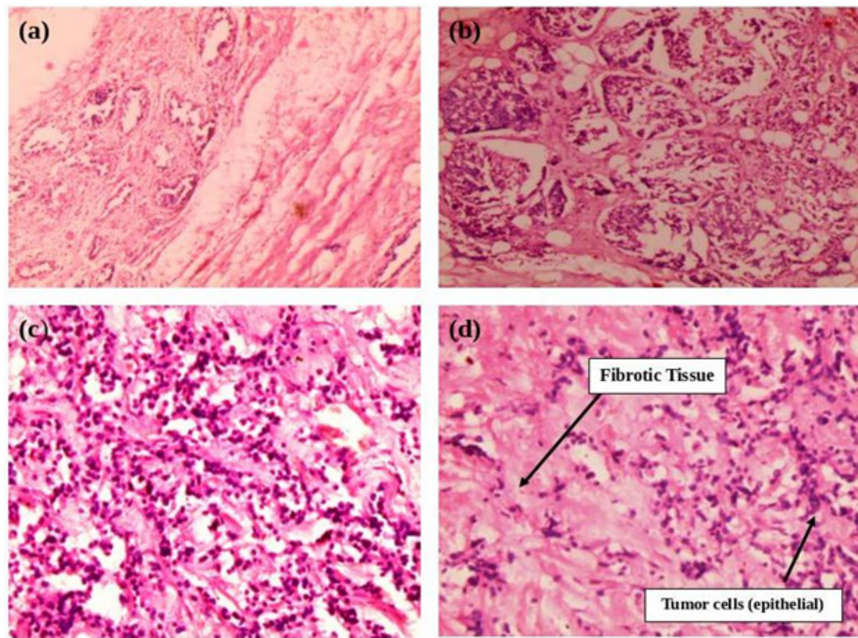

Figure 3. (a) Histologic Grade I Invasive Ductal Carcinoma: Well differentiated carcinoma consisting of small angulated glands with fairly uniform nuclei and mitotic figures are rarely seen (b) Histologic Grade II Invasive Ductal Carcinoma: Moderately differentiated carcinoma showing less tubule formation and some solid nests of cells with pleomorphic nuclei. Occasional mitotic figures are seen (HE stain, 40X). (c) and (d) Histologic Grade III Invasive Ductal Carcinoma: Poorly differentiated carcinoma showing absence of tubules, marked pleomorphism and prominent mitotic figures (HE stain, X40).

differentiated grade III showing no tubule formation, marked pleomorphism and prominent mitotic figures.

(Table 4) presents genotype frequencies for the *GSTM1* and *GSTT1*. The genotype frequencies in BC patients obtained for *GSTM1* (non-null, 36.19%), *GSTM1* (null, 63.81%) and in controls *GSTM1* (non-null, 55.86%), *GSTM1* (null, 44.14%). The *GSTM1* null genotype was most commonly found in the cases (63.81%) when

Table 6. Frequency Distribution and Association between the *GSTs* Genotypes in Control and BC Patients on the Basis of Menopausal Status

Genotype	Cases n (%)	Control n (%)	p-value	OR (95% CI)
Pre-menopause				
GSTM1				
Null	34 (79.1)	26 (48.1)	0.002*	4.068 (1.641-10.089)
Non-null	9 (20.9)	28 (51.9)	1	1
GSTT1				
Null	20 (46.5)	30 (55.6)	0.377	0.696 (0.311-1.555)
Non-null	23 (53.5)	24 (44.4)	1	1
GSTP1				
Ile/ Ile	23 (53.5)	13 (8.9)	1	1
Ile/ Val + Val/Val	23 (53.5)	41 (75.9)	0.001*	0.251 (0.105-0.597)
Post-menopause				
GSTM1				
Null	20 (33.0)	71 (67.0)	0.0001*	0.134 (0.065-0.278)
Non-null	42 (67.7)	20 (32.3)	1	1
GSTT1				
Null	10 (16.1)	20 (22.0)	0.373	0.683 (0.295-1.580)
Non-null	52 (83.9)	71 (78.0)	1	1
GSTP1				
Ile/Ile	15 (24.2)	60 (65.9)	1	1
Ile/ Val + Val/Val	47 (75.8)	31 (34.1)	0.0001*	6.065 (2.937-12.523)

*All p-values <0.05 were considered statistically significant.

Table 7. Analysis of Impact of GST Genotype Combination and BC Risk

Genotype Combination			Cases	Control	p-value	OR (95% CI)
GSTM1	*GSTT1*	*GSTP1*	N (%)	N (%)		
Non-Null	Non-null	Ile/Ile	18 (17.1)	39 (26.9)	1	1
Null	Null	Ile/Ile	5 (4.7)	6 (4.1)	0.377	1.808 (0.486-6.703)
Null	Non- null	Ile/Ile	11 (10.5)	16 (11)	0.412	1.490 (0.576-3.849)
Non-null	Null	Ile/Ile	4 (3.8)	12 (8.3)	0.615	0.722 (0.204-2.551)
Non-null	Non-null	Ile/Val + Val/Val	19 (18.1)	35 (24.1)	0.688	1.176 (0.534-2.591)
Null	Null	Ile/Val + Val/Val	9 (8.6)	3 (2.1)	0.005*	6.500 (1.570-26.918)
Null	Non-null	Ile/Val + Val/Val	32 (30.5)	23 (15.9)	0.005*	3.014 (1.390-6.536)
Non-null	Null	Ile/Val + Val/Val	7 (6.7)	11 (7.6)	0.569	1.379 (0.459-4.142)

*All p-values <0.05 were considered statistically significant.

compared with the controls (44.14%) indicating strong association between incidence of BC and *GSTM1* gene deletion. However, no significant variation in genotype frequencies of *GSTT1* was found between controls and BC cases. The odd ratios found for *GSTM1* (OR= 2.231; 95% CI= 1.332-3.737; p-value = 0.002) exhibited strong association whereas *GSTT1* (OR = 1.368; 95% CI =0.806-2.319; p-value = 0.245), does not appear to influence BC susceptibility in the tested North-Indian population.

The genotypic results of *GSTP1* are presented in Table 5. Among BC patients, 51.4% were homozygous for the (Ile/Ile) wild type allele, 32.4% were heterozygous (Ile/Val) and 16.2% homozygous for the mutant allele (Val/Val). In the control group, 37.2% of the subjects were homozygous for the *GSTP1* wild type allele, 55.17% were heterozygous and 7.6% homozygous for the mutant allele. The *GSTP1* (Val/Val) genotype (OR=1.545; CI=0.663-3.605; p-value= 0.314) was found to enhance the risk BC but the association was not statistically significant.

Table 6 presents the relation between *GST* polymorphisms and menopausal status. The results obtained from our study demonstrated that *GSTM1* gene deletion increased the risk for predisposition of BC in pre-menopausal women (OR = 4.068; 95% CI = 1.641-10.089; p-value = 0.002), but it showed a protective effect in post - menopausal women (OR = 0.134; 95% CI = 0.065–0.278; p-value = 0.0001). However, in our study no significant association was observed when comparing *GSTT1* gene deletion with risk of BC among pre- and post-menopausal women. The *GSTP1* (Ile/Val+Val/Val) genotype caused a six-fold increase in BC risk among post-menopausal women (OR=6.065; CI=2.937-12.523; p-value= 0.0001) whereas in case of pre-menopausal women it reduced the risk of BC (OR= 0.251; CI= 0.105-0.597; p-value= 0.001).

We also evaluated gene–gene interaction study to further examine whether the *GSTs* genotypes are associated with BC risk. Table 7 shows the risk of BC with each genotype combination. The reference group included individuals with all three putative low-risk genotypes, that is, the presence of *GSTM1* and *GSTT1* genotypes and the homozygous Ile/Ile genotype for *GSTP1*. We found six times increased BC risk in individuals who carry three high risk genotypes *GSTM1* null, *GSTT1* null and *GSTP1* Ile/Val+ Val/Val (OR = 6.500; 95% CI = 1.570-26.918; p-value= 0.005) and three times more risk of developing BC in individuals lacking *GSTM1* and having at least one mutant allele in *GSTP1* (OR = 3.014; 95% CI = 1.390-6.536; p-value= 0.005). Individuals with wild type genotype in *GSTM1* and *GSTP1* and with missing *GSTT1* were found to be at lower risk for BC (OR= 0.722; 95% CI=0.204-2.551; p-value= 0.615).

Discussion

Every year around 2.1 million fresh BC cases are reported, resulting in 600,000 deaths, which is the primary cause of mortality in females across the globe. According to reports, by the year 2050, the number of BC cases in women is expected to reach 3.2 million worldwide (Momenimovahed and Salehiniya 2019). The evaluation of different genetic variants and their relation with the development of BC has contributed in establishing the role of genomics in cancer research. Genetic polymorphism or SNPs, are such common genetic variations that are present in majority of the human genome. The study of these single nucleotide polymorphisms can help to prognosticate an individual's reaction to certain drugs, vulnerability to environmental factors, and risk of developing diseases including cancer development (Chang-Sheng et al., 2018). Additionally, the other factors that lead to commencement of a neoplasm and metastasis include accumulation of cellular mutations, like inhibition of tumor suppressor enzymes, resistance to apoptosis and increase in angiogenesis. The current case-control study aimed to explore genetic variability in *GSTM1*, *GSTT1* and *GSTP1* genes and its susceptibility to BC in a sample of North-Indian population and to analyze the occurrence of the four subtypes of BC based on molecular classification.

We found that *GSTM1* null genotype is significantly associated to BC risk in ethnic North-Indian population. However, no significant association was found between *GSTT1* null genotype and susceptibility to BC. Our findings were in alignment with a similar study conducted in northeastern Mexico which showed an increased BC risk associated with the *GSTM1* (null) genotype, whereas no association was found between the *GSTT1* (null) genotype and overall BC risk. The *GSTM1* null genotype resulted in two-fold (95%CI = 1.50-3.21) increase in the BC risk in the Mexican population (Jaramillo-Rangel et al., 2015). Conversely, there are some studies that have

linked *GSTT1* gene deletion with an increased risk of BC susceptibility (Fang et al., 2013). Chen et al. performed a meta-analysis which included 17,254 cases and 21,163 control subjects from 48 studies and investigated the association between the *GSTT1* polymorphism and BC risk. The result associated that *GSTT1* null genotype to a low-penetrant risk factor for BC risk (Chen X et al., 2011). Tagoe et al. performed a similar study in the Ghanaian population, which demonstrated a strong association between *GSTT1* null genotype and BC risk. The frequency of *GSTT1* (null) genotype in Ghanaian patients came out to be 42.9% and a significant association with BC was found (OR=2.84, 95% CI=1.52–5.29, p=0.001) (Tagoe et al., 2017). Khedhaier et al. reported a significant association between *GSTT1* gene deletion and the risk of BC development at an early stage. On the other hand, a number of authors couldn't find any significant association between *GSTM1* and *GSTT1* genes deletion and the risk of BC (Reis M, 2006, Anton et al., 2010). A study carried out by Morais et al. disclosed that about 35% of cases had *GSTM1* gene deletion and around 14% of the cases had *GSTT1* gene deletion, but they could not relate this deletion with the possibility of BC (Morais et al., 2008). Another study conducted in the Nigerian population indicated that the *GSTM1* and *GSTT1* homozygous gene deletion is a potential risk factor for the emergence of BC (Ogunlana et al., 2018).

Our data demonstrated that the *GSTP1* (Val/Val) genotype might alter the risk of BC but the association was not found to be statistically significant. Similarly, Ge et al., (2013) also documented a positive association between *GSTP1* (Ile105Val) polymorphism and BC risk (Ge et al., 2013). In constrast to our findings, many studies investigating the association of the *GSTP1* polymorphisms with BC risk found no association between both of them. For instance, (Samson et al., 2007, Unlu et al.,2008, Millikan et al., 2000, Zhao et al., 2001) have reported a non-significant increase in the risk of BC.

Previously reviewed literatures have shown contradictory reports on *GST* polymorphism and menopausal status. In our study we observed that the *GSTM1* gene deletion enhanced the risk of BC four times in pre-menopausal women, while in post- menopausal women it reduced the risk of BC. *GSTP1* (Ile/Val+Val/Val) genotype caused a six-fold increase in the BC risk in post-menopausal women in comparison to pre-menopausal women. Helzlsouer et al. and Charrier et al. observed positive associations for the *GSTM1* null genotype and post-menopausal women (Helzlsouer et al., 1998, Charrier et al. 1999). Ambrosone et al. performed a case-control study, which included 216 post-menopausal Caucasian women with incident BC and 282 community controls, results showed a positive association between *GSTM1* null genotype and younger pre-menopausal women. On the other hand, Bansal et al. reported no association between *GSTT1* and *GSTM1* gene deletion and menopausal status in the development of BC (Bansal et al., 2015). Similar to our finding, Helzlsouer et al. reported no association between *GSTT1* gene deletion and risk of BC in pre- or post-menopausal women while Garcia-Closas et al. reported no association between *GSTM1* and *GSTT1* gene deletion and BC risk, with null *GSTT1* being protective in pre-menopausal women. (Garcia-Closas et al.,1999).

Presence of null genotype in both *GSTM1* and *GSTT1* together with homozygous mutant *GSTP1* was termed as high risk genotype combination whereas presence of *GSTM1*, *GSTT1* alleles alongwith homozygous genotype in *GSTP1* were termed as low risk genotypes. Previously, many studies have reported that homozygous mutant individuals have a significantly higher risk of BC (Saxena et al., 2009, Zhang et al., 2011). Analysis of all the genotypes demonstrated significantly higher BC risk. We observed a six-fold increased BC risk in women who carry null genotype both in *GSTM1* and *GSTT1* and mutant genotype in *GSTP1*. Our findings were in harmony with the previously published reports (Gudmundsdottir et al. 2001; Park et al. 2004).

In our study, IDC was the most commonly observed histological type of breast carcinoma accounting 81.9% of the total cases. On the basis of a clinical database research, Wang et al demonstrated that 90.1% of breast carcinoma was of invasive ductal type which correlates with our study (Wang et al., 2014). Similarly, Kakarala et al. revealed that Asian Indian/Pakistani women had more IDC and less ILC in comparison to Caucasians (Kakarala et al., 2010).

Several studies have assessed the association of GST polymorphism with BC risk, but due to lack of conclusive evidence the association still remains ambiguous and has not been well established. There are multiple discrepancies in the association study reports which can be attributed to the different populations which may have been exposed to varied environmental risk factors. Moreover, the dissimilarities could also arise due to distinct methodology of study such as the study design, variation in sample size, genetic analysis, control selection as well as particular gene–environment interactions. Preliminary studies have already pointed that there is a undeniable association between the GST gene polymorphisms and BC. Therefore, further studies with larger sample sizes are compulsorily needed to authenticate our findings.

Author Contribution Statement

The authors confirm contributionto the paper as follows: study conception and design: Priyanka Gautam and Munish Kumar; Data collection: Priyanka Gautam, Sonia Tiwari and ZainabFeroz; Analysis and Interpretation of results: Munish Kumar, Sivakumar Vijayaraghavalu and Girish C. Shukla; Draft manuscript preparation: Priyanka Gautam, Munish Kumar and Sivakumar Vijayaraghavalu. All the authors reviewed the results and approved the final version of the manuscript.

Declarations of Conflicts of Interest

The authors declare no conflict of interest.

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