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RESEARCH ARTICLE
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COMPARISON OF VARIANT GENETICS IN GSTP1 GENE IN LOCAL BREAST CANCER PATIENTS AND HEALTHY INDIVIDUALS

Naila Rafiq[1], Sadia Falak[2], Irum Javid[3], Naheed Sajjad[4], Farah Naz Channa[4], Farrukh Bashir[5], Tanzila Sahar[1*], Shamaila Rafiq[6], Fiza Munir[1]

[1]Department of Biochemistry, Government College Women University Faisalabad-38000, Faisalabad, Pakistan
[2]Department of Biochemistry, The University of Faisalabad-38000, Faisalabad,Pakistan.
[3]Department of Biochemistry, Sardar Bahadur Khan Women, University, Quetta-87300, Pakistan.
[4]Department of Biotechnology, Sardar Bahadur Khan Women, University, Quetta-87300, Pakistan.
[5]Department of Chemistry, Sardar Bahadur Khan Women, University, Quetta-87300, Pakistan.
[6]Departmetnt of BiochemistryUniversity of Agriculture, Faisalabad-38000, Pakistan.

***Corresponding author;** Dr. Tanzila Sahar
*Email: drtanzilasahar@gcwuf.edu.pk

ABSTRACT
The Glutathione S-transferases P-1 (GSTP1) was Phase II metabolic enzyme caused detoxification of xenobiotics. Polymorphism in this enzyme was seen in different ethnic groups and might be related to difference in metabolism. Some carcinogens and mutagens are considered directly or indirectly involved in developing distinct sort of carcinoma. Single nucleotide polymorphism might lead to varying metabolic activity of resulting enzymes and could be tested for relation with disease occurrence. GSTP1 had two polymorphic sites that had conversions usually in the exon 5, A→G transition by nucleotide 313 near to codon 105 (Ile105↔Val105). A Polymerase Chain Reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis was conducted by using Alw261. All the digests were electrophoresed on a gel with optimized to 3.5% ultrapure agarose. It was found that the mutant genotype G/G was significantly high in breast cancer patients compared to normal individuls

Key Words: Breast Cancer, GSTP1, Gentic Polymorphism, Alw26I, Ethnic Variation

INTRODUCTION
Breast cancer is a common disease and major cause of death mostly among women than in men. Genetic variations cause susceptibility in individuals towards specific kind of diseases. Some metabolic enzymes and gene regulation play important role in normal physiological functions and variation among these genes can be the major cause of disease occurrence. If we know these genetic variations and their part in disease susceptibility we can understand the reason of disease occurrence. GSTs having two super families, one is membrane bound microsomal and other is cytosolic GSTs. There are eight classes of soluble GSTs (alpha through omega)that are identified on the basisof sequence homology and substrate specificity[1].GST enzymes detoxify chemotherapeutic drugs and their metabolites and different compounds accidently administered byindividuals. Everybody can behave differently according to its own genetic composition so variant alleles can behave resulting in

disease-like breast cancer**[2]**The glutathione S-transferase P1 (GSTP1) genewhich is nearly 2.8 kb and occur at 11q13 and containing seven exons. The GSTP1 is associated with the detoxification of base propenals and catabolizes carcinogenic products that is benzo-(α)-pyrene dial epoxide and acrolein that may come from cigarette smoke. There are two polymorphic sites that are identified in GSTP1 gene, one is an A→G transition at nucleotide 313 at codon 105 (Ile105→Val105) in exon 5 and the other is C→T transition which is at nucleotide 341 at codon 114 (Ala114→Val114) in exon 6. Thus, the GSTP1 locus of human gene contains four different alleles such as GSTP1*A (wild type Ile 105→Ala114), GSTP1*B (Val105→Ala114), GSTP1*C (Val 105→Val 114) and the last one is GSTP1*D (Ile105→Val 114). The major role of GSTP1 is played in the inactivation of carcinogenic electrophiles**3.** Biochemicalstudies revealed that GSTP1 Ile105 allele had a higher thermal stability than GSTP1 Val105 allele.

With heterozygous displaying intermediate activity, Val homozygous had a decreased conjugating activity than Ile homozygous. People that have no less than one Val allele at codon105 of GSTP1 protein does not have capacity to separate both chemotherapeutics and the cancer-causing agent found in cigarettes. It was in this way guaranteed variety in carcinogen breakdown, among people frames an establishment for malignancy advancement inside people**[4]**and might have a fundamental inclination to disease when presented to environmentally determined or endogenously shaped GSTP1 substrates. Indeed, The GSTP1 codon 105Val allele was associated with a significantly increased risk of danger of bladder, lung, testicular and breast cancer. Positive associations have been determined between the *GSTP*1 I105V polymorphism and danger of oral and breast cancers[5].

The study was design to check the polymorphisms in genetic polymorphism of isozyme of Glutathione S-Transferase (GSTP1) in Healthy volunteers and Breast Cancer patients. The genetic polymorphism was determined by PCR amplification and gel electrophoresis. The research was conducted TUF (The University of Faisalabad) and GCWUF (GC Women University Faisalabad).

Study Subjects

The project is designed according to the good clinical practice and the ethical principles laid down in declaration of Helsinki andW.H.O. 2022.Consent forms were signed by all subjects. A total of 200 hundred local patient subjects took part in this study. The physical examination and laboratory tests wereconducted for each subject to check their health status. Healthy and patient subjects were above 18 years, non-alcoholics, non-smokers.

Collection of blood samples

Blood was collected after an overnight fasting from both healthy and Patient subjects. Blood sample was collected in EDTA containing centrifuge tubes for DNA extraction and samples were stored in refrigerator at -20°C till analysis.

Chemicals and reagents

DNA blood isolation kit was purchased from Vivantis (GF-1 Blood DNA Extraction Kit). *Taq* polymerase, dNTPs, 6x loading dye, DNA ladders were purchased from Fermentas. Primers were got synthesized from Gene Link, USA, Deionized distilled water was obtained (Advanced GS-590, Distillery and CPW-200, Japan).

Equipment and Instrumentation

Thermostat Water Bath (HH-4), Glass Distillery (GS-590, distillery and CWP-200), Microcentrifuge (Hettichi Germany), Electronic balance (Shimadzu, Japan), Centrifuge Machine (YJ03-043-4000China), Gel Electrophoresis (BioRad), Gel Documentation System (Syngene, UK) PCR (Perkin Elmer) and Gene Quant.

Sample preparation
To isolate DNA, blood was collected from all female healthy and patient subjects in and stored at -20 ^{0}C. Blood samples were defrosted and used for DNA isolation.DNA was quantified by nanodrop. Samples were then digested by enzyme and run on gel electrophoresis.

Primer sequences
GSTP1: 5'-GTAGTTTGCC CAAGGTCAAG-3'
5'-AGCCACCTGAGG GGTAAG-3'

Restriction Enzyme
Alw26I is restriction enzyme.

Polymorphism OF GSTP1
GSTP1 is known to have genetic polymorphisms (GP) in exon 5 guanine to adenine conversion in exon 5, the PCR and restriction fragment length polymorphism (RFLP) studies will be performed[6]. A PCR method was used with few modifications tosimultaneously amplify regions of GSTP1 in Genomic DNA[7,8] Briefly, the assay for the exon 5 variant uses the primer pair (5'-GTAGTTTGCC CAAGGTCAAG-3') and (5'-AGCCACCTGAGG GGTAAG-3'). The DNA will be separated from blood sample of each volunteer and subjected to PCR by applying the process included denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and elongation at 73°C for 60 sec, repeated for a total of 25 amplification cycles. The PCR products will be digested for 2 hours at 37°C with 5 units of. Z-Test was performed for significance of two grops.

RESULTS
In the following study, a total of 146 cases were included. The patients consist of 86 breast cancer women. Mean age of the breast cancer patients was 52 years. Between the groups, no significant age-related differences were detected. The frequencies of GSTP1 genotypes was observed as 75.6% AA (mutant type) and 24.4 % GG (wild type). The GSTP1 gene was analyzed using the PCR-RFLP technique. DNA fragments underwent amplification over 37 cycles with an annealing temperature of 60°C. The resulting amplification products were digested with the Alw26I restriction enzyme. The G allele was identified by the formation of 148 bp and 189 bp fragments (as shown in Fig. 5), while the A allele fragments remained uncut.

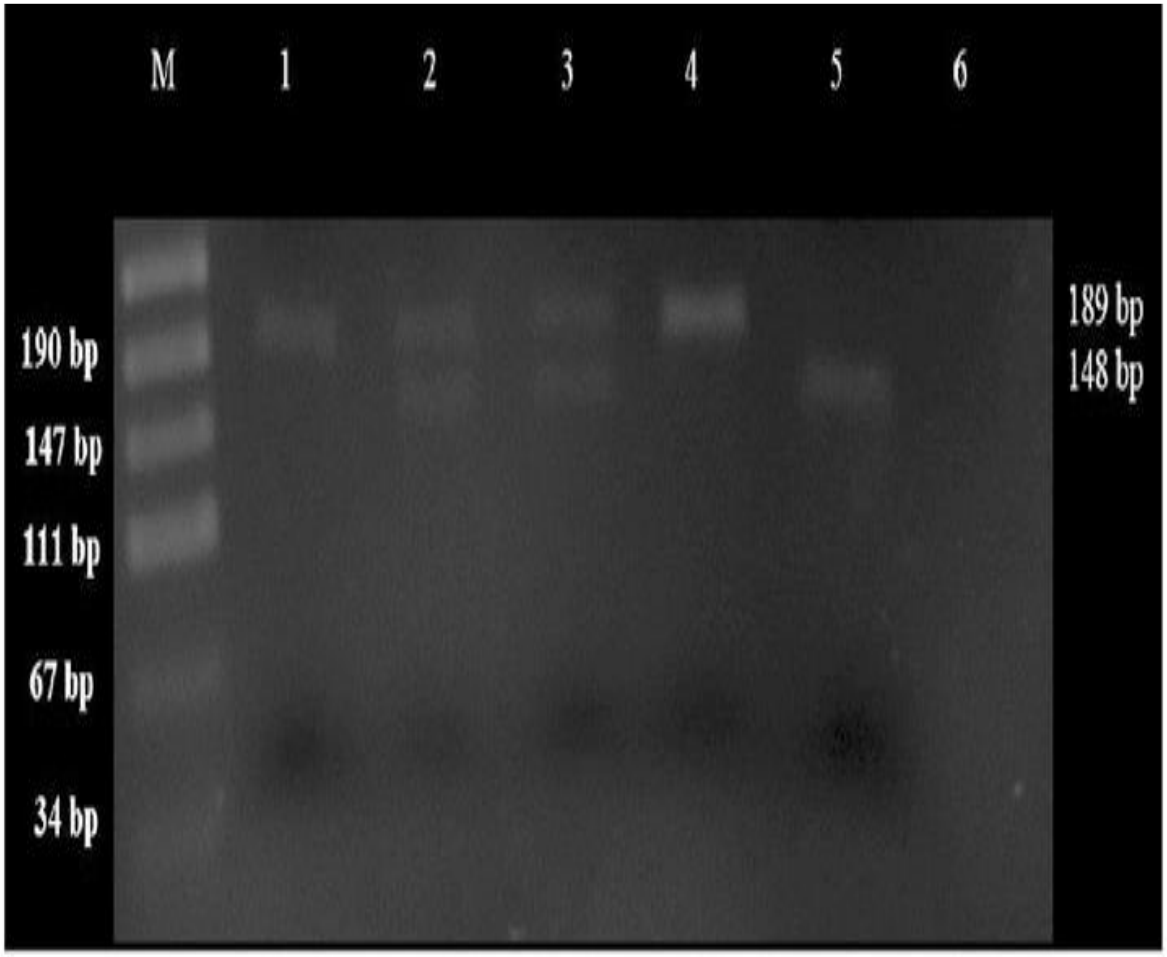

Fig1: Digestion products of GSTP1 by Alw26I restriction enzymeLane M represents the DNA ladder. Lanes 1 and 4 display the (AA), Lane 2 serves as the heterozygous positive control for digestion, Lane 3 corresponds to the AG genotype, Lane 5 shows the wild type (GG), and Lane 6 acts as the negative control.

The breast cancer groups, DNA was extracted from lymphocyte of patients with breast cancer to explain the polymorphism of GSTP1 gene by RFLP-PCR, A fragment containing polymorphism in exon 5 of GSTP1 gene was amplified as 148 and 189 bp bands.For detection of this polymorphism, Alw26I restriction enzyme was used to produce two bands 148bp and 189 bp.[6]

Allele and genotype frequencies of GSTP1

Allele frequencies of GSTP1 in the breast cancer groups showed in the Table 1. In this study, GSTP1 polymorphisms were analyzed in 156 subjects, including 60 healthy control subjects and 86 breast cancer. There was A and G allele frequencies of GSTP1 in the breast cancer patients. Significant difference was also found between these two groups. GSTP1 polymorphisms were examined in the 156 subjects. In the breast cancer groups, there were frequencies of two different genotypes, one was wild type and the other was mutant genotype of GSTP1. Between these two groups, significant difference was observed.All subjects in the breast cancer were genotyped for GSTP1 exon 5.

Relation between GSTP1 polymorphisms and breast cancer risk

GSTP1 was significantly higher with the mutant genotype than in helthy females and wild type genotype ws significantly more in healthy compared to patients, the mutant type has a decreased ability to detoxify mobilized carcinogens and thus their carriers have an elevated susceptibility to breast cancer. Respectively G/G genotype illustrated the non-A/A genotype. Subjects with the A/A genotype were remarked as a reference group to estimate the danger of breast cancer. Overall, 75%

of the patients in the breast cancer group carried the G/G genotype of GSTP1, which was higher than A/A genotype 24%. The ellivated level in breast cncer patients show susceptibility of these patients to breast cancer occurance.

Table: Showed the genotype distribution and allelic frequency of GSTP1 gene in breast cancer patients and healthy female volunteers. (*statically significant)

Genotypes /alleles	**Cases n = 86**	**Controls (%) n = 60**	**OR (CI)**	**Z- Value**
Val/Val or G/G (Mutant Type)	65 (75.6%)	33 (55.0%)	95%	2.6*
Ile/Ile OR A/A (Wild Type)	21(24.4%)	27 (45.0%)	95%	-2.56*

In breast cancer cases, family history of breast cancer was also found to be significantly different when they were compared to control, family history does not have much effect on breast cancer occurace because most of the patients had breast cancer without history of occurance in their family. There were (38%) of the patients women had positive history of breast cancer in their families while others are without history. Females that feed their children don't have any impact on cancer occurance according to this study. All patients were nonsmoker which means smoking does not effect on cancer occurrence. Female with rural ares have more breast cancer compared to urban female. Most of patients had A and B grade tumer. Married individual has more cancer compared to single.

Table 2: Explained the relation between the variables that is age group, dwelling, smoking, marital status, tumor grade, breast feeding with the breast cancer risk in the two groups of breast cancer patients.

	Brest Cancer Patients Females (n=86)			**P value/ Z score (±1.96)**
Age Group		**Group I ≤50 = 30 (34.9%)**	**Group II >50= 60 (23.5%)**	**1.77**
Val/Val	**65 (75.6%)**	**21 (0.32)**	**44(0.67)**	
lle/lle	**21 (24.4%)**	**09 (0.42)**	**12 (0.76)**	
Dwelling		**Rural = 59 (68.6%)**	**Urban = 27(31.4%)**	**4.87***
Val/Val	**65 (75.6%)**	**45 (0.69)**	**20 (0.31)**	
lle/lle	**21 (24.4%)**	**14 (0.66)**	**7 (0.33)**	
Smoking status		**Nonsmoker = 86 (100.0%)**	**Smoker = 0**	**3.125***
Val/Val	**65 (75.6%)**	**65 (0.10)**	**0(0.00)**	
lle/lle	**21 (24.4%)**	**21(0.10)**	**0**	
Tumor		**GradeA+ B 56 (65.1%)**	**Grade C+D= 30(34.8 %)**	**2.75***
Val/Val	**65 (75.6%)**	**45 (0.69)**	**20 (0.31)**	
lle/lle	**21 (24.4%)**	**11(0.52)**	**10 (0.47)**	
Breastfeeding		**Breastfeeding 49 (56.9%)**	**Non- Breastfeeding 37(43.0%)**	**1.84***
Val/Val	**65 (75.6%)**	**37 (0.57)**	**28 (0.43)**	
lle/lle	**21 (24.4%)**	**12(0.57)**	**9 (0.42)**	
Marital Status		**Single 30 (34.8%)**	**Married 56(65.1%)**	**1.19***
Val/Val	**65 (75.6%)**	**25 (0.38)**	**40 (0.61)**	
lle/lle	**21 (24.4%)**	**5 (0.24)**	**16 (0.76)**	
Family history of Breast cancer		**With History 33(38.3%)**	**Without History 53(61.6%)**	**-3.03****
Val/Val	**65 (75.6%)**	**26 (0.40)**	**39 (0.60)**	
lle/lle	**21 (24.4%)**	**7(0.33)**	**14 (0.66)**	

Table 1 and Table 2 that showed the relations between the variables and breast cancer risk and explained the mutant and wild type genotype. We found that, there were statistically significant differences between patients (Val/Val) genotype and healthy female on the otherhand (Ile/Ile) genotype is more in healthy females. This finding indicated that GSTP1 mutant type was linked to a higher risk of developing breast cancer.

DISCUSSION

Several investigations have been conducted in tumor cells to assess the role of enzyme activities as a contributing factor to drug resistance, with the GSTP1 enzyme playing a key role in the metabolism of various chemotherapy drugs. In modulating susceptibility to cancers, reactive metabolites of carcinogens may therefore be essential and are detoxified by GST enzymes principally GSTP1. There was significant increase in the GSTP1 (Val/Val) allele frequencies observed in breast cancer groups. Respective studies had also described significant association of this allele with susceptibility to form tumors of breast, bladder, lung, multiple myeloma and chronic myeloid leukemia. From our experiment, the (Val/Val) fragments remain uncut when we use our restriction enzyme as compared to (Ile/Ile) that bears two bands of 148bp and 189 bp.[6]

In Pakistan, the genotypic distribution of GSTP1, including the wild type A/A and the mutant type (G/G) was analyzed in women diagnosed with breast cancer. Among the affected individuals, 75% exhibited the Val/Val genotype (cut fragments), while 24% had the Ile/Ile genotype (uncut fragments) as wild type.The prevalence of mutant alleles (75%) in this study is comparable to findings from the Chinese population in Shanghai (50%) and Taiwan (53%) in Indian (60%), Slovakian (61.8%), European-American (68%), and African-American populations (65%). However, it is higher than the frequencies observed in English (28%) and Italian populations (30%).[7]

It is well-established that the allele frequency of this genes vary across human populations and can follow distinct ethnic patterns. Genetic variations in genes encoding metabolic enzymes have been linked to an increased susceptibility to breast cancer[8]. In this study, we found that carriers of the GSTP1 mutant alele had a higher risk of developing breast cancer compared to those with the homozygous wild type. These findings are consistent with studies conducted in American, Indian, and Chinese populations in Shanghai, Southeast China. However, studies in African-American, North Carolina, and Caucasian populations did not find significant differences between the GSTP1 mutant and wild type polymorphisms in relation to breast cancer risk[7,8].

Studies conducted on Finnish and Korean populations have suggested that the GSTP1 Val allele may be linked to a reduced risk of breast cancer. A meta-analysis finding reveilles that the GSTP1 mutant allele was associated with agreater probability of breast cancer in Chinese, but not in non-Chinese. The GSTP1 plays an important role in detoxifying and inactivating various toxic compounds[9]. This alteration occurs near the enzyme's substrate binding site, and the altered protein may contribute to the accumulation of carcinogens in the body, potentially leading to the development of a malignancy[7,10].

Environmental pollution in low-income countries, including Pakistan and India, has been linked to an increased risk of breast cancer, primarily due to rising population levels and changes in modern lifestyles. This risk is particularly heightened in individuals carrying the GSTP1 mutant allele. It was observed that breast cancer patients with the GSTP1 Val allele were more likely to present with lymph metastasis. Findings suggest that the mutant variant of GSTP1 leads to reduced or lost enzyme activity compared to the wild genotype, resulting in the dump of carcinogenic substances in the body. Tumors with harsh properties, such as poor differentiation and metastatic potential, arise when toxic damage to genomic DNA induces carcinogenesis. Thus, while the GSTP1 Val/Val genotype represents an unfavorable factor for healthy individuals, it may also enhance the cytotoxic effectiveness of chemotherapy in breast cancer patients. Consequently, those with the Val/Val genotype might have a better prognosis than individuals homozygous for the Ile/Ile genotype[7].Given that the G allele is associated with lower enzyme activity, we anticipated that the homozygous GG genotype would be linked to positive lymph nodes. In fact, the G allele appeared to be associated with negative lymph nodes when analyzed in a dominant model (GG vs. AA). A study in Shanghai reported that breast

cancer patients with the GSTP1 the mutant genotype experienced a 60% reduction in mortality risk following chemotherapy. Our research further indicated that the GSTP1 Val/Val genotype was associated with a more favorable prognosis for breast cancer patients in Pakistan who underwent chemotherapy based on CTX. However, this genotype was not found to be linked with breast cancer patient's prognosis receiving CTX-based chemotherapy in North America[11.7]

The GSTP1 single nucleotide polymorphism (SNP) leads to amino acid substitutions within the enzyme's substrate binding site. Research has shown that the GSTP1 Val variant exhibits lower temperature stability and changed catalytic activity toward various substrates when relevant to the GSTP1 Ile variant, resulting in a reduced ability to metabolize chemotherapeutic agents. This reduced metabolism leads to lower clearance and potentially enhanced therapeutic efficacy. It was further explored the impact of the GSTP1 Ile/Ile and Val/Val genotypes on drug sensitivity in breast cancer cells. Findings suggest that, in Pakistan, the GSTP1 mutant type is associated with a higher risk of breast cancer and more aggressive tumors. However, following CTX-based chemotherapy, individuals with the mutant allele appeared to have better survival outcomes. Therefore, the GSTP1 G/G genotype could potentially serve as a molecular marker to identify women at high risk for breast cancer, assess tumor aggressiveness. A meta-analysis also indicated that GSTP1 polymorphisms may be linked to an increased incidence of toxicities, particularly in patients undergoing chemotherapy or surgery[12.7]

CONCLUSION

Statistically significant differences between patients (Val/Val) genotype as well as (Ile/ Ile) genotype and this finding indicated that GSTP1 G/G genotype or mutant type was linked to a higher risk of developing breast cancer.Women with positive history of breast cancer found more responsive to breast cancer. On other hand factors such as number of pregnancies, breastfeeding and smoking were not associated with risk of breast cancer.

Conflict of Interest; Authors have no conflict of interest.

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