

Possible Association of Glutathione S-Transferase Pi 1 (GSTP1) Gene Polymorphism in Iraqi Patients with Breast Cancer

Hasan Alhayali¹ and Zohreh Hojati^{*1}

Abstract

Background: Investigating the role of glutathione S-transferase pi 1 (GSTP1) in breast cancer development and exploring genetic variations in GSTP1 that may contribute to susceptibility to the disease.

Methods: Blood samples were collected from 40 healthy control individuals and 75 breast cancer patients for genomic DNA extraction. PCR and bioinformatics analysis were used to examine the GSTP1 gene sequences.

Results: Gel electrophoresis confirmed the presence of a 433-bp amplified genetic locus with a 212C>A variation in the intron-4 region, identified as rs757152293. The Single nucleotide polymorphisms (SNP) exhibited variable distributions, with homozygous CC and heterozygous CA genotypes. Patient samples with the CA genotype were submitted to NCBI under accession numbers OL957029-OL957036, while those with the CC genotype were submitted under accession numbers OL957027-OL957034. Additionally, sequence analysis of the rs757152293 SNP in healthy individuals was submitted to NCBI under accession numbers OL957037-OL957041.

Conclusion: This study is the first to suggest a possible association between GSTP1 genetic polymorphism and breast cancer in the investigated population.

Keywords: Breast Cancer, Glutathione S-Transferase Pi 1, Iraq, Polymorphism.

Introduction

Cancer is a complex group of diseases where cells in the body grow uncontrollably and can spread to other parts of the body. Chemical agents, physical factors, hormones, oncoviruses, radiation, and hereditary predisposition are among the causes of cancer (1). Breast cancer (BC) is one of the most common malignancies and a major public health concern among women all over the world, while the prevalence varies by country and ethnic group (2). Age, family history, and various reproductive factors are all proven risk factors, although they only account for one-third of BC cases (3). It is important to understand that breast cancer is not solely determined by genetic factors, but also by

environmental exposures and lifestyle choices (4). Consequently, studies on gene polymorphisms have become increasingly understanding the molecular mechanisms underlying BC development (5, 6). Several genes have been established as potential tumor susceptibility genes in recent years. The Glutathione S-transferase (GST) gene family (GTSs) are essential genes that play a vital role in the detoxification of toxic, potentially carcinogenic chemicals as well as a variety of fundamental physiological functions in the human body (7, 8). In exon 5 of the glutathione S-transferase pi 1 (GSTP1) gene, a polymorphism involves a codon 105 A-to-G mutation, resulting in an

1: Department of Cell and Molecular Biology and Microbiology, Faculty of Biological Sciences and Technology, University of Isfahan, Iran.

**Corresponding author: Zohreh Hojati; Tel: +98 3137932128; E-mail: z.hojati@sci.ui.ac.ir.*

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isoleucine-to-valine substitution, which reduces enzymatic activity (9). As a result of their biological consequences, the three gene alterations may increase BC risk. The GSTP1 polymorphisms were related to BC risk (10). Nonetheless, the findings of these investigations were inconsistent. Individual GSTP1 polymorphisms and their impact on BC risk have been studied in 14 previous meta-analyses (11, 12). Additionally, numerous new studies have been published, necessitating a meta-analysis and re-analysis of earlier meta-analyses to understand the individual and combined effects of these genes on breast cancer risk. However, these meta-analyses did not examine the combined effects of the three genes on breast cancer risk, nor did they evaluate the validity of statistically significant associations (13-15).

In this study, the new breast cancer-related SNP finding may play a role in revealing the significance of genetic polymorphisms as well as breast cancer identity. The objective of the study is to enhance knowledge of the extent to which specific genes, such as GSTP1, influence the development of breast cancer, particularly when genetic variations arise from exposure to external factors like chemicals, radiation, and hormonal imbalances linked to genetic heterogeneity. In addition, Iraq has been exposed to many wars during the past forty years, which has led to widespread environmental pollution.

Materials and Methods

Subjects

Seventy-five blood samples were collected from breast cancer patients and forty blood samples were collected from healthy control subjects at the Oncology center, Merjan Hospital in Babylon Province/ Iraq. Blood samples were collected from subjects at different ages. The blood samples were preserved at -20 °C for molecular analysis.

Amplification of GSTP1

Genomic DNA was extracted from whole blood using the gSYNCTM DNA Extraction Kit and kept at -80 °C until use. The polymerase chain reaction (PCR) was performed using the Applied Biosystems (USA) PCR system with a reaction volume of 25 µL, including 5 µL of Master Mix, 1 µL of each primer pair for GSTP1 (Forward: 5' -GTA GTT TGC CCA AGG TCA AG-3', Reverse: 5' -AGC CAC CTG AGG GGT AAG-3'), 16.5 µL of nuclease-free water, and 1.5 µL of DNA. The amplification program was as follows: initial denaturation at 94 °C for 15 min; denaturation-2 at 94 °C for 120 sec., annealing at 60 °C for 40 sec, extension 1 at 72 °C for 11 min; and final extension at 72 °C for 10 min. The PCR products were subjected to 2% agarose gel electrophoresis at 70 volts for 30 min, and a UV transilluminator was used to visualize DNA bands.

Sequencing the PCR amplicons of the GSTP1

Following the sequencing company's instructions, the resolved PCR amplicons were commercially sequenced in both forward and reverse directions (Macrogen Inc. Geumchen, Seoul, South Korea). Sequence files were then analyzed, with annotation and variations identified using bioinformatics analysis. Additionally, the virtual locations and other characteristics of the retrieved PCR fragments were determined by comparing the observed DNA sequences from local samples with reference DNA sequences.

Results

Detection of GSTP1 Amplicon by Electrophoresis

Gel electrophoresis confirmed the presence of DNA bands corresponding to the GSTP1 gene extracted from breast cancer patients (Fig. 1).

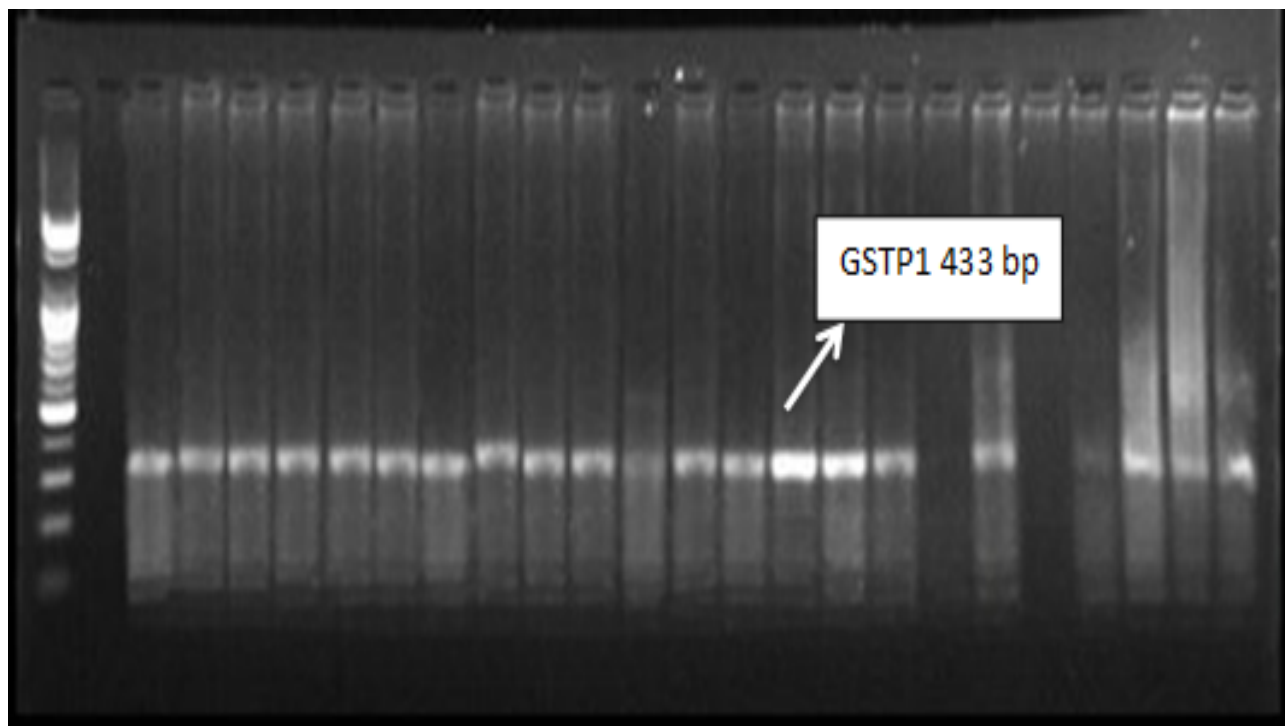


Fig. 1. PCR electrophoresis gel. M = Molecular weight marker (100 bp); 1-16, 18, 20-23) = one genotype GSTP1; 17 and 19 = null genotypes.

***GSTp1* sequencing**

The genetic sequences of GSTP1 in chromosome 11 were analyzed in fifteen samples, ten samples of patients (assigned S1 to S10), and five samples of control (assigned S11 to S15). One genetic locus was amplified in this study, which was made of 433 bp in length. The amplified fragment partially covered the intron-4 within the GSTP1 gene. Within the amplified locus, the genetic polymorphisms of the targeted locus were screened in the selected population.

The conducted sequencing experiments indicated the presence of one variation in the amplified fragments of the intron-4 region, namely 212C>A. The identity of this SNP was confirmed as it was deposited as rs757152293. This SNP exhibited variable distributions in the investigated samples, homozygous CC, and a heterozygous CA, while the homozygous (AA) pattern was not detected in the investigated population. The homozygous CC pattern was detected in the majority of samples, while the heterozygous CA pattern

was largely detected in the samples from patients. Thus, the heterozygous CA form showed a clear tendency to be localized in the patient samples. Statistical analyses indicated that this heterozygous form exerted a significant association with the progression of breast cancer.

Bioinformatics analysis of the GSTP1

The NCBI BLASTn engine showed more than 99% sequence similarities between the sequenced samples and the intended reference target sequences, which fully cover the intron-4 of this gene. The precise locations and other information of the obtained PCR fragments were determined by comparing the observed DNA sequences of these investigated samples with the retrieved DNA sequences.

The features of its sequences were highlighted, in terms of the placement of both forward and reverse primers, in the amplified 433 bp amplicons, after positioning the 433 bp amplicons' sequences within chromosome 11 (Table 1).

Table 1. The location and dimensions of the 433 bp PCR amplicons used to amplify the GSTP1 gene's intron-4 on chromosome 11 (GenBank accession number NG 012075.1). The forward and reverse primer positions were indicated by the highlighted sequences.

Amplicon	Reference locus sequences (5' - 3')	length
DNA sequences within the <i>GSTP1</i> gene	*GTAGTTTGCCCAAGGTCAAGCCTGGGTGCCTGCAAT CCTTGCCCTGTGCCAGGCTGCCTCCCAGGTGTCAGGTG AGCTCTGAGCACCTGCTGTGTGGCAGTCTCTCATCCTTC CACGCACATCCTCTTCCCTCCTCCCAGGCTGGGGCTCA CAGACAGCCCCCTGGTTGGCCCATCCCCAGTGAAGTGTG TGTTGATCAGGCGCCAGTCACGCGGCTGCTCCCCCTC CACCCAACCCAGGGCTCTATGGGAAGGACCAGCAGG AGGCAGCCCTGGTGGACATGGTGAATGACGGCGTGGA GGACCTCCGCTGCAAATACATCTCCCTCATCTACACCA ACTATGTGAGCATCTGCACCAGGGTTGGGCACTGGGGG CTGAACAAAGAAAGGGGCTTCTTGTGCCCTCACCCCC TTACCCCTCAGGTGGCT**	433 bp

* Sequencing of the forward primers, placed in a forward direction.

** Sequences refers to the reverse primer placed in a reverse complement direction.

In comparison to the referenced reference DNA sequences, the alignment results of the 433 bp samples showed that several of the investigated samples only contained one variation.

The sequencing chromatogram of the identified variation region, and its detailed annotations, were documented, and the chromatogram of the observed variation was shown according to its positions in the PCR amplicons. SNP was detected in the investigated samples, in which Cytosine was replaced with Adenine at position 212 of the amplified PCR products, namely C212A, or 212C>A. This SNP was detected in only two polymorphic patterns, CC, and CA. The homozygous C/C status was observed in most of the samples, while the heterozygous C/A pattern was observed in S3, S4, S5, S9, and S10.

This polymorphic position form was observed to exert a relatively high prevalence in the investigated samples. Thus, it is mandatory to explore further details of this SNP in its corresponding positions in the genomic DNA sequences of the *GSTP1* gene deposited in the dbSNP database. To elucidate the positions of the targeted SNP concerning their deposited SNP database of the sequenced 433 bp fragments, the corresponding position

of the *GSTP1* gene was retrieved from the dbSNP server (<https://www.ncbi.nlm.nih.gov/projects/SNP/>). To check the possible novelty of the observed SNP, graphical representations were performed concerning the *GSTP1* dbSNP database within chromosome 11 using the GenBank Acc. No. NC_000011.10. By reviewing the dbSNP engine, it was found that the position of the detected 212C>A SNP was previously known in the genome as they were deposited as rs757152293.

Many pieces of evidence suggest that the glutathione S-transferase pi 1 plays an essential role in the development of breast cancer. Thus, the genetic polymorphism of the *GSTP1* gene in breast cancer is mandatory to be understood in terms of breast cancer disease progression. Therefore, it is necessary to expand our understanding of the role of the *GSTP1* gene in this aspect. This expansion has come from our ability to understand the pattern of the genetic polymorphism of this genetic fragment.

The observed rs757152293 SNP is recognized in the targeted *GSTP1* gene due to its previous deposition in this server. Although no previous study reported an association between rs757152293 SNP and the progression of this disease, this study showed

that this SNP was strongly associated with the progression of breast cancer. This was due to the high prevalence of this SNP in the samples of patients with breast cancer, while its occurrence in the control samples was not

observed.

Furthermore, the novelty rs757152293 SNP heterozygous and homozygous in Iraqi women patients was submitted to NCBI under the accession numbers presented in Table 2.

Table 2. rs757152293 SNP result in breast cancer patients and healthy controls with the GenBank accession numbers.

Sequences	Accession Number	SNP result
Seq 1	OL957027	homozygous CC Patients
Seq 2	OL957028	homozygous CC Patients
Seq 3	OL957029	heterozygous CA Patients
Seq 4	OL957030	heterozygous CA Patients
Seq 5	OL957031	heterozygous CA Patients
Seq 6	OL957032	homozygous CC Patients
Seq 7	OL957033	homozygous CC Patients
Seq 8	OL957034	homozygous CC Patients
Seq 9	OL957035	heterozygous CA Patients
Seq 10	OL957036	heterozygous CA Patients
Seq 11	OL957037	Healthy controls
Seq 12	OL957038	Healthy controls
Seq 13	OL957039	Healthy controls
Seq 14	OL957040	Healthy controls
Seq 15	OL957041	Healthy controls

Discussion

The currently observed rs757152293 SNP is not the only SNP in the investigated 433 bp *GSTP1* gene. Instead, many other SNPs are also present in the same targeted PCR amplicons. It was found that these SNPs occupy various positions in intron 4 and may have variable effects on patients. Based on the highest records of the available allele frequency data in the dbSNP, such as GnomAD exome, GnomAD genome, TopMed, ALFA projects, and ExAC, it was found that these SNPs have been deposited in extremely low frequencies. Due to the extremely low frequency of these SNPs, no polymorphism was found for all these loci in the currently investigated population. This

observation may explain the absence of any detectable polymorphism for these SNPs in this study. However, this observation was not only concerned with these SNPs as the currently observed rs757152293 SNP in this study was also deposited in an extremely low frequency within the investigated 433 bp amplicons.

This low frequency of deposition in the dbSNP database is different from our observation, which found that the heterozygous C/A pattern of this SNP was found in several samples of the patients. This discrepancy may be attributed to the type of the observed allele (C/A) in this study which was different from the deposited heterozygous allele in the dbSNP database (which is C/T). These data indicated that the currently

observed allele A was not previously deposited in the dbSNP and that this form of heterozygosity is a new polymorphic form in the rs757152293 SNP.

This study revealed that the AA genotype of the rs757152293 SNP was not present in the population under investigation. Additionally, the homozygous A/A form of the rs757152293 SNP was not observed in this study.

Nevertheless, the observed C/A heterozygous form of the rs757152293 SNP exhibited a high tendency to be localized mainly within the samples of patients, as it was observed in S3, S4, S5, S9, and S10. Meanwhile, the control samples were not found to exhibit this heterozygous variant. However, the results of this study indicated a significant association between individuals having the heterozygous form of this SNP and the development of breast cancer. Despite the medical importance of the rs757152293 SNP in this study, it was not reported in the clinical variation (ClinVar) database as no previous deposition had taken place of this SNP in this server. However, the substitution of C to A was found to be associated with possible benign as well as malignant consequences in breast cancer development.

The documentation of this observation entailed more potential importance for this in the *GSTP1* gene than the other SNPs deposited in the same amplified locus within the *GSTP1* gene in terms of its association with breast cancer. Thus, the substitution of Cytosine to Adenine in the position of 212 (212C>A) in this SNP seems to cause a possible effect on the resulting mRNA transcript via inducing a possible alteration in the transcript. Due to this alteration, altered products would be formed in patients with breast cancer. According to our knowledge, no previous study has associated this SNP with the diagnosis and prognosis of breast cancer. Therefore, it can be stated that this study is the first one that detects a possible association between the genetic polymorphism of the *GSTP1* gene with breast cancer in the investigated Iraqi population. However, the limited number of the studied samples represented a crucial problem against for any

large-scale statistical association with the development of breast cancer.

However, the pathological status of the studied area in terms of the recorded phenotypic parameters could not be excluded from our explanation. As well, further genetic fragments within the same gene are highly important to be amplified and sequenced in these cases to assess the extent to which the other portions of the *GSTP1* gene are associated with this pathological status. These portions may represent coding as well as other noncoding sequences, however; they have to be investigated and exposed to extended sequencing reactions to give a final impression of the actual status of the genotype-phenotype association in the investigated population.

Although the etiological causes of breast cancer are unknown, estrogen levels, lifestyle choices, particularly food, oxidative stress, and carbonyl stress have all been linked to an increased risk of breast cancer (BC). Numerous enzyme systems in the body guard against genotoxic damage, either directly through the removal of free radicals by enzymes like glutathione S-transferase (*GSTP1*), or indirectly through the reduction of possible substrates for the generation of free radicals, such as like cytochrome P450 (*CYP17*) (16). The *GSTP1* gene's polymorphisms are thought to be important determinants of cancer susceptibility to hazardous or environmental substances (17). Therefore, to better understand the interaction between potential carcinogenic environmental exposure and genetic factors in the pathogenesis and predisposition to BC disease risk, we identified the gene polymorphisms of *GSTP1* in a control population and a population of patients with breast tumors.

In this study, we found that heterozygous individuals (CA) for the intron 4 of *GSTP1* have a protective effect against breast cancer, while those with the primary risk for breast cancer were linked to a similar pattern likely due to tissue-level enzyme activation. Experimental data suggested that the C/A variation in *GSTP1* may result in either lower or higher specific activity and

affinities depending on the substrate. Our results may differ from other studies due to varying environmental exposures and dietary practices, which could explain the conflicting findings regarding the elevated risk associated with the A/A variation in women. This study is the first to investigate the GSTP1 C/A variation in relation to breast cancer risk. Free radicals and oxidative stress from external and internal carcinogens are known to increase the risk of breast cancer. Gulyan et al. (17) found that homozygous deletion of GSTT1 (GSTT1-0) may be a low-penetrant risk factor for breast cancer, while Li et al. (18) reported that the GSTM1 polymorphism plays a complex role in breast cancer patient survival and response to chemotherapy. Patients with breast cancer who have the GSTM1-present genotype may not progress. Hashemi et al. (19) found that genetic polymorphisms in GSTM1 and GSTP1, but not GSTT1, are associated with an increased risk of breast cancer (19).

Concerning GSTP1, our study found that women with the GSTP1/CC genotype had a lower risk of developing breast cancer compared to those with the GSTP1/CA genotype. This could be attributed to the fact that the C to A substitution leads to an enzyme with enhanced detoxification activity, which is beneficial in combating oxidative stress and lipid peroxidation byproducts that can contribute to cancer development. When comparing women with breast cancer to controls, the presence of the GSTP1 212A variation was also associated with an increased risk of breast cancer. Our results suggest that the GSTP1 C212A polymorphism may have a distinct impact on the development of breast cancer. Furthermore, the activity of the glutathione S-transferase enzyme may be influenced by various factors such as environmental conditions, dietary habits, and lifestyle choices in addition to genetic factors. It is worth noting that our findings differ from those of a previous study on the association between the GSTP1-212C variant and breast cancer risk. Therefore, this study represents the first investigation of the

GSTP1 C212A SNP polymorphism in Iraq and globally.

We also calculated the GSTP1 polymorphism frequencies for each group (control and patients), utilizing the ALFA project's low frequency (0.000015), test the frequencies. Additionally, for the majority of the SNPs under investigation, the frequencies of the mutant allele among controls did not differ significantly from those seen among women in other published research (16, 20). Genetic drift, migration, inbreeding, cultural characteristics based on religious convictions and socioeconomic requirements, the recent origin/introduction of the polymorphism, the absence of random mating, and/or a stratification bias are further explanations for the observed anomalies. As a result of our sample collection including people of known ethnicity, the final scenario can be ruled out. Since our current sample set is insufficient to make any judgments about the selection forces at work in the population, the other factors cannot be completely ruled out. After sequence analysis, we found that the rs757152293 SNP in this study revealed that the homozygote CC had a concentrated risk of BC in women but the heterozygote CA genotype is associated with the risk of breast cancer. In comparison, the homozygous AA form of the rs757152293 SNP was not observed in this study. Due to the presence of the heterozygous CA form of the rs757152293 SNP in five samples of patients, it can be stated that this novel allele is highly associated with the development of breast cancer disease in the investigated population. This observation emphasizes the novel importance of this SNP to be used as a potential marker for this highly serious disease. While the study of Farmohammadi, et.al. (21) revealed two SNP polymorphisms, the first rs1695 SNP had AA, AG, and GG genotypes in patients. The homozygote genotype GG is associated with an increased risk of breast cancer but the AG genotype was not related to the risk of breast cancer. Although carriers of allele G had high frequency in the patient population (Farmohammadi, et.al. 2020) (21). The second

rs1138272 SNP had the control of CC, CT, and TT genotypes. The homozygote TT and heterozygote CT genotypes are not associated with the risk of breast cancer. Also, the carriers of allele T had high frequency in the healthy population. Likewise, we found an interesting SNP in this study, in which Cytosine was replaced with other Adenine in position 212, namely C212A, or 212C>A. However, the study of Sergentanis & Economopoulos (22) revealed the GSTT1 and GSTP1 genes associated with elevated breast cancer risk in a Chinese population in position 105 namely G105G.

According to our knowledge, no previous study has associated this SNP with the diagnosis and prognosis of breast cancer. Therefore, it can be stated that this study is the first to detect a possible association between the genetic polymorphism of the GSTP1 gene and breast cancer in the investigated Iraqi population.

Based on the analysis of the GSTP1 gene, one SNP was detected in the analyzed population: the rs757152293 SNP, which exists in two zygosity states - homozygous CC and heterozygous CA. The presence of the heterozygous CA form of the rs757152293 SNP in five patient samples suggests a strong association with the development of breast cancer in the studied population. This finding highlights the potential significance of this SNP as a marker for the disease.

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The GSTP1 gene investigated in this study exhibited highly informative polymorphic patterns in the population under study. The identified rs757152293 SNP in the GSTP1 gene showed a significant association with breast cancer development. This study is the first to establish a potential link between the genetic polymorphism of the GSTP1 gene and breast cancer in Iraqi women patients, as there is no prior research connecting this SNP to the detection and prognosis of breast cancer.

Ethics

The procedures adhered to scientific integrity guidelines, considering ethical, human, and scientific factors during sample collection. Approval was obtained from official authorities, and patients provided written consent (Grant Code: IRAQ CO1126).

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Conflict of Interest

The authors declare that they have no competing interests.

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