GENETIC POLYMORPHISMS OF GSTP1 (RS1695) AND GSTA1 (RS3957357) GENES IN THE BREAST CANCER PATIENTS

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ABSTRACT

Breast cancer is the commonest cancer affecting women worldwide. Different studies have dealt with the etiological factors of that cancer aiming to find a way for early diagnosis and satisfactory therapy. The present study investigated the relationship between genetic polymorphisms of GSTP1. This investigation was carried out on 70 patients (all were females) who were confirmatory for breast cancer by histopathological examinations attended from tumors center in Mirjan medical city in Babylon and 30 of apparently healthy women were used as a control. The GSTP1 (rs1695) gene polymorphism was studied in breast cancer cases and control. The distribution observed in GSTP1 (rs1695) gene polymorphism in cases group and control group. The highest genotype in control group was mutant homozygote AA (80%) followed by GA heterozygote genotype (16.7%) and homozygote genotype GG(3.3%) and mutant homozygote AA (80%). In breast cancer disease, the highest genotype was mutant homozygote AA (82.8%) followed by GA heterozygote genotype (8.6%) and GG homozygote genotype (8.6%). Conclusion: There are no significant association between polymorphisms of GSTP1 gene with breast cancer risk, while these findings indicate that the GSTA1 rs3957357 A/G/T (52803891), as well as other GSTA1 variants, may be play a role in breast cancer susceptibility.

Keywords: Breast cancer, rs1695, rs3957357, Single nucleotide polymorphism.

I. INTRODUCTION

Breast Cancer (BC) is one of the most important cancers in women worldwide, according to the last global cancer statistics. And it was the second-leading cause of cancer-related deaths in 2018 [1]. Chemotherapy is seldom used for treating BC, but in specific cases, it may be recommended [2]. Usually, BC is classified into molecular subtypes, and for some of them, chemotherapy is an option. Among the molecular subtypes, triple-negative BC is considered one of the most aggressive, and its chemotherapy response rate is considered higher when compared to the others. However, despite adequate chemotherapy, the overall survival of these patients is still poor [3]. Since chemotherapy is usually used for triple-negative, inflammatory and advanced-stage BC, new strategies and molecular predictive markers are required to increase the patient’s prognosis [4]. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS), and antioxidants reaction capacity which stimulate the development of a disease such as breast cancer [5], [6]. Antioxidant defenses protect against free radicals, but these defenses are not completely adequate and systems that repair damage by ROS are also necessary [7]. While some ROS are necessary and play important physiological roles, ROS can also cause harm. Excess oxidative species can directly damage DNA, proteins and lipids. Furthermore, reactive oxygen species (ROS), such as superoxide anions and hydrogen peroxide induced lipid peroxidation, play a major role in malignant transformation and tumor cell proliferation and invasion [8]. Antioxidants can be divided into two systems: enzymatic and non-enzymatic. The enzymatic system involves enzymes produced by the organism itself, as superoxide dismutase (SOD), catalase (CAT). The enzyme SOD acts as a defense against superoxide, while the enzyme catalase act on H2O2 [9]. Free radicals, primarily the reactive oxygen species, superoxide and hydroxyl radicals which are highly reactive having an unpaired electron in an atomic or molecular orbit are generated under physiological conditions during aerobic metabolism and can damage the almost all kind of molecules in living cells. As free radicals are potentially toxic, they are usually inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. The human body has a complex antioxidant defense...
system that includes the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GSHPx), and catalase (CAT). These block the initiation of free radical chain reactions [10]. When free radicals are generated in excess or when the cellular antioxidant defense system is defective, they can stimulate chain reactions by interacting with proteins, lipids and nucleic acids causing cellular dysfunction and even death. Enzymes SOD and CAT have a vital role in the follow up of breast cancer disease. These enzymes counteracts the deleterious action of Reactive Oxygen Metabolites (ROMs) such as singlet oxygen (1O2), super oxide anions (O2-), Hydroxyl radical (OH) hydrogen peroxide (H2O2)[11], [12]. The present study aimed to determine changes The GSTP1 G/G genotype has been associated with better survival after treatment with chemotherapy in breast cancer patients [13].

II. MATERIAL AND METHODS

Study Subjects

The research was carried out between September 2020/ April 2021. Seventy breast cancer patients were adopted from tumors center in Mirjan medical city in Babylon, including 70 case of breast cancer patients and 30 from healthy. The peripheral blood for DNA isolation was collected using anticoagulants EDTA tubes.

DNA extraction and genotyping

DNA of blood was extracted and purified using extraction and purification kit from Geneaid company (UK). The genotyping of the study groups was performed using the PCR-RFLP technique after DNA extraction from blood samples. PCR for The polymorphism of the GSTP1 was analyzed using the PCR-RFLP Technique. Primers for the 5’region of GSTP1 (5’ TCCCCAGTGACTGTGTGTG 3’) and the 3’ region (5’ GAAGCCCTTTCTTGTCA 3’) PCR was carried out in 20 μl reaction volumes containing 1 μl of each forward and reverse primer, 12.5 μl of Green Master Mix, 2 μl of Genomic DNA, and 8.5 μl of nuclease-free water to bring the reaction volume up to 25 μl. Amplification was conducted in a thermocycler (Biometra, Germany) with the following settings: 5 min pre-denaturation at 94°C; 30 cycles with denaturation for 30 seconds at 94°C, primer annealing at 60°C for 30 seconds, polymerization at 70°C for 30 seconds, 30 PCR cycles were done and a final elongation step of 72°C for 7 minutes. Amplification leads to a 224 bp band and visualized by staining with ethidium bromide [14].

RFLP Detection of GSTP1 Polymorphisms

The A to G polymorphism of GSTP1 introduces a restriction site recognized by the BsmA1 restriction enzyme. Digestion of the PCR product with 1 μl BsmA1 in a 3 μl volume for 45 minute at 37°C. Results either in retention of the 224 bp product or complete digestion to 146 bp and 78 bp fragments corresponding to individuals homozygous for the Ile or Val alleles, respectively. The presence of all three fragments corresponded to individuals heterozygous at codon 105, Products were separated on 1.0% agarose and visualized with ethidium bromide and red safe stain.

Statistical analysis

All the statistical analyses were done with the SPSS statistical software (version 23; SPSS Inc., Chicago, IL). p<0.05 was considered statistically significant.

SSCP-PCR technique to identify genotypes

After extracting DNA from blood samples, the study groups were genotyped by the SSCP-PCR method. A Geneaid abstraction and purification kit was used to extract and purify DNA from blood. The targeted DNA sites were amplified using design specific primers obtained from Macrogen company in South Korea,

Primers for the 5’region of GSTA1 (5’-GCATCAGCT TGC CCT TCA-3’) and the 3’ region (5’-AAACGC TGT CACCGT CCTG-3’) were used to amplify a 400 bp fragment. PCR was carried out in 20 μl reaction volumes containing 1 μl of each forward and reverse primer, 12.5 μl of Green Master Mix, 2 μl of Genomic DNA, and 8.5 μl of nuclease-free water to bring the reaction volume up to 25 μl. Amplification was conducted in a thermocycler (Biometra, Germany) with the following settings: 5 min pre-denaturation at 94°C; 30 cycles with denaturation for 30 seconds at 94°C, primer annealing at 60°C for 30 seconds. polymerization at 70°C for 30 seconds, 30 PCR cycles were done and a final elongation step of 72°C for 7 minutes. Following initial denaturation at 94°C for 5 minutes, amplification conditions include strand separation at 94 °C for 30 seconds, primer annealing at 60°C for 30 seconds, polymerization at 70°C for 30 seconds, 30 PCR cycles were done and a
final elongation step of 72°C for 7 minutes. Amplification leads to a 400 bp band and visualized by staining with ethidium bromide [15]. PCR products were electrophoresed in 1 percent agarose at 75 V using gel electrophoresis (cleaver science – UK) and visualized with ethidium bromide. A gel documentation system (Cleaver Scientific – UK) was used to take photos. After electrophoresis of GSTA1 PCR fragment, the clear and bright bands were found. These amplified fragments are then suitable for SSCP experiments.

As an ideal non-expensive method of detecting unidentified single nucleotide polymorphisms (SNPs), the PCR-SSCP technique was used to conduct post-PCR screening experiments for all 100 PCR amplicon [16]. The critical PCR-SSCP procedure was followed as defined by [17]. Followed by 30 cycles of PCR amplification, an equivalent volume of SSCP denaturing-loading buffer was applied to 10 of each PCR amplicon (95 percent formamide, 0.05 percent bromophenol blue, 0.05 percent xylene cyanol, and 20 mM EDTA, pH 8). After being denatured for 8 minutes, PCR amplicons were directly put on ice and kept freezing for at least 10 minutes. The samples were then loaded onto polyacrylamide gels that were neutral. Afterward, gels were stained with a silver staining procedure that was highly sensitive [18]. PCR-SSCP gel electrophoresis, the existence of one conformational DNA polymorphisms was found according to the number of bands: 1-bands.

III. RESULTS AND DISCUSSION

The genomic DNA (Fig.1) was extracted from the blood samples as a first step to amplify the target region of some Antioxidant genes (GSTP1).

![Electrophoresis pattern of genomic DNA extracted from blood samples of breast cancer patients and healthy control groups.](image1)

Figure (1): The electrophoresis pattern of genomic DNA extracted from blood samples of breast cancer patients and healthy control groups.

Lane 1 - lane 10 refers to genomic DNA from blood samples; Electrophoresis conditions, 1% agarose, 75 V, 20 mA for 1h, stained with ethidium bromide.

Genotyping of GSTP1(rs1695) Gene Polymorphisms

The results revealed that the presence a single band (224bp) of the target sequence of GSTP1(rs1695) gene in agarose gel as in figure (2).

![Agarose gel electrophoresis of an amplified product patterns of Glutathion S-Transferase Pi 1 (GSTP1) with specific primer.](image2)

Figure (2) Agarose gel electrophoresis of an amplified product patterns of Glutathion S-Transferase Pi 1 (GSTP1) with specific primer.

M: refers to DNA size marker(50bp); lanes 1 - 7 refer to PCR products of GSTP1 (224bp) of breast cancer patients and lanes 8 - 10 healthy control groups. Electrophoresis conditions: 1% agarose concentration 1%; 75 V, 20 mA for 120 min. Staining method; ethidium bromide.
After that, the PCR products of the GSTP1 (rs1695) target sequences were digested with BsmAl (5’ GTCTCN…3’) restriction enzyme to detect the rs1695 SNP in GSTP1 gene (Fig.6). The genotypes of the studied subjects have been distributed into three groups based on the presence or absence of the polymorphisms: A/A homozygous 224 bp, A/G heterozygote demonstrated 224 bp, 146 bp, 78 bp and G/G homozygous 146 & 78 bp (Fig 3).

Fig. (3): Electrophoresis patterns of allelotyping of GSTP1 (rs1695) gene of breast cancer patients and healthy control groups using BsmAl enzyme by PCR-RFLP method

M: DNA ladder (50 bp); Lanes 1,2,3 refer to heterozygous allele (GA) had 3 bands with 224, 146 & 78 bp; Lanes 5, 6, 7 refer to homozygous allele (GG) had a two band with 78, 146 bp molecular size; Lanes 4, 8, 9 refer to a homozygous allele (AA) had a single band with 224 bp molecular size.

The Genotypes Distribution of Rs1695 Polymorphisms with Allele Frequency in Control and Case Groups.

The GSTP1 (rs1695) gene polymorphism was studied in breast cancer cases and control. The distribution observed in GSTP1 (rs1695) gene polymorphism in cases group and control group are showed in Table (1).

Table (1): Genotype distribution and odd ratio of rs1695 polymorphisms between the patients vs healthy control

<table>
<thead>
<tr>
<th>Genotype rs1695</th>
<th>Patients No.(%</th>
<th>Control No.(%</th>
<th>Significance level</th>
<th>O.R</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>58 (82.8%)</td>
<td>24 (80%)</td>
<td></td>
<td>0.2832</td>
<td>0.4966</td>
</tr>
<tr>
<td>AG</td>
<td>6 (8.6%)</td>
<td>5 (16.7%)</td>
<td></td>
<td>0.4114</td>
<td>2.4828</td>
</tr>
<tr>
<td>GG</td>
<td>6 (8.6%)</td>
<td>1 (3.3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total No. Allele</td>
<td>70</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allele Frequency</td>
<td>0.87</td>
<td>0.88</td>
<td></td>
<td>0.233</td>
<td>1.1171</td>
</tr>
</tbody>
</table>

P ≤ 0.05 ; OR=(95%CI); * reference

GST enzymes involved in the detoxification of reactive metabolites of carcinogens may therefore be important in modulating susceptibility to cancers, especially, P1(GSTP1). Many researches indicate that these gene alterations are associated with certain cancers, for example GSTM1 null genotype as associated with ovarian cancer [19], and the GSTM1 and GSTT1 null genotypes were reported to involve in increasing risk of hepatocellular carcinoma [20]. GSTP1 polymorphism was found to link to the development of colorectal cancer [21]. GSTP1 (glutathione S-transferase pi), located on chromosome 11, prevents cells from carcinogen and cytotoxin [22], [23]. GSTP1 c.313A > G (rs1695) determines the amino acid substitution Ile105Val and lower specific activity in GSTP1 enzymes containing Val compared with Ile have been reported [24]. The GSTP1 G/G genotype has been associated with better survival after treatment with chemotherapy in breast cancer patients [25]. Several studies of GSTP1 expression in breast tumors have been conducted [26], [27]. However, the results are not conclusive and the contribution of GSTP1 to the inactivation of chemotherapy drugs and their metabolites in breast cancer tissue
remains unknown as well as how much this inactivation may account in survival and treatment outcome since GSTP1 is expressed in many other tissues as liver and red blood cells. The GSTs are expressed in a tissue-specific manner [28]. GSTP1 is the major GSTs expressed in breast tissue [29]. We have observed that GSTP1 expression varies significantly among breast cancer molecular subtypes. It is well established that breast cancer is not a single disease and breast cancer molecular subtypes have specific clinicopathological characteristics with different prognoses [30], [31], and sensitivity to chemotherapeutic agents [32], [33]. However, the relative benefit of anthracyclines has not been tested for each specific breast cancer molecular subtype. In the same way, the potential clinical use of predictive factors should be evaluated for each breast cancer molecular subtype especially if, as in case of GSTP1, the expression of the gene varies significantly among molecular subtypes [34].

Genotyping of GSTA1 (rs3957357) Gene Polymorphisms

for GSTA1 (rs3957357) genotyping, the genomic DNA was amplified using specific primers and accomplished by the Thermo-cycler apparatus under the optimal conditions. The results revealed that the presence a one bands (400bp) of the target sequence of GSTA1 (rs3957357) gene in agarose gel (Fig. 4).

![Figure 4](image)

Figure (4): Agarose gel electrophoresis of an amplified product patterns of Glutathion S-Transferase alpha 1 (GSTA1), with specific primer.

![Figure 5](image)

Figure( 5): GSTA1 gene polymorphisms of Breast cancer patients and healthy control subjects according to the number of the bands using PCR-SSCP method.

Inherited polymorphisms in enzymes that activate or detoxify chemotherapy drugs are thought to account for some of the variability in toxicity and efficacy of cancer treatment.[35] The GST enzymes catalyze the glutathione-dependent detoxification of several chemotherapeutic drugs or their metabolites.[36], Polymorphisms that result in reduced (e.g., GSTP1 single nucleotide polymorphisms (SNP) or no (e.g., GSTM1 and GSTT1 deletion polymorphisms) activity of certain GST enzymes are recognized. These polymorphisms may alter the metabolism of chemotherapeutic drugs and modify the effectiveness of therapy, as suggested by reports that GST
polymorphisms predict differences in outcomes of treatment for cancers including breast cancer[37, 38], leukemias [39, 40] and colorectal cancer[41]. GSTA1 and other GSTs of the class are the predominant GSTs in human liver[42, 43], the major site of drug metabolism, and are also expressed in other tissues.[44, 45]. In vitro studies have shown that among human GSTs, GSTA1 has the highest catalytic activity for glutathione conjugation of nitrogen mustard chemotherapy agents,[4] including metabolites of cyclophosphamide (CP), which is used in combination chemotherapy for breast cancer. A polymorphism that influences the hepatic expression of GSTA1 has recently been described. [47] Liver cytosols from individuals who carried the variant GSTA1*B allele, which consists of several linked SNPs in the proximal promoter region of the GSTA1 gene, had reduced levels of GSTA1 enzyme.[48] Because of the importance of the GSTA1 enzyme in metabolism of chemotherapeutic drugs, it can be hypothesized that individuals carrying the low expression GSTA1*B allele may have altered responses to chemotherapy. [49] To our knowledge, no studies have considered genotypic variants affecting _ class GST enzymes in relation to outcomes of cancer treatment, Other study determined genotypes at the GSTA1 proximal promoter polymorphism in normal DNA from women who received CP as part of combination chemotherapy for breast cancer and evaluated overall survival in relation to GSTA1 genotype.

**Sequencing**

According to Macrogen sequencing laboratory instructions, each PCR amplicon representing a specific PCR-SSCP banding pattern was submitted for Sanger sequencing reactions from both the forward and reverse termini (South Korea). All observed PCR-SSCP banding pattern sequences have been aligned with the program DNA Bio Edit 7.2.5 and edited.

Glutathione S-transferases (GSTs) detoxify toxic molecules by conjugation with reduced glutathione and regulate cell signaling. In other study, Single nucleotide polymorphisms (SNPs) of GST genes have been suggested to affect GST functions and thus to increase the risk of human hepatocellular carcinoma (HCC). As GSTA1 is expressed in hepatocytes and the rs3957357C>T (TT) SNP is known to downregulate GSTA1 mRNA expression, study were: (i) to explore the relationship between the TT SNP in GSTA1 and the occurrence of HCC; (ii) to measure GSTA1 mRNA expression in HCCs [48]. Glutathione S-transferases (GSTs) are phase II enzymes that are involved in the detoxification of a wide range of carcinogens, Another study showed the novel GSTA1*A and GSTA1*B genetic polymorphism results in differential expression, with lower transcriptional activation of GSTA1*B (variant) than that of GSTA1*A (common) allele. Considering that cruciferous vegetables induce GSTs, which metabolize tobacco smoke carcinogens, hypothesized that the variant GSTA1*B genotype may predispose women to breast cancer, Particularly among low cruciferous vegetable consumers and among smokers. Thus, we evaluated potential relationships between GSTA1 polymorphisms and breast cancer risk, in relation to vegetable consumption and smoking status in the Long Island Breast Cancer Study Project (1996-1997), a population-based case-control study.
REFERENCE


