

RESEARCH ARTICLE

The genotypes of glutathione peroxidase 1 (GPx1) (Rs1050450) affect some biomarker levels in the breast cancer patients

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Polymorphisms in the antioxidant enzymes have a role in the development of breast cancer. Glutathione peroxidase 1 (GPx1) is one of the antioxidant enzymes that play an effective role in oxidative stress resistance. Pro198Leu (C → T) polymorphism affected GPx1 effectiveness, which might further play a vital role in cancer development. This study aimed to recognize the influence of the GPX1 (rs1050450) gene polymorphism on breast cancer progression and levels of certain biomarkers in patients by using a collection of blood samples from each subject. After extraction of genomic DNA, the SNP Rs1050450 analysis was performed by using polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and agarose gel electrophoresis. The results were visualized under UV light and analyzed with SPSS software (version 23). Additionally, this study revealed that the heterozygote CT (93.3%) genotype was higher than TT genotype in the control group, while the CT genotype was higher (55.7%) than CC and TT (38.6% and 5.7%) in patient's group, respectively. The genotypes TT (OR = 0.0252, 95% CI = 0.0015-0.4305, P = 0.0110) and CT (OR = 0.0327, 95% CI = 0.0.0013-0.7995, P = 0.0360) were less likely to develop breast cancer (BC) sequentially. The frequency of the T allele demonstrated insignificant differences between breast cancer patients and control groups (OR = 0.4422, 95% CI = 0.2387-0.8193, P = 0.0095). The CT genotype caused an increase in glutathione (GSH) concentration and catalase (CAT) activity, while the CC genotype caused an increase in malondialdehyde (MDA) concentration and superoxide dismutase (SOD) activity in patients with BC. However, the CT genotype caused an increase in GSH concentration in the healthy control group. The results found that the genetic variation of GPX1 (rs1050450) was unrelated to BC. The rs1050450 SNP which involved in a reduced risk of BC increased levels of SOD, GSH, CAT, and MDA. The results suggested that it was necessary to monitor oxidative stress and the level of antioxidants for cancer patients.

Keywords: breast cancer; antioxidant polymorphism; RFLP-PCR; GPX1 (rs1050450); glutathione; malondialdehyde; catalase.

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Introduction

Breast carcinoma (BC) is the dominant illness in women accounting for one-third of all

malignancies in females and is the world's second leading cause of death in the health sector, preceded by lung cancer [1]. Oxidative stress has an essential role in carcinogenesis as it affects the

intracellular signal transmission system and damages the DNA. Reactive oxygen species (ROS) is also an important factor in cancer development because it drives the oncogenic of cancers [2]. When ROS and antioxidant responses are out of balance, oxidative stress occurs and promotes the progression of breast cancer [3]. Because of their crucial roles in a wide range of physiological situations as well as their involvement in a wide variety of diseases, free radicals and other oxidants have gained significance in the study of biology [4]. ROS in excess can cause damage to protein, DNA, and lipids. Antioxidants can be divided into two categories including nonenzymatic and enzymatic. Catalase (CAT) and superoxide dismutase (SOD) are created by human body. SOD breaks down superoxide to protect the body against H_2O_2 [5]. Free radicals can adversely alter lipids, proteins, and DNA, and have been implicated in aging and a number of human diseases. Glutathione peroxidase, superoxide dismutase, and catalase are antioxidant enzymes which were part of the human body's antioxidant defense system and inhibit the effect of free radicals' reaction [6]. The protein produced by GPx gene is a member of the glutathione peroxidase (GPx) family, which catalyzes the depletion of biotic hydroperoxides and hydrogen peroxide (H_2O_2) by glutathione and so safeguards cells from oxidative damage. Other research indicates that H_2O_2 is also required for growth factor-mediated signal transmission, mitochondrial function, and the maintenance of thiol redox balance. Hence, glutathione peroxidases are also engaged in controlling these activities by restricting H_2O_2 buildup [7]. GPx1 is one of the antioxidants that play an important role in mitigating the stress damage caused by antioxidants. The Pro198Leu (rs1050450) gene polymorphism has been linked to involvement in the development of different malignancies, while the results are conflicting [8]. The depletion in GPx1 activity is caused by the substitution of proline (Pro) with leucine (Leu) as a result of nucleotide substitution (cytosine (C) → thymine (T)), which is known as rs1050450 [7]. Multiple single nucleotide polymorphisms (SNPs) in the DNA sequences of the GPX1 gene have been

reported and the Pro198Leu (rs1050450 was indicated in the NCBI database as position 197) polymorphism has been widely studied. Previous research has established a link between low levels of circulating GPx1 and an increased risk of cancer in a variety of malignancies including breast cancer [9]. Breast cancer risk was shown to be substantially related to the GPX1 (rs1050450, Pro198Leu) gene polymorphism, which impacted the variation Leu allele. The GPx1 polymorphism is a key element in modulating the oxidative response to stress in breast cancer [10]. Another study found no correlation between levels of GPx1 and malondialdehyde (MDA) activity in cancer tissue, despite both parameters being much higher when comparing to benign tumors [11]. Several studies have indicated that CAT gene polymorphism plays an essential role in the pathogenesis of cancer. High lipid peroxidation is an important risk factor for breast cancer. On the other hand, lipid peroxidation induced by oxygen-free-radical can led to malignant transformation [5, 7]. It has been established that glutathione (GSH) deficiency or a change in glutathione/glutathione disulfide (GSH/GSSG) ratio increases the vulnerability of cells to oxidative stress, inflammation, and tumor progression. However, elevated GSH levels increase antioxidant capacity and resistance to oxidative stress. Breast cancer risk was significantly associated with GPX1 rs1050450 (Pro198Leu) polymorphism, showing a protective effect of variant (Leu) allele. As compared to the control subjects, lipid peroxidation and GPx1 activity were significantly higher in the breast cancer cases, whereas ceruloplasmin activity was decreased [10]. The objective of this investigation was to assess the deleterious effects of the GPX1 SNP (rs1050450) genotype on SOD and CAT activity, BC susceptibility, and survival in Iraqi patients, which could contribute to early detection of disease or as an indicator of the occurrence of cancerous diseases.

Materials and methods

Blood sample collection

The blood samples of 70 breast cancer patients from Merjan University Hospital (Hilla city, Babylon Province, Iraq) with the age from 26 to 80 years old were collected through the time of September 2021 to January 2022. All patients were provided written permission forms before being enrolled into this study. This research was approved by the ethical committee at the University of Babylon (Babylon, Hillah, Iraq) (Approval No. DMS-PF-4532). Additional 30 healthy females aged 20-71 years old were enrolled as the normal control group. 4 mL of venous blood was taken from each participant. The blood sample was divided into two portions equally with 2 mL of blood transferred into a EDTA-containing tube for molecular analysis, and the other 2 mL of blood centrifugated at 3,000 rpm for 15 mins at 4°C for serum separation and stored at -20°C for future use [12, 13].

Biochemical analysis

(1) Superoxide dismutase (SOD) assay

The SOD activities were assessed through Pyrogallol self-oxidation [14]. Briefly, the xanthine/xanthine oxidase system was used to generate the superoxide anion. This anion reduced nitroblue tetrazolium (NBT) to formazan, which was monitored at 560 nm. SOD in the sample removed the superoxide anion and inhibited the reduction. The level of this reduction was used as a measure of SOD activity. The final concentrations of xanthine, xanthine oxidase, and nitroblue tetrazolium in the assay were 50 mM, 10 U/mL, and 0.125 mM, respectively. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of the reduction of NBT to formazan observed.

(2) Catalase (CAT) assay

The CAT activity was assayed by the method of Claiborne, *et al.* [15]. The assay mixture consisted of 1.99 mL of phosphate buffer (0.05 M, pH 7.0), 1.0 mL of 0.019 M H₂O₂, and 10 µL of 10% PMS (w/v) in a total volume of 3.0 mL. The decrease of the absorbance, which was the indication of disappearance of H₂O₂, was recorded at an interval of 30 sec for 3.0 mins at 230 nm. The activity was expressed in nmole of H₂O₂

consumed/min/mg protein using the extinction coefficient of 0.081 x 10³/M·cm.

(3) Glutathione (GSH) assay

The glutathione's content was determined by forming a yellow-colored compound by dithionitrobenzene (DTNB) with acid-soluble sulfhydryl groups [16]. 1.0 mL of the homogenate was precipitated by using 1.0 mL of 4% sulphosalicylic acid. The samples were then kept for 1 hour at 4°C and centrifuged at 1,200 x g for 15 min at 4°C. The assay mixture consisted of 0.1 mL of above supernatant, 2.7 mL of phosphate buffer (0.1 M, pH 7.4) and 0.2 mL of freshly prepared 5,5'-dithiobis-2-nitrobenzene (DTNB) (40 mg in 10 mL of 0.1 M phosphate buffer) in a total volume of 3.0 mL. The color developed due to the formation of a yellow-colored complex (5-thio-2-nitrobenzoate) was immediately measured at 412 nm. The activity was calculated by using GSH as standard and expressed as µmole of GSH/g tissue.

(4) Lipid peroxidation assay

Lipid peroxidation was estimated by using the thiobarbituric acid assay for malondialdehyde (MDA) concentration based on the method reported by Hu, *et al.* [17]. Serum MDA level was determined through thiobarbituric acid (TBA) method. Briefly, 500 µL of serum was mixed with 1 mL of 0.67% TBA and 3 mL of 1% phosphoric acid, and then, incubated for 45 mins before extraction with 3 mL of butanol and centrifugation at 3,000 rpm at 4°C for 10 mins. The absorbance was measured by using a spectrophotometer at 532 nm.

Extraction of DNA and polymerase chain reaction (PCR)

The genomic DNA (gDNA) was extracted from blood samples by using the Genomic DNA Mini Kit (Blood/Cultured Cell) (Genaid, Taipei, Taiwan, China) following the manufacturer's instructions. The absorbances of 260 nm and 280 nm were obtained to determine the quality of extracted gDNA by using NP80 Nanophotometer (Schatzbogen, München, Germany). All gDNA samples were maintained at -20°C for

subsequent tests [18, 19]. DNA-targeted sites were amplified by using a specifically designed primer to detect and recognize the GPx1 (Rs1050450) gene. The designed forward primer (5'-TGT GCC CCT ACG CAG GTA CA-3') and reverse primer (5'- AGG ACC AGC ACC CAT CTC-3') were synthesized by Macrogen company (Gangnam-gu, Seoul, Republic of Korea). The polymerase chain reaction (PCR) was performed with a reaction volume of 20 μ L (1 μ L of each primer, 12.5 μ L of master mix, 3 μ L of gDNA, and 2.5 μ L of DNase-free water). The amplification was carried out by using Biometra thermal cycler (Rudolf-Wissell-Str. Lower Saxony Goettingen, Germany) with the program of 95°C for 5 mins, 35 cycles of 94°C for 30 sec, 60 °C 25 sec, and 72°C for 30 sec, followed by 72°C for 5 mins.

Restriction analysis of PCR fragment length polymorphism

The PCR products were digested by using 2 units of candidate endonuclease Apal (Promega, Madison, WI, USA) at 37°C for 15 minutes. The enzyme was inactivated by at 65°C for 20 minutes before checking the restriction digestion results using agarose gel electrophoresis.

Statistical Analysis.

IBM SPSS software (version 23.0) was employed in this study. The t-test was applied to compare the sample means from two related groups. The whole number of incidences of the tested allele in the population was divided by the whole number of alleles to compute allele frequencies. The odds ratio (OR), 95% confidence intervals (95% CI), and P values of genotype distributions and allele frequencies were calculated by using the Hardy-Weinberg equilibrium assumption and a Chi-square test. The $P < 0.05$ was denoted as statistically significant [20, 21].

Results and discussion

GPX1 (rs1050450) genotyping and gene polymorphisms

A single band (239 bp) of the GPX1 gene target sequence (rs1050450) was amplified by PCR. The

PCR products of GPX1 (rs1050450) gene were digested by using Apal restriction enzyme which cut the sequence of 5'-GGGC[↓]C-3' to recognize rs1050450 SNP in the GPX1 gene (Figure 1). Genotyping was classified into three categories according to the presence or absence of polymorphism including T/T homozygote with one band as 239 bp, C/T heterozygote with three bands as 239 bp, 158 bp, and 81 bp, C/C homozygous with two bands as 158 bp and 81 bp.

Distribution of GPX1 (rs1050450) genotypes with allele frequency

The CT genotype (93.3%) was the most common one in the control group, followed by the TT genotype (6.7%) and the mutant CC (0%). In breast cancer patient group, the heterozygote CT genotype was the most prevalent (55.7%) one, followed by the homozygote CC genotype (38.6%) and the mutant TT genotype (5.7%). The individual carriers of C/T (OR = 0.0252, 95% CI = 0.0015-0.4305, P = 0.0110) and T/T (OR=0.0327, 95% CI=0.0013-0.7995, P = 0.0360) genotypes were less likely to develop BC. Also, there were insignificant differences between breast cancer patients and control groups in frequencies of the T allele of the GPX1 gene (rs1050450) (OR=0.4422, 95% CI=0.2387-0.8193, P = 0.0095).

Studies on breast cancer and GPX1 (rs1050450) gene polymorphism were still conflicting, with allele variation demonstrating no connection or an elevated risk of breast cancer [22]. Hu and his colleagues' meta-analysis that included 5,509 breast cancer patients and 6,542 healthy controls from six case-control studies found that there was no link between GPX1 polymorphism and BC among white people while the incidence of breast cancer rose with gene variation exclusively among African people [17]. Salimi and colleagues suggested that the effect of the Leu allele was that patients with reduced ROS activity scavenging enzymes might have a better prognosis after treatments (chemotherapy, radiotherapy, and immunotherapy) based on ROS generation [23]. The GPX1 (rs1050450) alleles' frequencies and genotypes were not different between thyroid carcinoma patients

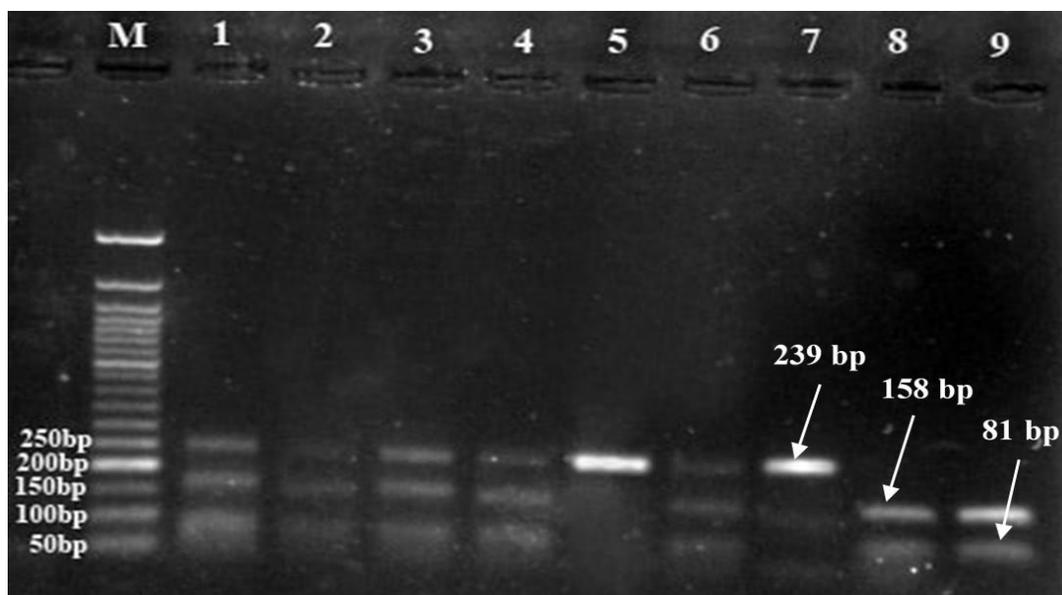


Figure 1. GPX1 gene (rs1050450) allelotyping in patients with breast cancer and healthy control after Apal restriction digestion of PCR products. Lanes 1-4 and 6: CT heterozygous allele with three fragments at 239, 158, 81 bps. Lanes 5 and 7: TT homozygous allele with a single band of 239 bp. Lanes 8 and 9: CC homozygous allele with two bands of 158 and 81 bps. M: DNA marker.

and the healthy control group, and also insignificant related between GPX1 (rs1050450) gene polymorphism and clinical, pathological, and demographic features of the disease [24]. Other studies found that the rs1050450 CT+TT and CC genotypes had a 1.7-to-1.6-fold greater risk of preeclampsia comparing to the CC homozygote genotype with the statistical values as (OR = 1.7, 95% CI = 1.1-2.7, P = 0.01) and (OR = 1.6, 95% CI = 1.1-2.4, P = 0.02), respectively. The prevalence of the CT genotype was high in preeclampsia patients with rs1050450 gene polymorphism, indicating that these variations contributed to preeclampsia risk [25].

Another study revealed that people who carried the variant T allele had a significantly increased chance of developing urinary tract cancer. This study observed that the rs1050450 C → T polymorphism was highly associated with an increased risk of BC but not pancreatic cancer in the stratified analysis [19, 26]. In some genetic models, the results of the complete population revealed a substantial connection between GPX1 (rs1050450) polymorphism and cancer risk. The GPX1 (rs1050450) polymorphism was found to

be associated with the development of bladder cancer, as well as head and neck cancer and brain cancer in a stratified analysis by cancer type. These data provided credence to the notion that the GPX1 gene polymorphism(rs1050450) might be a viable marker for cancer risk [27]. Another meta-analysis study found evidence that the SNP (rs1050450 C → T) might contribute to genetic susceptibility to malignancies, including bladder cancer, lending credence to the idea that the polymorphism might serve as a possible tumor-predicting biomarker [8].

The current investigation found that the CT genotype caused an increase in GSH concentration of $10.0166 \pm 6.198 \mu\text{g/mL}$ and CAT activity of $11.5057 \pm 6.975 \text{ U/mL}$ while the CC genotype caused an increase in MDA concentration of $1.8381 \pm 1.58271 \mu\text{mol/mL}$ and SOD activity of $52.3503 \pm 25.399 \text{ U/mL}$ in BC patients. Further, the CT genotype caused an increase in GSH concentration of $15.895 \pm 12.442 \mu\text{g/mL}$ in the healthy control group (Table 1).

According to the results of this study, the GPX1 polymorphism might be a significant factor in

Table 1. The studied biomarkers level according to GPX1 (rs1050450) genotypes.

Group	Genotype of GPX1 (rs1050450)	Mean \pm SD			
		SOD activity (U/mL)	GSH (μ g/mL)	CAT activity (U/mL)	MDA (μ mol/mL)
Patients	TT	36.0135 \pm 22.434 ^a	9.1138 \pm 10.039 ^a	7.9929 \pm 3.6730 ^a	1.2979 \pm 1.08649 ^a
	CT	32.3460 \pm 20.148 ^a	10.0166 \pm 6.198 ^a	11.5057 \pm 6.975 ^b	1.3605 \pm 0.93806 ^{ab}
	CC	52.3503 \pm 25.399 ^b	4.5340 \pm 4.1309 ^b	7.4788 \pm 2.6370 ^a	1.8381 \pm 1.58271 ^b
Control	TT	54.9959 \pm 59.038 ^a	3.2754 \pm 1.7360 ^a	16.0094 \pm 6.023 ^a	2.0242 \pm 0.48672 ^a
	CT	59.5221 \pm 23.181 ^a	15.895 \pm 12.442 ^b	16.8475 \pm 8.607 ^a	1.8729 \pm 1.41557 ^a
	P value	0.931	0.000	0.878	0.755

changing the oxidative response to stress in breast cancer. Because the alleged link might have a major influence on tumor development or treatment, these findings do need further investigation [10]. Harboring the GPX1 polymorphism variation was found to have a significant influence on plasma MDA concentrations, with wild-type homozygotes having higher levels than persons carrying at least one polymorphic allele ($P = 0.0320$). The relationship between GPX1 polymorphism and MDA to the disease state was investigated by using a multivariate regression model that included age, BMI, and smoking status [10, 24]. Several investigations have discovered higher levels of different lipid peroxidation markers (malondialdehyde) in breast cancer patients' urine, plasma, serum, and, in certain circumstances, cancer tissue [26, 27]. Notably, the relationship between erythrocyte glutathione peroxidase and plasma lipid peroxidase appeared to be conditional, being positive in healthy people for example [28]. The oxidative stress state was evaluated by measuring MDA as an indicator of lipid peroxidation and SOD and GPX as the indicator of antioxidants in BC patients to achieve a defined pattern of oxidative stress in breast cancer pathophysiology. The results of this study could be useful as diagnostic and therapeutic finding for the patients.

Conclusion

This study showed that the genetic variation of GPX1 (rs1050450) was unrelated to BC. A GPX1 gene polymorphism (rs1050450) was involved in a reduced risk of BC. In addition, this SNP played a role in the increased levels of SOD, GSH, CAT, and MDA in breast cancer patients.

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