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Research article

Effect of Rs5746136 genotypes on SOD activity and biomarkers levels in breast cancer patients

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Abstract: Oxidative stress factors are among the most common carcinogens, Superoxide dismutase enzyme-2 (SOD2) is an endogenous antioxidant involved in the scavenging of superoxide anions. This study aimed to investigate the effect of the SOD2 gene polymorphism (rs5746136) on SOD activity and biomarker levels in breast cancer patients. This study aimed to investigate the effect of SOD2 gene (rs5746136) polymorphisms on SOD activity and biomarkers levels in breast cancer patients. The spectrophotometry methods were used to detect malondialdehyde (MDA) and Catalase (CAT), Superoxide dismutase (SOD), and Glutathione (GSH) levels, which reflect antioxidant capacity, and the genotypes of rs5746136 were detected utilize PCR and RFLP. According to the current findings, the GA genotype of the control group was the most common (70%), followed by GG and AA genotypes (26.7% and 3.3%) respectively. In the patient group, the most common genotype was GG (45.6%), followed by the GA genotype (42.8%) and then the AA genotype (11.4%) The frequency of heterozygous genotype G/A compared to the homozygous genotype (G/G) [OR = 0.3571, 95% CI = 0.1375–0.9277, P = 0.0345]. The AA genotype is significantly associated with an increased risk of developing BC [OR = 2.00, p = 0.5403, CI: 0.2175–18.3883]. No significant differences were found in frequencies of the A allele between patients and control groups [OR = 0.7872, 95% CI = 0.4198–1.4762, P = 0.4558]. In addition, there are modest (P 0.05) relationships between serum biochemical parameters levels and rs5746136 genotype in breast cancer patients, but a substantial association between serum SOD activity and GSH concentration and GA and GG rs5746136 genotype in the control group. In conclusion, the current investigation suggests that the AA genotype of (rs5746136) in the MnSOD gene may be

associated with an increased risk of breast cancer. The chosen SOD2 variants (rs5746136) play a crucial role in controlling the activity of the SOD enzyme.

Keywords: breast cancer; antioxidant polymorphism; MnSOD; Rs5746136; SOD2; RFLP-PCR

1. Introduction

The most common cancer among women is breast cancer (one-third of all female cancers), it is the second-highest cancer-causing death after lung cancer and the first on the list causing death in American women between 40–55 years old [1]. In general, the accumulation of damaged DNA affects signal transduction pathways within cells, and oxidative stresses are the main factors that cause cancer development [2]. Reactive oxygen species have been linked to the emergence of cancer in numerous well-established research (ROS). One of their most notable known traits is that they are extremely reactive, created by various diseases, pharmaceutical medications, UV radiation, and ROS-trigger inflammatory cells.

ROS are considered inducers of malignancy because they can promote the transformation of oncogenes and increase cell proliferation, survival, and migration due to the accumulation of damaged DNA.

Oxidative stress increases when the body produces ROS and cannot remove them effectively, which can cause serious health problems [3–5]. Superoxide dismutase (SOD), which is found in the mitochondria, peroxisomes, and cytoplasm, is the body's main ROS-activated antioxidant defense system [3,6].

SOD enzyme catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide [7–9]. Three families of superoxide dismutase are generated according to the type of metal cofactor: copper/zinc Cu/Zn (SOD1, OMIM No. 147450); ferritin/manganese Fe/Mn (SOD2, OMIM No. 147460); and nickel Ni (SOD3, OMIM No. 185490) [10]. The increased or decreased activity of antioxidant enzymes such as GPx, CAT, SOD, GST, and GR has become a significant and necessary tool for understanding the development of cancer and therapy [11]. Several studies have shown some indication that genetic variants in the MnSOD gene may be connected to an increased chance of breast cancer in Chinese women with high-rise levels of oxidative stress or reduced antioxidant consumption [7,11]. The mutations in the SOD2 gene are risk factors for tumor progression [12]. Several investigations have indicated the possibility of polymorphisms in the MnSOD gene, affecting gene expression and/or protein function, referring to the predicted relationship between SNPs in the enzyme gene and cancer development [1,2,13].

The rs5746136 C > T or G > A is associated with the development of premature [3], cardiovascular illnesses [4], diabetes mellitus type 2 (DM2) [5], and cancer [7,10,13], some of the variants are linked to reduced enzymatic activity. The rs5746136 variants are situated around one kilobase upstream from SP1 and the NF-kB transcription element sequences and 65 base pairs downstream of the poly-A site in the 5th intron, close to 3′UTR. The biological impact of the variations is unknown, but some theories have suggested that it may influence how genes are expressed, transport mRNA to the cytoplasm, and affect mRNA half-life. In the Chinese population, bladder cancer risk is correlated with genetic variations in N6-methyladenosine [11]. The SOD2 gene

variant might limit BC susceptibility, but the association Educations that studied the rs5746136 variants and BC risk remain unknown. For this reason, we deliberate it important to govern the frequency of the rs5746136 variants, and whether there is a correlation between SOD2 gene polymorphisms and Iraqi women with BC.

2. Materials and methods

2.1. Ethical statements

Written consent forms have been given by volunteers. The University of Babylon's ethical committee accepted the study and authorized the collection of samples.

2.2. Population of study

The study included 70 patients with breast cancer at Merjan University Hospital aged a rounded 25–81 years old, the healthy control group consist of 30 healthy females (20–71 years old). Samples start collecting between September 2021 and January 2022. Every individual provided a written authentication.

2.3. Blood samples

About four milliliters of venous blood sample was collected from each subject in this study. Each blood sample was divided into two parts 2 ml for each: the first part was collected into EDTA-containing tubes to use for genetic analysis, and the second part was used to separate the serum by centrifugation at 3000 rpm for 15 min and then kept in Eppendorf tubes at –20 ℃ until used [14,15].

2.4. Biochemical analysis

2.4.1. Superoxide dismutase (SOD) assay

According to [12], superoxide dismutase activities were assessed through Pyrogallol self-oxidation. pyrogallol rapidly autoxidizes in the existence of molecular oxygen, in an alkaline medium, to produce numerous intermediate products. The principle of this procedure is based on the mass production of pyrogallol-quinone through a reactive intermediate, the semiquinone radical, and the capability of SOD to block this reaction by radical dismutation. Pyrogallol-quinone is nut brown and absorbs visible light at 420 nm. One unit of SOD activity is defined as the quantity of enzyme required to block the oxidation of pyrogallol autoxidation by 50% per min per ml of the assay combination.

2.4.2. Catalase (CAT) assay

Serum catalase activity was measured using hydrogen peroxide as a substrate assay based on forming a stable complex with ammonium molybdate [16,17]. The test for glutathione content was based on the formation of a yellow-colored compound by dithionitrobenzene (DTNB) with acid-soluble sulfhydryl groups, as described by [18].

2.4.3. Lipid peroxidation

Lipid peroxidation was estimated using the thiobarbituric acid assay for malondialdehyde (MDA) concentration based on [19]. Malondialdehyde (MDA), the main product of lipid peroxidation, and thiobarbituric acid (TBA) interact in the assay to create MDA-TBA2 adducts known as TBARS. A reddish-pink tint produced by TBARS can be observed spectrophotometrically at 532 nm.

2.5. Molecular assay

2.5.1. DNA extraction

Following the manufacturer's instructions, gDNA was extracted from blood samples using the Genomic DNA Mini Kit (Blood/Cultured Cell) (Catalog number GB100/300, Genaid, Taiwan). The absorbance at two wavelengths (260 and 280) on a (Nanophotometer NP80, Implen, Germany) at the Advanced Microbiology Lab, biology department, College of Science, University of Babylon, Babylon, Iraq, was used to assess the quality of the DNA sample. For subsequent tests, all DNA samples were maintained at a temperature of 20 ℃ [15,16,20].

2.5.2. Polymerase chain reaction (PCR)

DNA-targeted sites were amplified using a specifically designed primer to detect and recognize the MnSOD (rs5746136) gene (Macrogene, South Korea). Forward primer 5′-GATGCCTTTCTCCTATTC-3′ and the reverse primer was 5′-TCAGTCACCTGCTACATT-3′. The Polymerase Chain Reaction PCR technique was performed with a reaction volume of 20 µl (1 µl of each primer, 12.5 µl of green master mix, 3 µl of DNA, and 2.5 µl of DNase-free water. Amplification was carried out by thermal cycler (Biometra, Germany) as the following program: 95 ℃ for denaturation for 5 minutes, 35 cycles for 30 seconds at 94 ℃, annealing for 25 seconds at 60 ℃, extension for 30 seconds at 72 ℃ and final extension for 5 minutes.

The product of PCR was electrophorized in 1% agarose gel at 75 V (cleaver sciences, UK) and then observed by using ethidium bromide. Gel documentation system (Cleaver science-UK) used to capture photographs.

2.5.3. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

According to the manufacturer's instructions, the PCR product was digested by two units of candidate endonuclease TaqI for 14 hrs at 65 ℃ (Promega). PCR products were electrophorized with 1% agarose at 75 V (Cleaver science-UK) and stained with ethidium bromide for visualization.

2.6. Statistical analysis

The statistical analyses in this study were carried out using IBM SPSS statistics version 23.0. To compare sample means from two related groups, the dependent samples t-test is utilized. 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, and P values of

the genotype distributions and allele frequencies were calculated using the Hardy-Weinberg equilibrium assumption and a Chi-square test. Duncan's test was used to investigate the correlation between blood biochemical parameter levels and rs1050450 genotypes. All $P < 0.05$ were considered statistically significant [21–23].

3. Results

As a preliminary step in amplifying the target area of the SOD1 gene, genomic DNA was isolated from Blood samples.

3.1. Genotyping of SOD2 (rs5746136) gene polymorphisms

The results of gene polymorphism show the presence of a unique band (253 bp) of the SOD2 gene target sequence (rs5746136).

SOD2 (rs5746136) gene PCR products were digested by TaqI restriction enzyme which cut the sequence (5′TCG3′) to recognize rs2576178 SNP in the SOD2 gene as shown in Figure 1. Genotyping was classified into three categories according to presence/absence of polymorphism: G/G homozygote with two bands as 130 and 123 bp, G/A heterozygote with three bands as 253, 130, 123 bp, and AA homozygous with a single band as 253 bp.

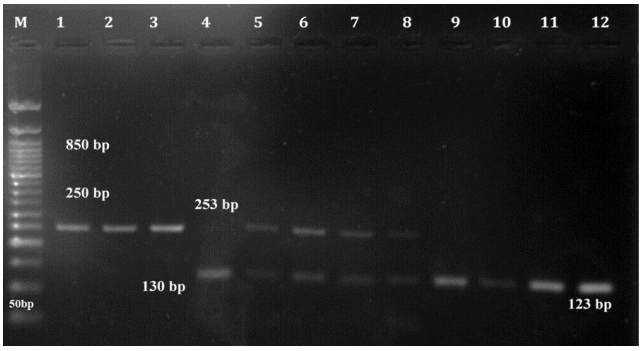

Figure 1. SOD2 gene (rs5746136) allelotyping in patients with breast cancer and healthy individuals depending on using TaqI restriction enzyme according to PCR-RFLP technique showed as follows: lane 1–3: present the AA homozygous allele as a single band of 253 bp, lane 5–8: refer to GA heterozygous allele as three bands of 253, 123, 130 bp, lane 4, 9–12: represent the GG homozygous allele as two bands of 130 and 123 bp.

3.2. The genotypes distribution of SOD2 rs5746136 polymorphisms with allele frequency in control and case groups

SOD2 gene (rs5746136) polymorphism distribution in study groups as shown in Table 1, the GA genotype (70%) was the greatest distributed in the control group, then the GG genotype (26.7%), followed by the mutant AA (3.3%). Instantly, the homozygote GG was the most common genotype (45.6%), then followed by the heterozygous GA genotype (42.8%), and mutant AA genotype (11.4%). The frequency of the G/A heterozygous genotype in comparison with the G/G

homozygous genotype (OR: 0.3571, 95% CI: 0.1375–0.9277, P value: 0.0345). The AA allele of rs5746136 was remarkably associated with an increased chance of developing BC [OR = 2.00, p = 0.5403, CI: 0.2175–18.3883]. Polymorphism in the MnSOD2 (rs5746136) gene shows unimportant variations in allele A frequency among patients and the control group (OR: 0.7872, 95% CI: 0.4762, P value: 0.4558).

Table 1. Distribution of SOD2 (rs5746136) genotype and odd ratio among patients and control group.

Genotype rs5746136	Patients No. (%)	Control No. (%)	P-value	O. R	CI (95%)
GG[a]	32 (45.6%)	8 (26.7%)			
GA	30 (42.8%)	21 (70%)	0.0345	0.3571	0.1375–0.9277
AA	8 (11.4%)	1 (3.3%)	0.5403	2.0000	0.2175–18.3883
Total No.	70 (100%)	30 (100%)			
Allele	Frequency	Frequency			
G	0.67	0.61			
A	0.33	0.39	0.4558	0.7872	0.4198–1.4762

$P \leq 0.05$; OR = (95% CI); [a] reference.

The current findings revealed that the SOD2 (rs5746136) AA and GA genotypes promote a decrease in SOD activity (40.037129.62) U/ml and (40.037129.62) U/ml, respectively. GSH, MDA levels, and CAT activity were not altered in BC patients. Furthermore, as listed in Table 2, the GA genotype reduces SOD activity (51.588630.42) U/ml in the healthy control group.

Table 2. Biochemical parameters levels in association with SOD2 (rs5746136) genotype.

Group	Genotype of SOD2 (rs5746136)	Mean ± SD SOD activity	GSH	CAT activity	MDA
Patients	AA	40.0371 ± 29.62a	6.1871 ± 4.64a	11.964 ± 11.21a	1.467 ± 1.014a
	GA	40.3042 ± 22.38a	7.7625 ± 6.3533a	9.5076 ± 5.693a	1.561 ± 1.380a
	GG	42.2578 ± 24.72b	8.5037 ± 7.2248a	9.4274 ± 3.9164a	1.476 ± 1.168a
Control	AA	29.4433a	20.1317a	8.0887a	1.1083a
	GA	51.5886 ± 30.42a	13.2198 ± 12.54a	17.1516 ± 7.905a	1.84 ± 1.585a
	GG	63.5457 ± 22.14b	19.2335 ± 12.52a	17.5300 ± 9.772a	2.07 ± 0.6787a

4. Discussion

Contrasting theories have been proposed regarding the function of the SOD2 enzyme in the modulation of oxidative stress that ROS generates (tumor promoter) in the cell and the progress of cancer. Furthermore, the SOD2 gene has binding sites for disparate transcription elements that behave as a ligand to activate the transcription and participate as a cell defense system as opposed to agents that persuade oxidative stress [7,24]. Studies linking the SOD2 rs5746136 variation to BC have revealed a variety of susceptibilities, including those associated with risk [25,26], some with protection [27], and still others without a connection [14,28]. The relationship between the SOD2 rs5746136 variations and BC susceptibility in the Iraqi population is not well understood, yet. In the current investigation, there were statistically significant differences between BC patients and controls

in the frequency of the GA and AA genotypes of the SOD2 rs5746136 variations, which were linked to an increased risk of developing BC ($p < 0.05$).

This is the first study to investigate the relationship between SOD2 rs5746136 variations and BC in the Iraqi population. When compared to the wild-type homozygous genotype, variation in the A allele of the MnSOD genotype was found to have a 2-fold (0.2175–18.3883 percent CI) greater risk of breast cancer. The findings presented in the current study credibly support the idea that MnSOD genotypes may influence breast cancer risk [15]. In this instance, numerous studies have examined the expression of SOD2 in BC; nonetheless, it is still unclear how BC is regulated to develop. T alleles from SOD2 (rs5746136) changes are likely to alter the inadequate activity of the SOD2 enzyme. The cellular defense systems and antioxidant defense capabilities are hence ineffective. Gene regulatory pathways can foresee and start the development of BC because oxidative stress is produced [29].

Many other studies linked the A/a genotype form to increased risk of cancer, like bladder cancer, Gastric Cancer, and primary open angle glaucoma [15,30,31]. With the help of this information, it can be concluded that the AA genotype of the rs5746136 variant confers a favorable susceptibility to BC. On the other hand, the development of cancer is complex and depends on interactions between various genes engaged in various metabolic pathways, epigenetic processes, and environmental variables rather than just being tied to the monogenic inheritance of a protein variant [32,33]. There was a study revealed that the rs-4880 and rs-4244285 and rs1001179 polymorphisms also play a crucial role in breast cancer development in the Iraqi population, but the rs-1801274 and rs2070424 were shown an insignificant association with the risk of breast cancer [25,34].

The study looked at selected variants in SOD2 30UTR A > G (rs5746136), genes that have previously been identified as having essential roles in the control of the oxidative stress process. MnSOD2 (rs5746136) gene is situated 65 bp downstream of the poly-A tail site in the 3′UTR of the SOD2 gene. Also, about 1kbp upstream of the NF-B and SP1 transcription factors sequences [35]. Because of environmental factors [36–38], and polymorphism in regulatory areas of the genes [39]. affected the expression levels of the superoxide dismutase family members, including SOD2, in this situation, they may play key roles. The positions of the rs5746136 suggest that these genetic variations are probably capable of controlling the expression of the SOD2 gene. We, therefore, proposed the possibility that these genetic variants could be linked to the risk of BC.

Previous research suggested that these polymorphisms were linked to the risks of multifactorial features related to oxidative stress [40,41]. The AA genotype of rs5746136 variants increased with the risk of heroin addiction and the haplotype AA was associated with the increase in heroin addiction, there was no correlation between genotype studies and the level of SOD2 expression [42].

There are several limitations to this study. First, the SOD2 gene contains a variety of other polymorphisms. Further study of the impact of SOD2 polymorphisms on mRNA levels should be conducted concurrently. Second, it has been noted that certain environmental factors, such as electromagnetic fields and medications, are linked to the mRNA levels of some antioxidant genes (such as catalase, SOD2, SOD1, *etc.*) [43]. More study with bigger sample numbers is needed to confirm the role of the rs5746136 polymorphisms in the pathophysiology of BC.

5. Conclusions

This study found that the AA genotype in the MnSOD gene (rs5746136) was associated with a

higher risk of breast cancer than the GG and GA genotypes. The MnSOD2 gene variation is crucial in modulating SOD enzyme activity, which can lead to uncontrolled ROS and oxidative stress, which is one of the stimulation and risk factors for cancer formation. The rs5746136 variant is located in the 5th intron and has the potential to affect gene transcription, RNA splicing, and mRNA half-life.

Conflict of interest

The authors declare no conflict of interest.

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