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Characterization of *ABCG2* Gene and Detection of Reactive Oxygen Species (ROS) and Some Cytological Alterations among Iraqi Breast Cancer Women in Maysan Province

Zainab Zamil Gataa Allami[1,*], Maytham A. Dragh[1]

[1] Department of Biology, College of Science, University of Misan, Maysan, Iraq
*Corresponding author
Email: zainabzamil@uomisan.edu.iq

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Abstract:

The emergence of breast cancer (BC) patients could be influenced by common polymorphisms in the ABCG2 gene which impact chemotherapeutic response. Reactive oxygen species (ROS) and altered redox conditions are two prevalent biochemical features that might be linked to cytological changes in breast cancer (BC) patients. This case-control study compares apoptosis, mitochondrial ROS levels, cytoplasmic ROS levels, and mitochondrial morphology with control for evaluating the relationship between mutations in the ABCG2 gene and the development of BC. It also evaluates oxidative stress in BC patients following diagnosis. The findings indicated that TT genotype was not linked to an elevated risk of BC (P more than 0.05) however, there has been a significant relationship between CT and CC and BC risk (P less than 0.05). A total of 11 new genetic mutations (SNPs) have been found; 6/11 (G71T), (T141C), (T148C), (G150C), (T169C), and (G172C) were non-synonymous mutations that altered the 3D protein's structure due to the amino acids in BC patients changing. Three of these mutations (G2559C), (G38A), and (G96428A) are synonymous mutations; two of these (163 G^{Del}), and (167 A^{Del}) are frameshift mutations that altered the 3D protein's structure. When put to comparison with normal tissue, the reaction to fluorescence dyes was more pronounced in BC tissue. According to our research, in addition to cytological changes, mutations and polymorphisms in the ABCG2 gene have been associated with the development of BC and its susceptibility to drugs. Additional validation of such results in a sizable population is required.

Keywords: Breast cancer; Polymorphisms; ATP-binding cassette superfamily G member 2 (*ABCG2*) gene, cytological alteration.

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1. Introduction:

The term "cancer" is presently utilized to describe a group of diseases that are defined through abnormal as well as uncontrolled cellular growth, primarily brought on through genetic mutations. (1, 2). Generally speaking, DNA damage causes cancer cells to develop from the normal. While DNA damage can usually be repaired by the body, in cancer cells, DNA damage is not repaired, and the damaged cells disregard the normal signals that tell them to stop dividing as well as avoid programmed cell death (apoptosis) (3). The most frequent type of cancer in women worldwide, BC is responsible for 15% of cancer-related fatalities in women and is the death cause for females globally (4,5). Based on the Iraqi Cancer Registry Board, in 2004 BC accounted

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for one-third of all female cancers registered, making it the most common type of malignancy in Iraq (6, 7). Drug resistance to anti-cancer drugs is a complex process which results from variations in gene targets. A person's genetic variations, particularly in tumoral somatic cells, may contribute to the resistance of the cancer cells to anti-cancer drugs. Several processes, like apoptosis suppression, multi-drug resistance, modifications in drug metabolism, and drug targets, could lead to drug resistance. Along with the creation of targeted therapies, advancements in DNA microarray as well as proteomics technologies have recently provided fresh approaches to combat drug resistance (8). ABCG2 gene encodes the protein known as breast cancer resistance protein (BCRP), which is one of the main ATP binding cassette (ABC) transporters that are involved in multi-drug resistance (MDR) of BC (9). The human chromosome's location 4q22.1 is home to the ABCG2 gene, which has a genomic sequence of about 141 kb 16 exons, and 15 introns (10, 11, 12). There are 655 amino acid proteins encoded by ABCG2 (13). ATP-binding cassette, superfamily G, member 2 (ABCG-2) genetic polymorphisms have been linked to individual variations in the chemotherapeutic response and an increased risk of developing cancer (14). Single nucleotide polymorphisms (SNPs) regarding the ABCG2 gene could alter gene expression and/or decrease its products' activity, which might impact a person's susceptibility to xenobiotics and the development of cancer (15). ROS are oxygen-containing chemically reactive species. They include singlet oxygen (1O2), hydrogen peroxide (H_2O_2), hydroxyl radicals (·OH), and superoxide anions (·O2-, which include a radical electron (16). Oxidative stress is one of the major factors in the development of BC because it arises from an imbalance in reactive oxygen species production (i.e., the free radicals, or ROS) and antioxidant defenses. (17). ROS generation is one of the features which was involved in malignancy progression through mitochondrial malfunction, and mitochondria have a critical impact on the redox redox signaling by electron liberation from the electron transport chain (ETC). The amplified gene from the gene sequencing study as well as breast tissue from the cytological alteration study were utilized in the presented investigation. In addition to examining the association between cytological changes and BC in the Maysan region, south of Iraq, the presented research intends to assess the genetic polymorphisms, mutations regarding the ABCG2 gene, and the link between genetic mutations of ABCG2 gene and drug resistance in women patients who have BC.

2. Materials and Methods:

2.1. Study sites

The research has been carried out at Misan University's genetic engineering lab, which is part of the biology department and College of Science. In the Health Directorate of Maysan, AL-Sader Teaching Hospital, AL-Shifaa Tumor Treatment Center, and AL-Amarah City (subdistricts of Maysan province/south of Iraq), a total of (20) blood and tissue samples have been obtained from BC patients and (20) control.

2.2. Sample collection and identification

Two to three milliliters of blood have been drawn from each BC patient and control group. Each female patient and control had her blood drawn into EDTA-containing tubes via the median cubital vein or, depending on her health, through the palm vein. The samples have been kept at -20°C until the DNA has been extracted. Following public health regulations, tissue samples from patients with BC were obtained and placed into cups containing formalin.

2.3. Genomic DNA isolation

Over three months, all samples from female patients with BC and the control group were taken at various times (September to November). Every sample was maintained in the same way. With the use of gSYNCTM DNA Extraction Kit Functional Test

Data (Geneaid, Taiwan), DNA has been extracted from samples of whole blood.

2.4. Polymerase chain reaction for isolated PCR product (specific *ABCG2* gene)

Genomic DNA samples (isolated DNA) from all of the female patients and controls have been created through combining an equal amount of each person's genomic DNA. PCR method was used to amplify the DNA fragments of the ABCG2 gene PCR product. The subsequent elements: for every 25 µl reaction mixture in the PCR tube, the following were added: 2.5µl primer F, 2.5µl primer R, 2.5µl Nuclease free water, 12.5µl GO Taq ®G2 Green master mixture, and 5µl DNA template. The table displays primer sequences (1).

Table1: *ABCG2* primer sequence, GC% and length.

Primers	Sequences	GC%	Length (Base)		Reference
ABCG2	5-AAAT GTTCATAG CCAGTTTCTTGGA3 3-ACAGTAATGTCGAAGTTTTTATCGCA-5	35.29 %	F:25	R:26	14

Three crucial steps were followed in the 35-cycle PCR reaction program: primer annealing ABCG-2 (45sec at 53.50–58.5°C gradient), primer extension (1min at 72 °C), and initial denaturation (5min at 94 °C), DNA denaturation (1min at 94°C). A final 7min. extension cycle at 72°C has been conducted. A 1.6% agarose gel, 1X TBE buffer containing 5% Et Br, and electrophoresis run at 80 V for 60 minutes were used to measure the amplified PCR product's size. The bands were captured on camera using Gel-Doc technology and seen under UV transillumination. The nucleotide sequencing process subsequently employed a PCR product with the clear band.

2.5. RFLP Method:

With the use of the Restriction enzyme kit technique (MboI) (Promega/USA), the generated PCR fragment of 302 bp was digested with MboI restriction enzyme to distinguish CC, CT, and TT (ABCG2) polymorphisms. To cut PCR products regarding the ABCG2 gene and detect genotypes, this work was the first in the world to use the restriction enzyme (MboI) rather than the BseMI enzyme. With 16.5µl of the sterile deionized water, 2µl RE 10X Buffer, 0.4µl Acetylated BSA (10µg/µ), 3.0µl (ABCG2 PCR product 1 µg/µ), and 0.6 µl MboI Restriction Enzyme (10 U/µL), the reaction mixture's final volume was 22.3 µl. In an Eppendorf tube, the reaction mixture has been gently mixed and spun down for a brief time. Subsequently, the reaction vials were maintained in a water bath at 37 °C for four hours, and they were seen on a 2.0 % agarose gel stained with ethidium bromide through using an ultraviolet transilluminator and gel documentation system.

2.6. Nucleotide sequencing

The PCR product of the *ABCG2* gene was chosen for nucleotide sequencing and sent to Macrogen Korea company to analyze the sequences of nucleotide bases. The three-dimensional structures of the proteins (3D protein) were drawn using the bioinformatics programs. Converting DNA sequences in FASTA format to amino acid

sequences in the same format using the Blast program on the NCBI website (18). Using Phyre 2 V.2.0 program to convert amino acid sequences from FASTA format to protein database (PDB) format and predict the three-dimensional shape of the protein (19). Using the EzMol V.1.22 program (www.Sbg.bio.ic.ac.uk/ezmol) to draw three-dimensional protein shapes with the determination of the location of the genetic mutation on the protein (20).

2.7. Tissue preparation

The tissue preparation for cytological alteration study according to Dragh et al. (21) with some modifications. Fixation done in 10% formalin solution was used to stabilize the tissue samples until use. The samples were washed with running water to remove the formalin. Transferred the tissue sample under a stereo (dissecting) microscope then took a small portion the size of a pinhead with forceps and placed it in a concave glass slide. Fixation with 4% Diethyl Pyrocarbonate (DEPC) fixation for 30 minutes. Samples were treated with 300 µl permeabilization solution (10 µl Phosphate Buffer Saline +10 µl Tween 20) for 15-20 minutes. Samples have been washed with the use of the Phosphate Buffer Saline 3 times for 5 minutes. Added 300µl of 4′,6-diamidino-2-phenylindole (DAPI) ($C_{16}H_{15}N_5$) stain for 20 minutes. Samples have been washed with Phosphate Buffer Saline 3 times for 5 minutes. Added 300.0µl of 2',7'-Dichloro-dihydro-fluorescein diacetate (DCFH-DA) ($C_{24}H_{14}Cl_2O_7$) stain for 20 minutes. The samples were washed with Phosphate Buffer Saline 3 times for 5 minutes. Added 300 µl of Mitotracker stain ($C_{32}H_{32}Cl_2N_2O$) for 20 minutes. The samples were washed with Phosphate Buffer saline 3 times for 5 minutes. Added 300 µl of Mitosox stain ($C_{43}H_{43}N_3IP$) for 20 minutes. The samples were washed with Phosphate Buffer Saline 3 times for 5 minutes. The samples were examined under the fluorescent microscope under magnification power (400_X) and photographs were captured by a computer and Corel Capture and J Image software were used.

2.8. Statistical analysis

For comparing the variations in genotype between patients and controls, Chi-square test has been used. SPSS version 22 was used for statistical analysis regarding the data, with a significance threshold of P ≤0.05 probability for significant differences, with the use of MedCalc statistical program (version 20.0111) (https://www.Medcalc.net/tests/odds ratio php), we evaluated the relationships regarding the alleles as well as genotypes with BC risk through Odds ratios (ORs) with 95% confidence intervals (CIs). (22, 23). Through the use of ANOVA/Dunnett, we assessed the relationships between the cytological changes and the risk of BC. GraphPad Prism v. 7 has been utilized for performing the statistical analyses of the cytological alteration study to determine the statistical differences in ROS as well as mitochondrial deficiency between the tissues of BC patients and controls based on the image that was obtained.

3. Results:

We discovered that ABCG2 was connected to both drug resistance and BC. Using a standard PCR experiment, we produced a single band at about 302 bp, as Figure (1) illustrates.

In the RFLP-PCR technique, the results of electrophoresis and digestion of the PCR products of the *ABCG2* gene by MboI restriction enzyme showed three genotypes CC, CT, and TT. The PCR/RFLP of the ABCG2 genotypes is shown in Figure (2).

For ABCG2, there have been statistically significant variations in the genotype distribution. Table 3 displays the odds ratio (OR) of the ABCG2 gene polymorphisms in BC patients and controls. Every OR is computed concerning persons who possess the genotypes CT, CC, and TT. In comparison with the cancer population, the control group had a much higher frequency of CT genotype. The risk of BC in

women with the CT genotype was significantly lower (OR=0.0201, 95%CI=0.0011 - 0.3789, P=0.0091). In the control group, the CT genotype protected against the risk of BC. The risks of BC have been significantly higher in women with CC genotype (OR=11.0000, 95%CI= 1.9976 - 60.5734, P=0.0059). On the contrary, the distribution regarding TT genotype with BC risk did not differ significantly between the two groups for persons with this genotype (OR=5.5405, CI=0.2494 - 123.0857, P=0.2792).

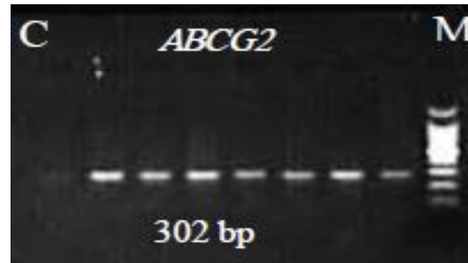


Figure (1): Amplification of the *ABCG2* 302 bp. The first lane from the right (M lane) is the DNA ladder marker 100-1500 bp in size used as DNA molecular weight marker, the last lane from left (C lane) is negative control (PCR mixture without DNA) and all other lanes are PCR product of *ABCG2* gene depended on molecular weight.

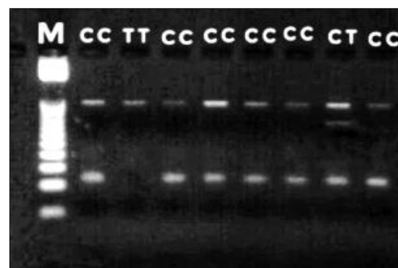


Figure (2): PCR RFLP results for *ABCG2* gene after digested with MboI restriction enzyme. The first lane (M) on the left is a ladder (25-300) bp in size, fragments have been 300bp and 62bp for wild-type homozygote CC, while RFLP-PCR product 300 bp, 150bp, and 62bp for the heterozygote CT, 300bp for Homozygote mutant variant TT.

Table (2) and Figure (3) present statistical analysis results with the use of the Chi-square test, which indicated significant differences (p less than 0.05) in the genotypes of ABCG2 between BC patients and controls.

Table (2): Genotype frequency statistical analyses of *ABCG2* gene amongst the breast cancer patients and controls.

Genes	Genotypes	Total	Cases	Control	X^2	P-value
ABCG2	CC	27	18	9	16	0.000***
	CT	11	0	11		
	TT	2	2	0		
	Total	40	20	20		
Significance *P<0.050,P<0.010,***P<0.005, NS =No significance P>0.050**						

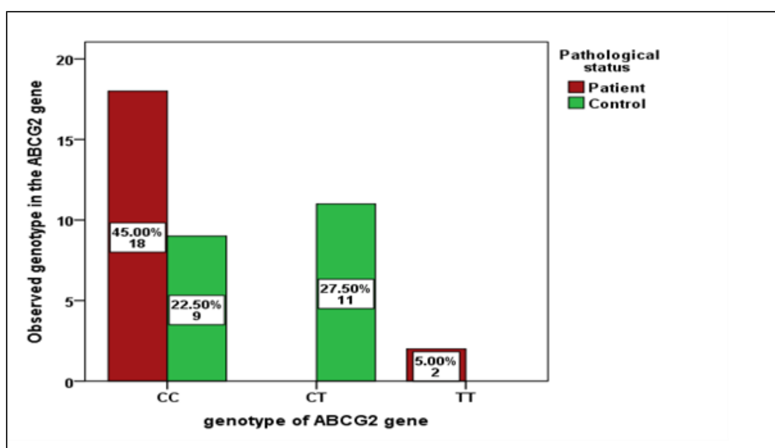


Figure3. The comparison among the numbers and percentages of total CC, CT, and TT genotype frequency values of the *ABCG2* gene between BC patients and controls with the use of the Chi-square testing.

Table 3(A): Alleles frequency of *ABCG2* gene among breast cancer patients and control.

Gene	Allele	Case %	Control %	OR	CI (95%)	P-value
ABCG2	C	36 (90)	29(72.5)	3.4138	0.9834 - 11.8501	0.05*
	T	4(10)	11(27.5)			
% Percentage, OR Odd Ratio, CI Confidence Interval, C (Allele), T (Allele)						
Significance *P<0.05,P<0.01,***P<0.005, NS =No significance, P>0.05**						

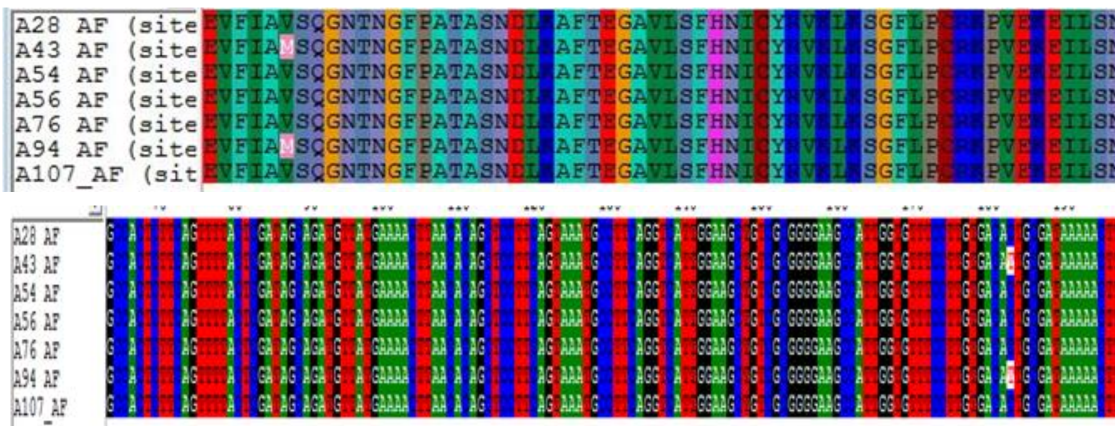
Table 3 (B): Genotype distribution and frequencies of *ABCG2* polymorphisms among BC cases (Patient) and healthy (control).

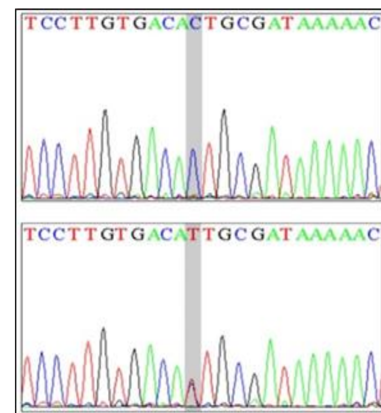
Genes	Genotypes	Cases%	Control%	OR	CI (95%)	P-value
ABCG2	CC	18 (90)	9 (45)	11.0000	1.9976 - 60.5734	0.0059***
	CT	0 (0)	11 (55)	0.0201	0.0011 - 0.3789	0.0091**
	TT	2 (10)	0 (0)	5.5405	0.2494 - 123.0857	0.2792
% Percentage, CI Confidence Interval, OR Odd Ratio, CT heterozygous, CC homozygous, TT homozygous						
Significance *P<0.050,P<0.01,***P<0.005, NS=No significance P>0.050**						

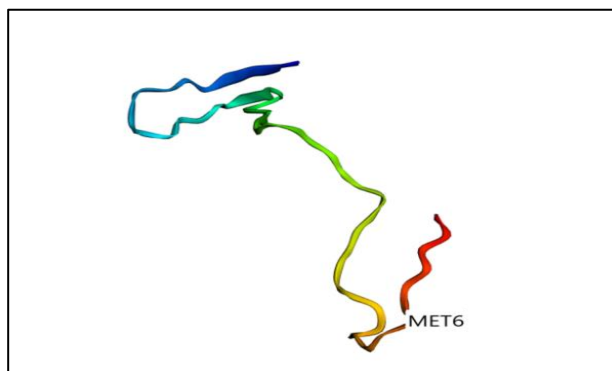
Table (4): Nucleotide changes, the type of mutations (SNPs), and resulting amino acid changes as well as their effects on the *ABCG2* gene translation process.

Gene	Site of SNP / InDel polymorphism	Nucleotides (SNPs)	Amino acids	Type of mutation	Effect of mutation on translation	Missense mutation%	Silent mutation%	Non sense mutation %	Frameshift mutation %
ABCG2	38	G>A	E>E	Transition	Silent	54.54	27.27	-	18.18
	71	G>T	S>Y	Transversion	Missense				
	141	T>C	M>S	Transition	Missense				
	148	T>C	M>V	Transition	Missense				
	150	G>C	P>A	Transversion	Missense				
	163	G	-	Deletion	Frameshift				
	167	A	-	Deletion	Frameshift				
	169	T>C	M>V	Transition	Missense				
	172	G>C	P>A	Transversion	Missense				
	2559	G>C	I>I	Transversion	Silent				
	96428	G>A	E>E	Transversion	Silent				

SNP:single nucleotide polymorphism ; InDel: insertion /deletion polymorphism ; C: Cytosine ; T:Thymine ; A:adenine ; G :Guanine ; M: Methionine ; A: Alanine ; P: Proline ; V: Valine ; Y: Tyrosine ; S: Serine ; I: Isoleucine; E: Glutamic







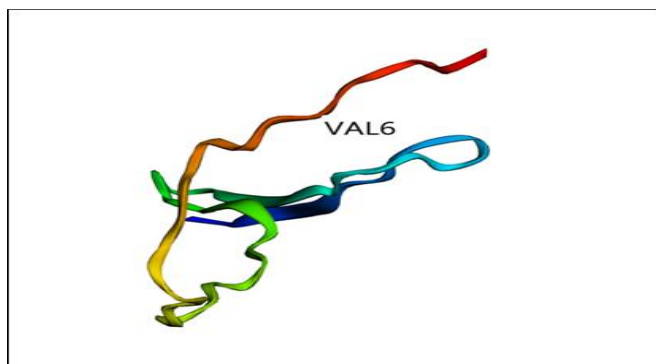


Figure (4): Single nucleotide polymorphisms (SNPs) at the studied sites, besides three-dimensional shapes of the *ABCG2* protein (3D protein) in BC patients compared with control.

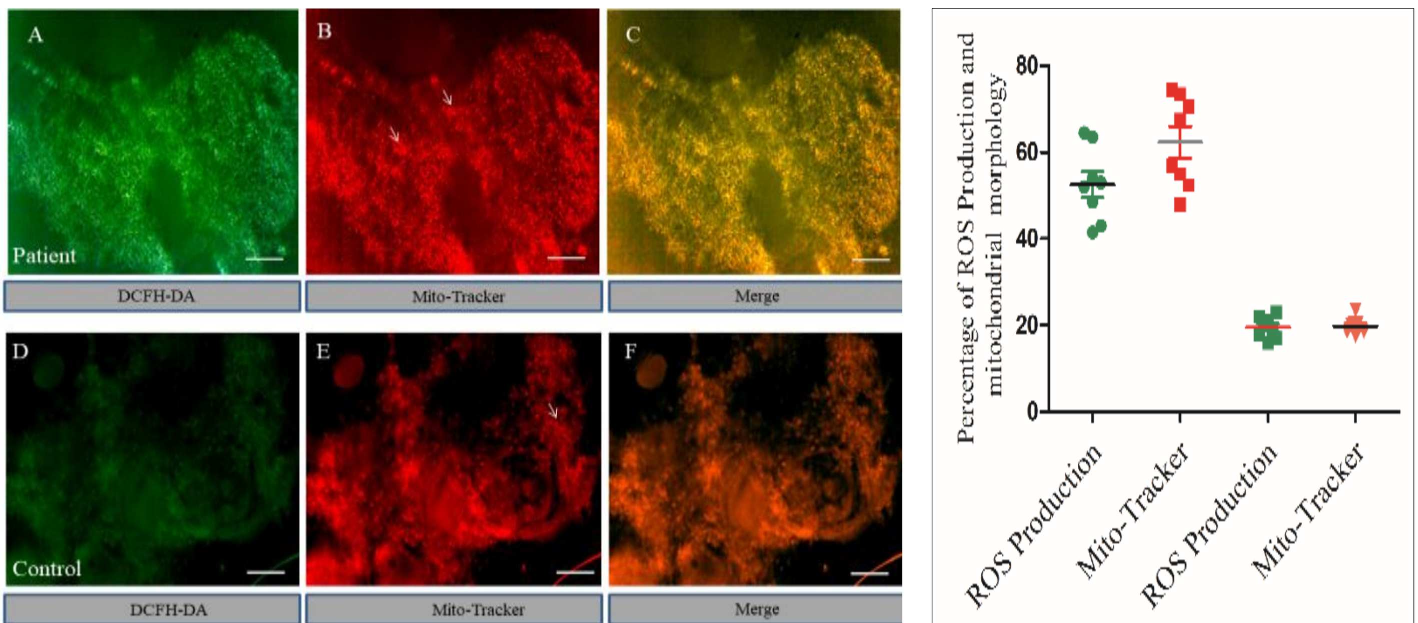


Figure (5): Fluorescent microscopy. (A, D) DCFH-DA stained cell with green fluorescence is an indication of the amount of ROS**,** arrows indicate high. **(B, E)** Fluorescent microscopy of Mito-Tracker stained mitochondria, red fluorescence indicates a mitochondrial morphology defect, and arrows represent mitochondrial structure. **(C, F)** Merged images. Error bars are an indication of the SEM. Analyses of variance (ANOVA/Dunnett: * P <0.050, ** P <0.010, *** P <0.001).

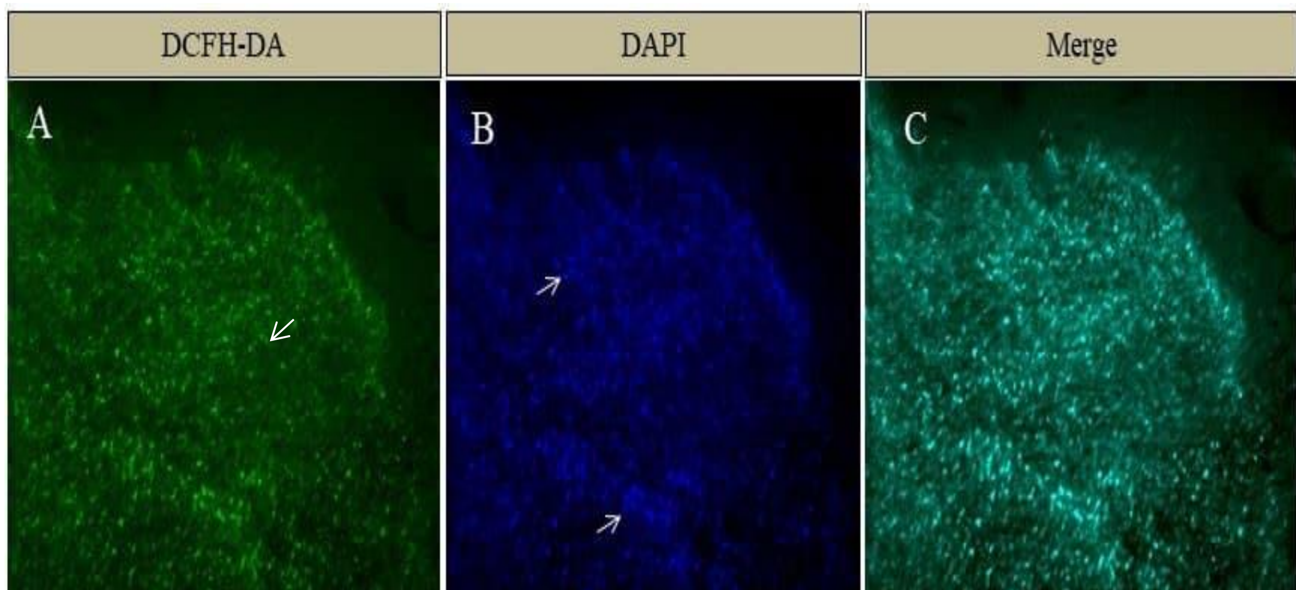


Figure 6: Fluorescent microscopy. (A) DCFH-DA stained cell, green fluorescence is an indication of ROS amount, white arrows represent high ROS. (B) nucleus stained with DAPI, blue fluorescence indicates the nucleus and white arrows indicate the abnormal nucleus. (C) Merged images.

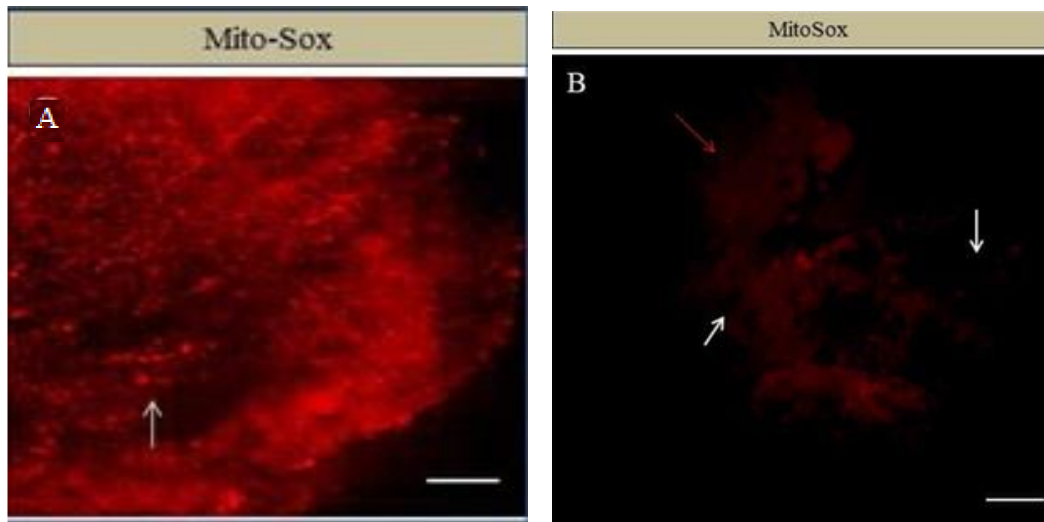


Figure (7): (A) Mito-Sox stained cell, red fluorescence indicates the amount of mitochondrial reactive oxygen species (mtROS), arrows indicate high mtROS. (B) Mito-Sox stained cell, (White) arrows indicate the normal mitochondrial reactive oxygen species (mtROS). Error bars are an indication of the SEM. ANOVA/Dunnett: * P <0.050, ** P <0.010, *** P <0.001.

4. Discussion

As far as we are concerned, this is the first research to use RFLP, nucleotide sequencing, and fluorescence techniques to quantify the association between genetic polymorphisms in the ABCG2 gene as well as cytological changes deficit in BC patients in comparison with control. ABCG2 gene's genetic polymorphisms were previously studied using the BSMI restriction enzyme. This work was the first in the world to use the MboI restriction enzyme in place of BseMI for the RFLP method to explore genetic polymorphisms regarding the ABCG2 gene. As a result, the genotypes and alleles seemed to differ significantly from those found in the earlier research. Wu et al. (14) reported that the BseMI restriction enzyme was used to digest the amplified ABCG2 gene at 55 ºC for an entire night. According to this study, those who have the CC genotype are at a lower risk of developing BC. On the other hand, those who have the CT genotype are more likely to get BC. While discrepancies in HWE were caused by significant differences between observed and predicted frequencies (P <0.05), Hardy-Weinberg equilibrium did reveal disparities between the case and control groups. According to Carroll and Ward (24), HWE tests assess how much the observed genotype and predicted frequencies differ from one another. In the case when these differences are substantial, the test becomes statistically significant and indicates a departure from the HWE assumption. In this research, the genotype distribution in ABCG2 polymorphism had shown a significant difference between case and control groups, indicating that heterozygote genotype (CT) can decrease the risk of BC. The allele distribution in the ABCG2 gene indicated a significant difference between normal allele and mutant allele in case group in comparison with the controls. This means that normal alleles can lead to reducing breast cancer risk. Wu et al. (14) discovered a substantial correlation between the polymorphisms in the ABCG-2 gene and an elevated risk of BC.

In the present study, 11 new genetic mutations (SNPs) were obtained, 6/11 (G71T), (T141C), (T148C), (G150C), (T169C), (G172C) were non-synonymous mutations that caused changes in the structure of the three-dimensional protein as a result of changing amino acids (S>Y, M>S, M>V, P>A, M>V, P>A) in breast cancer. (163 G^{Del}), (167 A^{Del}) are frameshift mutations that caused changes in the structure of the three-dimensional protein located in exon 16 of the *ABCG2* gene, encoding ATP-binding

cassette super-family G 2 transporter, the key protein in chemotherapeutic response. Zeliha et al. (25) reported that the connection between the SNPs of the *ABCG-2* gene with breast cancer risk was found in several studies. The SNPs (G71T, T141C, T148C, G150C, T169C, G172C) gave missense mutation due to a change in the genetic code, that led to a change in amino acids at the level of the resulting protein from S to Y, M to S, M to V, P to A, M to V, P to A). Kadioglu et al. (26) mentioned that the missense and frameshift mutations of the transporter gene, lead to altered binding of anti-cancer drugs in the molecular docking methods. Robey et al. (27) stated that modern studies revealed that mutations at amino acids in some sites of the *ABCG-2* gene influence the substrate specificity of the protein and may probably undermine the activity of *ABCG2*.

It is anticipated that the current study's employment of bioinformatics programs for the prediction-dependent ABCG2 gene and protein as a treatment assistant will improve treatment outcomes. According to Lima et al. (28), drug metabolism is linked to genetic alterations in genes that can mimic therapeutic responses. Pharmacogenomics thus results in the provision of a particular medication based on particular genetic data.

In this work, DAPI (4′,6-diamidino-2-phenylindole $C_{16}H_{15}N_5$) stained cells have been examined under a fluorescence microscope to examine the apoptosis in BC cells. When compared to normal cells, BC cells (found in many patients who do not respond to treatment and develop resistance to anti-cancer drugs) did not exhibit chromatin fragmentation within the nucleus, and DAPI staining indicates that multidrug-resistant cells do not undergo apoptosis. According to research by Mohammad et al. (29), evading apoptosis might contribute to tumor growth, progression, and resistance to treatment. According to Safa et al. (30), some mechanisms, including increased expression of ATP-binding cassette membrane transporters, defective DNA mechanisms, enhanced immune evasion, and incompetence of mitochondrial-mediated apoptosis, contribute to the drug and apoptosis resistance phenotype in many cancer types.

Using 2',7'-Dichloro-dihydro-fluorescein diacetate (DCFH-DA $C_{24}H_{14}C_{l2}O_7$), we have been able to measure total ROS in BC cells and compare them to normal to identify oxidative stress. Our findings showed that the increased concentration of ROS in BC tissues caused an increase in the green color signal when compared to control tissues. In the case when DCFH stain interacts with ROS in breast tissue, it transforms into DCF. According to Kim and Xue (31), cells absorb the DCFH-DA after cellular esterase cleaves off the acetyl groups, which leads to the creation of DCFH. When ROS oxidizes DCFH, the chemical changes into DCF, which emits green fluorescence with an emission wavelength of 530nm and an irritation wavelength of 485nm. Levels of cytoplasmic ROS in BC cells and the control group differed statistically significantly. When comparing the BC patient to a control group, notable differences were seen in the following areas: Oxidative stress (ROS) is higher in Breast Cancer cells as compared to the controls. According to Batool et al. (32), an increase in ROS is typically linked to BC cells' increased metabolism. As a result, oxidative stress is typically exacerbated by a high ROS and low antioxidant content.

In the presented work, we used MitoSOX Red superoxide indicators ($C_{43}H_{43}N_{3I}P$) to examine the connection between mitochondrial ROS generation and BC. When put to comparison with normal tissue, we discovered that the BC tissue had a notably higher level of mitochondrial ROS. Shining red fluorescence is produced when mitochondrial superoxide oxidizes the mitosox indicator. According to Kauffman et al. (33), superoxide and other mitochondrial ROS are commonly detected using the MitoSOX-based assays. The development and growth of cancer are largely dependent on mitochondria. As a result, increased mitochondrial ROS (mtROS) production, the accumulation of mt DNA mutations, and the evolution of mitochondrial

malfunction are all caused by the high demand for mitochondrial efficiency in cancer. Inigo and Chandra (34). Increased metabolic rate, relative hypoxia, and gene mutation all contribute to an increase in the generation of ROS in cancer cells Perillo et al. (35).

Mitotracker ($C_{32}H_{32}C_{12}N_2O$) was utilized in this investigation to determine the mitochondrial morphology in BC tissue in comparison to control. The mitochondrial morphological deficit in BC tissue was identified using the Mito-Tracker stain in comparison to normal tissue. Previous research has detailed the variations in mitochondrial activity and shape between BC cells and a non-tumorigenic cell line Sarmiento-Salinas et al. 18. According to Ma et al. (36), dysregulated mitochondrial dynamics have been linked to the development and progression of many human cancers, impacting factors like drug resistance and cancer spread.

Conclusions:

Our findings demonstrated the accuracy with which bioinformatics as well as molecular genetics methods could be applied to medical diagnosis, including the identification of an appropriate course of treatment and the genetic diagnosis of BC. Additionally, this research revealed ROS in BC patients relative to the control group, which could be associated with genetic mutations. According to our research, in addition to oxidative stress, mutations and polymorphisms in the ABCG2 gene were associated with the development of BC in Maysan Iraqi women. These associations also included aspects of drug resistance and cytological alterations. Additional validation of these results in a sizable population is required.

Authors contributions:

Conceptualization, ZZG; methodology, ZZG, MAD; software, ZZG, MAD; validation, ZZG; formal analysis, ZZG, MAD; investigation, ZZG; resources, ZZG; data curation, ZZG; writing—original draft preparation, ZZG; writing review and editing, ZZG; visualization, ZZG; supervision, ZZG, MAD; project administration, ZZG. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board:

Before blood samples were taken, AL-Shifa Tumor Treatment Center and the Misan Health Directorate were both made aware of the study's aims, and they both attest to their willingness to participate (255;13/9/2020). The investigation complies with Misan University of Iraq's guidelines for scientific research.

Informed Consent:

Every participant in the study gave their informed consent. Oral consent was given by the patient.

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