RESEARCH ARTICLE

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Development of a T-ARMS-PCR Assay for Detecting Genetic Polymorphism in the Catalase (*rs7943316*) Gene in the Iraqi Population with Breast Cancer

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Abstract

Numerous investigations have demonstrated that oxidative stress is markedly increased in breast cancer patients compared to their healthy counterparts. Catalase (CAT), a crucial antioxidant enzyme, plays a pivotal role in safeguarding cells against oxidative damage initiated by reactive oxygen species (ROS). The CAT (*rs7943316*) gene encodes catalase, and certain genetic variations in this gene have been observed to modify catalase activity and levels. Such changes can lead to an altered response to oxidative stress, potentially increasing the risk of breast cancer. In light of this, a novel tetra-primer amplification-refractory mutation system (T-ARMS)-PCR assay was developed to investigate the possible correlation between the CAT (*rs7943316*) gene polymorphism and the development of breast cancer in patients. This method employs a one-step PCR, which is faster, more cost-effective, and more precise than existing techniques. Sanger sequencing was performed to validate the accuracy of our findings. The T-ARMS-PCR assay revealed a significant association between the A/T allele of the CAT (*rs7943316*) gene and breast cancer. Specifically, individuals with the TT genotype had a higher risk of developing breast cancer than those with the AA genotype. The T allele frequency was greater among breast cancer patients than in the control group, and genotype frequencies were consistent with the principles of the Hardy-Weinberg Equilibrium. This study is the first to showcase a rapid, cost-effective, and high-throughput method for detecting the SNP in the CAT (*rs7943316*) gene. This method has the potential to be employed in large-scale clinical trials.

Keywords: Breast cancer- Catalase- T-ARMS-PCR- single nucleotide polymorphism- CAT (*rs7943316*) gene

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Introduction

Breast cancer is a malignant tumour that arises from the cells of the breast tissue. Breast cancer occurs when the cells in the breast tissue begin to divide and grow uncontrollably, eventually forming a mass or tumour that can invade nearby tissues and potentially spread to other parts of the body. Although both men and women can develop breast cancer, women are at a significantly higher risk than men (Feng et al., 2018; Wisam et al., 2019). The typical manifestations of breast cancer include the presence of a lump or thickening in the breast or armpit, alterations in the size or shape of the breast, dimpling or puckering of the skin, discharge from the nipple, or changes in the texture or colour of the breast or nipple skin. However, it is worth noting that not all individuals with breast cancer may experience these symptoms, and regrettably, some may remain asymptomatic (Allison, 2012). Age, gender, family history of the disease, exposure to certain hormones, obesity, alcohol consumption, and a sedentary lifestyle are all recognized risk factors for breast cancer. Nevertheless, many cases of breast cancer occur in individuals without any identifiable risk factors (Colditz et al., 2012; Ferdous and Wisam, 2018).

Oxidative stress arises when the body's ability to detoxify and eliminate reactive oxygen species (ROS) is overwhelmed by its production (Vona et al., 2021). ROS are highly reactive molecules containing oxygen that can damage cellular structures, such as lipids, proteins, and DNA (Janssen-Heininger et al., 2008). Under normal conditions, the body generates antioxidants to counteract the harmful effects of ROS. However, when there is excessive ROS production or a deficiency in the antioxidant defense system, oxidative stress can occur (Eddaikra ans Eddaikra, 2021; Habtemariam, 2019). Numerous studies have linked oxidative stress to various diseases and conditions, including cancer, cardiovascular disease, diabetes, neurodegenerative disorders, and ageing. Moreover, oxidative stress has also been implicated in the development of inflammation and tissue damage,

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thereby underscoring its pervasive impact on human health. Oxidative stress can lead to genetic mutations and alterations in gene expression, which can affect the development and progression of breast cancer (Pizzino et al., 2017). For example, oxidative stress can activate signaling pathways that promote cell proliferation and survival while inhibiting pathways that trigger cell death and DNA repair (Sosa et al., 2013). Consequently, this can lead to the accumulation of DNA damage and mutations, ultimately leading to the development of cancer. Additionally, oxidative stress can promote angiogenesis, which allows tumours to form new blood vessels and grow (Clerkin et al., 2008). Therefore, reducing oxidative stress in breast tissue is an important strategy for preventing and treating breast cancer.

Catalase, a vital enzyme that plays a crucial role in cellular homeostasis, is involved in the degradation of hydrogen peroxide, a ROS, which can cause oxidative stress when present in excess. When hydrogen peroxide levels are elevated, the body's defense mechanisms increase the production of catalase, which converts hydrogen peroxide into water and oxygen, consequently reducing oxidative stress and mitigating cellular damage (Alfonso-Prieto et al., 2009; Zucca et al., 2014). Various factors can influence catalase activity, including genetic mutations that alter its structure or function, or environmental factors that impact its expression or activity. For instance, exposure to certain toxins, drugs, or radiation may inhibit catalase activity and contribute to oxidative stress (Casp et al., 2002; Xiao et al., 2015). Deficiency or malfunction of catalase can disrupt the delicate balance between ROS production and scavenging, leading to oxidative stress and a host of pathological conditions. Thus, catalase plays a pivotal role in preventing oxidative stress-induced cellular damage by efficiently removing excess hydrogen peroxide, thereby maintaining cellular homeostasis (Nandi et al., 2019).

Breast tissue is particularly susceptible to oxidative stress, primarily due to its high-fat content and increased metabolic activity, which may contribute to the generation of ROS and reduce antioxidant defenses (Kryston et al., 2011; Lee et al., 2017; Ferdous and Wisam, 2017). Research has shown that markers of oxidative stress, including lipid peroxidation and DNA damage, are elevated in breast cancer tissue compared to healthy breast tissue. Furthermore, genetic and environmental factors associated with increased oxidative stress, such as exposure to environmental toxins, radiation, and high-fat diets, have been linked to an increased risk of breast cancer (Acevedo-León et al., 2022; AK et al., 2015).

Genotyping is a crucial process in molecular biology that allows for the identification and analysis of genetic variations, which can help to elucidate the genetic basis of complex diseases. Polymerase chain reaction (PCR) has emerged as a powerful tool for detecting naturally occurring and artificially produced genetic variations, owing to its high specificity and sensitivity. However, detecting single nucleotide polymorphisms (SNPs) by PCR can be challenging since the variant and normal alleles differ only by a single nucleotide. While commercial kits and Sanger sequencing can detect SNPs, they are low throughput, high cost and can have operational difficulties (Yang et al., 2014). In this study, we propose a simple primer design approach, tetra-primer amplification-refractory mutation system (T-ARMS), that enables one-step PCR-based SNP detection of CAT (*rs7943316*), which is flexible, rapid, precise and economical (Alyethodi et al., 2018). The Amplification Refractory Mutation System PCR (ARMS-PCR) is one of the most accurate tools in genetic disease diagnosis in recent days, ARMS is a very technique that can detect known mutations involving single base changes or small deletions, by using sequence-specific PCR primers (Wisam et al., 2022). The key feature of T-ARMS is its primer design strategy, which involves using a modified reverse primer that contains a mismatched base at the SNP position. This mismatched base allows for selective amplification of the variant or normal allele, depending on the primer used, thereby enabling accurate genotyping of the SNP (Heidar and Khatami, 2017). The successful implementation of this approach would not only aid in studying the correlation between this SNP and breast cancer but also enable its application in detecting other types of cancer or diseases.

Materials and Methods

Study Participants

This study comprised a cohort of 270 women, of which 150 were healthy, and 120 were diagnosed with breast cancer. Participants were aged between 40 and 70 years old, all of whom were receiving treatment at the teaching hospital located in Al-Dewaniyah city, Iraq, between June 2022 and January 2023. The research methods employed in the study were approved by the Ethical Committee of the Department of Biology/College of Science at the University of Baghdad, and the study was conducted in compliance with the Helsinki Declaration (ethical approval code identifier: CSEC/0123/0031). In addition, the study participants were fully informed about the purpose and procedure of the study and provided consent to participate by signing a consent form.

Blood Collection and Genomic DNA Extraction

Venous blood samples were collected from participants using anticoagulant tubes and were then transported in ice boxes from the hospital to the laboratory for further analysis. DNA was extracted from whole blood samples using a DNA extraction kit (AddBIO, South Korea) according to the manufacturer's instructions. DNA samples were prepared by lysing 200 µl of whole blood with 20 µL of proteinase K (20 mg/mL). The resulting lysate was mixed with an equal volume of binding buffer and added directly to a filter-spin column. After centrifuging the column at 13,000 rpm for 1 minute, the column was washed twice with 500 µL of washing buffer, followed by centrifugation at 13,000 rpm for 1 minute. The spin column was placed in a new 1.5 mL collecting tube with 50 µL of elution buffer to elute the DNA yield and centrifuged again at 13,000 rpm for 1 minute. The DNA yield was quantified using a Quantus fluorometer (Promega, USA), and the samples were stored at -20°C until further analysis.

Genotyping of CAT (rs7943316) A/T Polymorphism

The identification of the CAT (*rs7943316*) gene in the selected samples was accomplished through the use of T-ARMS PCR. The original software found on the website http://primer1.soton.ac.uk/primer1.html was utilised to design and construct the required tetra primers for this PCR technique. The SNP sites were positioned asymmetrically to the common (inner) primers, enabling easy differentiation of allele-specific amplicons with various product sizes using agarose gel electrophoresis. The tetra oligonucleotides used in this study are presented in Table 1 and were purchased from Macrogen (South Korea). For each sample, a total PCR reaction mixture volume of 20 µL was prepared, consisting of 2 µL of dNTPs, 2 µL of buffer, 1.5 µL (0.05 pmol/µL) of each tetra primer (forward and reverse), 0.2 µL of Taq, 7.8 µL of RNase-DNase free water, and 2 µL of template DNA (100 ng/µL). The optimal PCR conditions were as follows: Step 1: 94°C for 5 min; Step 2: (94°C for 30 sec, 63.5°C for 30 sec, 72°C for 50 sec) for 35 cycles; Step 3: (72°C for 5 min, infinity hold at 4°C). PCR products were visualised by loading them onto a 1.7% Tris-Borate-EDTA (TBE) agarose gel. PCR was performed with outer primers on samples from each genotype to validate the T-ARMS-PCR results. The PCR products were subsequently purified and sent to the Macrogen Company for Sanger sequencing, and the resulting data were analysed to confirm the homozygous and heterozygous genotypes.

Statistical Analysis

Descriptive statistics were performed using the Statistical Package for Social Sciences (SPSS), version 23. The chi-square test was utilised to determine the allelic frequencies and genotypes of CAT (*rs7943316*) in breast cancer patients and healthy controls. Odds ratios (OR) with corresponding 95% confidence intervals (CI) were calculated to further investigate the association between the CAT (*rs7943316*) gene and breast cancer.

Results

SNP of CAT (rs7943316) Gene and Risk of Breast cancer

The CAT (*rs7943316*) gene, responsible for the production of catalase enzyme, is located on the plus strand of chromosome 11, specifically between nucleotides 34459972 and 34460972 in the human genome, as shown in shown in Figure 1.

A A A A A G A A A A G C A T C C A
TCCATCCTTTGGTTGCAAATAATACTTACATTAGC
G T A T G G C A A A A T T T A A T T
T T G T A C A G A G T A A T T T A A
C C C A G G A T T G C T G A C T T T T
T A A G A G C T G A G A A A G C A T A
G C T A T G G A G C G C A A G G C C C C
A C C C A G C A G G G T C T A A G T A T
T C C G T C T G C A A A A C T G G C A G
G C C A C C A A C G G C C G C G T C C C A
G G G C G G C C T G A A G G A T G C T G A
T A A C C G G G A G C C C C G C C C T G G G T
T C G G C T A T C C C G G G C A C C C C G G G
C C G G C G G G G C G A G G C T C T C C A A T T
G C T G G G C C A G A G C G G G A C C C T T C C T
T T C C G C A C C C T C C T G G G T A T C T C C G G
T C T T C A G G C C T C C T T C G G A G A G C C C T
G C T C C G A G C C C A T T G G G C T T C C A A T C T
T G G C C T G C C T A G C G C C G A G C A G C C A A T
C A G A A G G C A G T C C T C C C G A G G G G G C G G
G A C G A G G G G G T G G T G C T G A T T G G C T G A G
C C T G A A G T C G C C A C G G W C T C G G G G C A A C A
G G C A G A T T T G C C T G C T G A G G G T G G A G A C C C

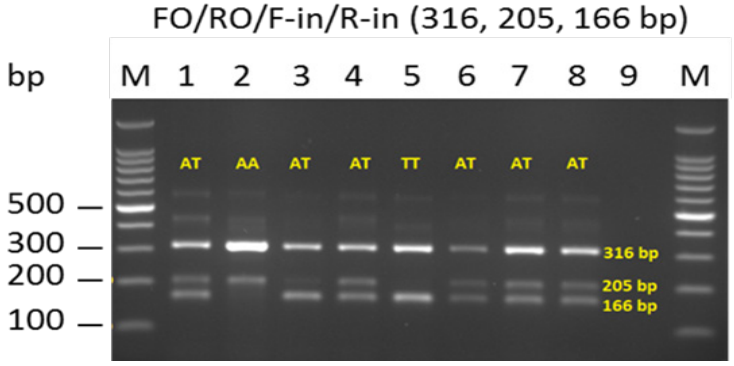

Figure 1. Gel Electrophoresis Image Following PCR Amplification of the CAT (rs7943316) Gene Polymorphism for the Control Group (G1). M: 100bp DNA ladder (GeneDirex, South Korea). Lane 1, 3, 4, 6, 7, and 8: heterozygous AT genotypes (316, 205 and 166 bp); 2: homozygous AA genotypes (316 and 205 bp); 5: homozygous TT genotypes (316 and 166 bp); 9: no template control.

Table 1. Oligonucleotides Used in this Study and Their Properties

Oligonucleotide name	Sequence '5->3'	Start	End	Tm (°C)	Genotype	Expected amplicon (bp)
Forward outer (FO)	CTTCGGAGAGCCCTGCTCCGAGCCCATT	362	389	78	Outer region	316
Reverse outer (RO)	GCTCGGGGAGCACAGAGTGTACCTGCGC	677	650	78		
Inner forward A (F-in)	GATTGGCTGAGCCTGAAGTCGCCACCGA	474	501	78	A-allele	205
Inner Reverse T (R-in)	CAGGCAAATCTGCCTGTTGCCCCGTGA	527	501	78	T-allele	166

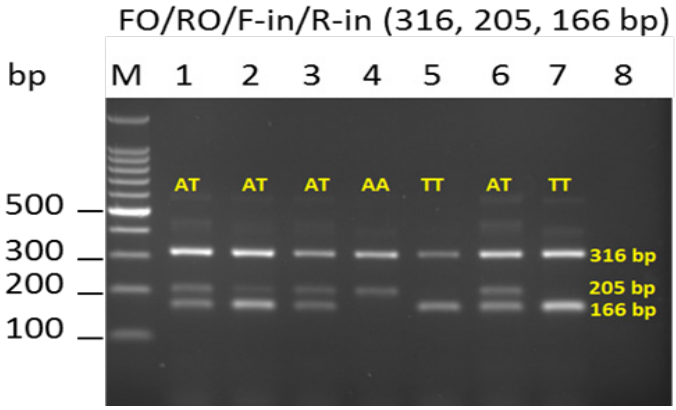

Figure 2. Gel Electrophoresis Image Following PCR Amplification of the CAT (rs7943316) Gene Polymorphism for the Patient Group (G2). M: 100bp DNA ladder (GeneDirex, South Korea). Lane 1, 2, 3, 6: heterozygous AT genotypes (316, 205 and 166 bp); 4: homozygous AA genotypes (316 and 205 bp); 5, 7: homozygous TT genotypes (316 and 166 bp); 8: no template control.

ACGAGCCGAGGCCTCCTGCAGTGTTCTGC
ACAGCAAACCGCACGCTATGGCTGACAGCC
GGGATCCCGCCAGCGACCAGATGCAGCACT
GGAAGGAGCAGCGGGCCGCGCAGGTACACT
CTGTGCTCCCCGAGCGGGCCCGAAGGTCCGTT
TAGAAAGCGGGGGCGTCGGCAAGTAAAGGCCC
GGCTTCCCCCGGGGCGGCGCTTGGAGGGACTG
TACCGCGGCTCACTGGGCAGGGGGGATCCCCT
TCGGTGCAGACGGACTTTTACATTCGCCGAAG
GGGAGGGGGGTCCGGGTAGTGGGGCGCGGG
ACTGCAGGCTTTGTTGTCCGCGACAGGCTCGGGT
GGTTGCTTCAGAATTTTGCACTTTTGCCAACTGG
ACAGAGGTCGCAGCTTGAGGACAGATTGAGGGC
GGGAAAGAGGCAGAGAGCTGCAGCTGCAAG
GAAGTCAGT.

Analysis of Catalase Variants

The T-ARMS-PCR technique was performed to determine the genotype of the CAT (*rs7943316*) gene, which encodes the catalase enzyme. T-ARMS-PCR involved the use of four primers, namely outer forward (OF), outer reverse (OR), inner forward (IF), and inner reverse (IR). The OF/OR primer pair generated the outer fragment of the SNP locus, which acted as an internal control for the PCR. Meanwhile, the IF/OR and OF/IR primer combinations generated allele-specific amplicons. The strategic placement of the inner primers at asymmetric distances from the corresponding outer primers ensured the production of amplicons with distinct sizes, easily distinguishable by gel electrophoresis.

The T-ARMS-PCR amplicons were subjected to gel electrophoresis on an agarose gel in TBE buffer and stained with SYBR-safe DNA gel stain (ADDBIO, South Korea). Based on the size of the PCR amplicons, the samples were divided into three subsets: homozygous T (TT), homozygous A (AA), and heterozygous (TA). Agarose gel electrophoresis images of the PCR analysis for the control samples (G1) and the breast cancer patient samples (G2) are presented in Figure 1 and Figure 2, respectively. Homozygous wild (AA) individuals displayed two bands: a 205 base-pair (bp) band for the A allele and an outer band of 316 bp, while homozygous

Table 2. Distribution of CAT (rs7943316) Genotypes between Control (G1) and Breast Cancer (G2) Patient Groups and Their Association with Breast Cancer

Polymorphisms	G1 (Control) N=150	G2 (Patients) N=120	X^2	P value	OR (95%CI)	P value
CAT (A/T)						
AA	40 (26.6%)	31 (25.8%)	12.072	0.002*	1.0 ref (1.0ref)	
AT	88 (58.7%)	51 (42.5%)			0.748 (0.418-1.339)	0.327
TT	22 (14.7%)	38 (31.7%)			2.229 (1.102-4.506)	0.025*
Allele frequency						
A allele	168 (56%)	113 (47.1%)			1.0ref (1.0ref)	
T allele	132 (44%)	127 (52.9%)	4.247	0.039*	1.430 (0.1017-2.013)	
Dominant model						
AA	40 (26.6%)	31 (25.8%)			1.0ref (1.0ref)	
AT & TT	110 (73.3%)	89 (74.2%)	0.024	0.877	1.044 (0.608-1.802)	
Recessive model						
TT	22 (14.7%)	38 (31.7%)			1.0ref (1.0ref)	
AA & AT	128 (85.3%)	82 (68.3%)	11.338	0.001*	2.717 (1.501-4.920)	

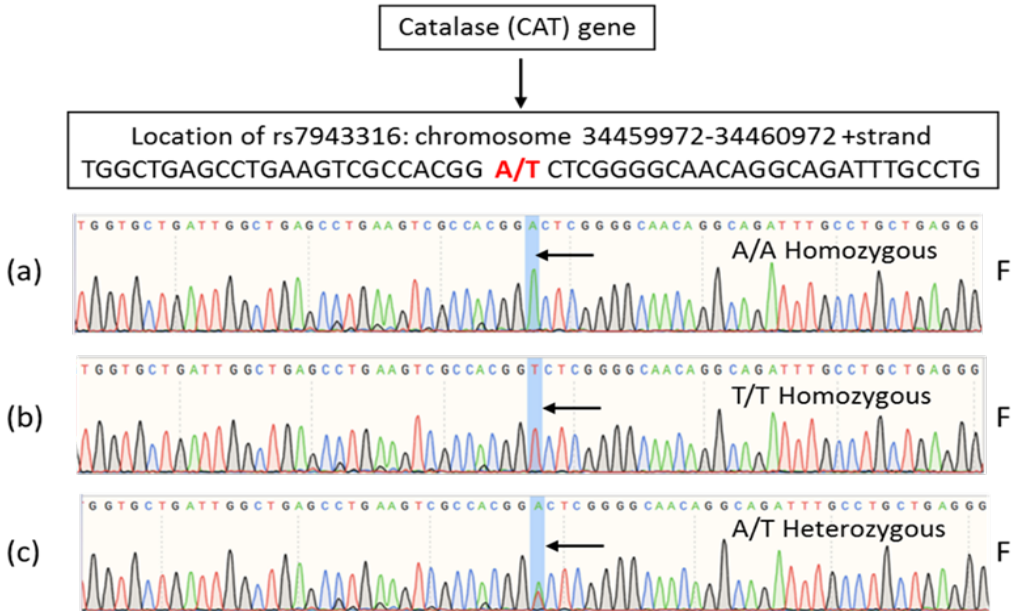

Figure 3. Chromatogram Data Analysis by Sanger Sequencing for the Forward Primer Represents Genotyping of the CAT (rs7943316) SNP. The region of interest, which is the anticipated SNP region is indicated by a black arrow and highlighted in blue. (a) Homozygous A is represented by the green curve; (b) homozygous T is shown by the red curve (c) heterozygous A/T is displayed by the red and green curves overlapping in the same position, indicating the presence of both alleles in the sequencing data.

mutant (TT) individuals showed a 166 bp band for the T allele and an outer band of 316 bp. Heterozygous mutant (AT) individuals showed bands for both the A (205 bp) and T (166 bp) alleles, along with the outer band (316 bp). Fifty percent of the samples were subjected to commercial Sanger sequencing to confirm the PCR results. Figure 3 shows a chromatogram representing the sequencing data for different samples.

Association of CAT (rs7943316) Gene with Risk of Breast Cancer

The results presented in Table 2 provide insight into the genotype frequencies and allele distribution of the A/T polymorphism of CAT (*rs7943316*) SNP in control (G1) and breast cancer (G2) patient groups. The data indicates a significant association between the A/T allele and breast cancer, as evidenced by the X2 value of 12.072 and a corresponding P value of 0.002. These findings suggest a relationship between the SNP and the risk of developing breast cancer. Furthermore, the study found that individuals carrying the AT genotype did not exhibit an increased risk of breast cancer compared to those with the AA genotype (OR 0.748, 95% CI=0.418–1.339; P= 0.327). However, the TT genotype was associated with a higher risk of breast cancer than the AA genotype (OR=2.229, 95% CI=1.102–4.506, P=0.025). These results suggest that individuals with the TT genotype may be at an increased risk of developing breast cancer compared to those with the AA genotype.

The results of this study provide evidence for an association between the CAT (*rs7943316*) A/T polymorphism and the risk of breast cancer. Specifically, the minor T allele was found to be more frequent in breast cancer patients than healthy controls, indicating that this allele may be a risk factor for breast cancer. The genotypic frequencies for both groups were in accordance with the Hardy-Weinberg Equilibrium (X^2= 4.247, P= 0.039), which suggests that the study population is representative of the general population. In the dominant model, results showed that the AT and TT genotypes of CAT (*rs7943316*) did not significantly increase the risk of breast cancer compared to the AA genotype (OR= 1.044, 95% CI= 0.608–1.802 and P value= 0.877). However, a significant association was found in the recessive model when comparing the TT genotype with the AT and AA genotypes (OR= 2.717, 95% CI= 1.501–4.920 and P value= 0.001).

Discussion

Breast cancer represents a significant public health challenge in Iraq, affecting a substantial number of women across the country. Recent data reported by AL-Hashimi highlight that breast cancer is the most prevalent cancer among Iraqi women, with an estimated incidence rate of new cases rising from 52.0 per 100,000 women in 2000 to 91.66 per 100,000 women in 2019 (AL-Hashimi, 2021). The high incidence of breast cancer in Iraq can be attributed to many factors, such as limited awareness and education about the disease, limited access to screening and early detection services, and inadequate treatment options. Breast cancer can arise from a complex interplay of multiple risk factors, including heritable components, such as a family history of the disease, as well as environmental and lifestyle factors, such as obesity, smoking, sedentary behaviour, alcohol consumption, and hormonal therapy (Admoun and Mayrovitz, 2022). As a crucial step towards decreasing the number of cases and mortality rates of breast cancer, the present study aimed to develop a simple, cost-effective and rapid molecular method for identifying individuals at risk of developing

breast cancer.

Studying gene variations associated with cancer is crucial for understanding the underlying causes of the disease. This research was conducted with the same objective: to investigate the possible association between the CAT (*rs7943316*) SNP and breast cancer susceptibility in the Iraqi population. The CAT (*rs7943316*) gene, which codes for the antioxidant enzyme catalase, is of particular interest due to its protective role in preventing oxidative damage (Ahn et al., 2005; Nandi et al., 2019). This genetic association study is the first of its kind to explore the relationship between the CAT (*rs7943316*) gene and breast cancer risk in Iraq. Previous studies have linked the presence of the CAT-262C/T polymorphism with an increased risk of breast cancer (Saadat and Saadat, 2015). The CC genotype has been linked to higher catalase activity in red blood cells and a 17% lower risk of breast cancer compared to the TT and TC genotypes. Other studies have demonstrated that the CAT-262C/T polymorphism can influence both transcriptional activity and catalase levels in red blood cells (Ahn et al., 2005; Quick et al., 2008). It is important to note that there is no clear consensus on the frequency of CAT (*rs7943316*) genotypes in breast cancer patients, which may vary depending on the specific population being studied and other factors such as age, lifestyle, and environmental exposures (Alwan et al., 2019; Jalal Rasheed shwana Shwana et al., 2020).

Overall, these findings suggest that the CAT (*rs7943316*) A/T polymorphism could be a useful biomarker for breast cancer risk assessment. However, it is crucial to emphasise that this study alone cannot provide a definitive conclusion on the relationship between the CAT (*rs7943316*) SNP and breast cancer risk. Further studies are needed to validate these results in larger and more diverse populations to gain a complete understanding of the connection between the CAT (*rs7943316*) SNP and breast cancer risk. Moreover, genetics is just one of many factors that can influence the development of breast cancer, and genetic testing alone cannot be used to diagnose or predict the disease. Therefore, a multifactorial approach incorporating environmental, lifestyle, and genetic factors is essential for a comprehensive understanding of breast cancer risk.

In conclusion, in this study, a T-ARMS-PCR technique was developed for detecting CAT (*rs7943316*) SNP. This technique boasts many advantages, including low-cost, high specificity, rapidity, flexibility and simplicity. The careful selection of primers, optimisation of PCR conditions, and use of a single tube for identifying both wild-type and mutant alleles have contributed to the efficiency and accuracy of this method. Compared to other detection methods such as ELISA, allele-specific PCR, and PCR-Restriction Fragment Length Polymorphism (PCR-RFLP), T-ARMS-PCR is a simpler, more cost-effective option that requires fewer reagents and less time. The association between environmental oxidative stress and genetic mutations underscores the importance of reducing exposure to these factors to minimise the occurrence of breast cancer. While genetics is just one of many factors influencing breast cancer risk, this research highlights the potential for molecular approaches to help identify at-risk individuals and inform prevention strategies. The successful implementation of this T-ARMS approach has significant implications for both research and clinical applications. Overall, the development of T- ARMS provides a valuable addition to the existing arsenal of genotyping techniques and has the potential to improve our understanding of the genetic basis of diseases. Moreover, the T-ARMS approach could potentially be adapted for detecting other types of genetic variations, expanding its utility beyond breast cancer research. Further research in this area is critical to advancing our understanding of the complex interplay between genetic and environmental factors in breast cancer.

Author Contribution Statement

All authors contributed equally in this study.

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Authorship Disclosure

The authors have stated that they have no conflicts of interest.

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