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Circulating tumor DNA as a dynamic tool for evaluating TP53 and BRCA2 alterations in breast cancer progression

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Abstract

Circulating cell-free tumor DNA (ctDNA) released from apoptotic or necrotic sold tumor cells, such as breast cancer, into the bloodstream. Breast cancer patients show high levels of circulating DNA shed from the tumor compared to healthy individuals. This case-control study involved 35 women diagnosed with breast cancer and 50 healthy controls. Blood samples were processed for serum and plasma, utilizing K3_EDTA tubes for ctDNA extraction and gel tubes for ELISA assays. The study assessed the evaluation of the TP53 and BRCA2 proteins from serum using ELISA kits. CtDNA was extracted from plasma using a special kit and real-time PCR (RT-qPCR) to quantify the levels of *TP53* and *BRCA2*. The purpose of this study is to explore the advancements in ctDNA detection technologies, monitor disease progression, and provide insight into the genetic landscape of breast cancer in Iraqi patients, suggesting the ctDNA of *BRCA2* and *TP53* potential as valuable biomarkers for diagnosis and treatment monitoring. The results identified several important findings regarding breast cancer patients and their healthy control counterparts, in terms of genetic markers, serum BRCA2 levels showed (327.02) in breast cancer patients which is significantly lower compared to controls (511.85) and serum TP53 levels followed a similar trend (198.41) in breast cancer group and (389.57) in control group. DNA concentration analysis which is ctDNA showed (2.440) DNA level of *BRCA2* gene in breast cancer patients, compared to controls (1.000). On the other hand, DNA level of *TP53* gene was (0.191), significantly lower in breast cancer patients compared to controls (1.000). These findings highlight the importance of using ctDNA and molecular markers in improving personalized cancer treatment.

Keywords: Circulating tumor DNA, Breast cancer, BRCA2, TP53, ELISA.

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Introduction

Cancer is the leading cause of mortality worldwide and second leading cause of death in developing countries Jemal [1]. In Iraq, cancer incidence has been rising based on recent data from the Iraqi Cancer Registry report 2022 indicate that breast cancer in females accounts as a top-one cancer for 8,184 recorded cases in the country [2].

Breast cancer is classified into several types based on the origin and characteristics of the cancer cells. At present, the selection of breast cancer treatment is based on the analysis of tumor biopsy. However, the information obtained from the tumor biopsy is not permanent and changes over time and the acquired resistance that can occur during cancer treatment cannot be evaluated or analyzed in the original tumor specimen. Studies have found up to 25% of changes in subtypes at or after progression to anticancer therapies [3,4]. There is a growing interest in improving precision medicine by characterizing and monitoring the tumor genome in blood samples [5], known as liquid biopsy. A liquid biopsy can contain circulating tumor cells (CTCs), circulating cell-free tumor DNA (ctDNA), and exosomes that can help to understand tumor evolution, resistance, and heterogeneity during treatment [6]. Furthermore, in some cases, a tumor biopsy may not be feasible, and a liquid biopsy would be the only method to obtain a diagnosis or knowledge of the tumor biology.

The study of ctDNA gives a promising advancement in diagnostic and therapeutic strategies of cancer treatment. Circulating DNA comprises short fragments of DNA that are released into the bloodstream by tumor cells it is a part of cell-free DNA, most of which are around 180 bp. These fragments carry genetic information that reflects the unique mutations and alterations present in the tumor genome, making them a valuable biomarker for cancer detection and monitoring. The half-life of ctDNA is short. However, recent studies on cfDNA collection and processing have found that cfDNA levels are stable for 24 h at room temperature or even for 3 days stored at 4 °C using EDTA tubes [7]. These factors need to be taken into account if ctDNA detection is to be progressively implemented in routine clinical practice in most hospitals. It is well known that cancer patients exhibit higher levels of circulating DNA (ctDNA) in their bloodstream compared to healthy individuals. This circulating DNA, derived from both normal and tumor cells, serves various diagnostic and monitoring purposes [8].

CtDNA is more frequently detectable in metastatic breast cancer compared to early-stage disease. Studies, such as the one by Zhou [9], found that 85.71% of stage IV patients carried tumor-derived mutations in their blood, compared to 57.81% in non-metastatic stages. This makes ctDNA a valuable tool in monitoring advanced disease. Unlike traditional prognostic markers (e.g., tumor size, histological grade), the percentage of ctDNA strongly correlates with overall survival and treatment response. Mutations like *BRCA2* and *TP53* found in ctDNA can predict patient outcomes earlier than standard biomarkers, allowing for the detection of tumor progression, relapse, or treatment resistance, leading to more timely and personalized therapeutic interventions [10]. Studies have shown that analyzing these mutations in ctDNA can offer valuable prognostic information, especially in cancers like breast cancer, where ctDNA reflects the genetic landscape of the tumor and its metastatic spread [11]. This study aims to evaluate the utility of circulating tumor DNA as a biomarker for diagnosis, early detection, monitoring tumor evolution, and predicting treatment response in breast cancer patients, focusing on genes like BRCA2 and TP53.

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Materials and Methods

Study design and sample collection:

The current study is a case-control study that extended through eight months during the period from October 2023 to May 2024 included samples collection from thirty-five diagnosed women with breast cancer. Blood samples were collected at routine follow-up visits for treatment to their physicians at Al-amal National Hospital for Cancer Treatment. Inclusion criteria of case choices included: age from 25-70 years, family history of cancers. The current study also included fifty apparently healthy controls and they were selected based on some criteria: the presence of a family history of cancer or not, smoking, as all of the samples were non-smokers, and the normal range of a carcinoembryonic antigen (CEA) test to prove that the controls are cancer-free [12] and had no inflammation at the time. The samples included the collection of peripheral blood from the participants, using two types of tubes K3_EDTA for circulating tumor DNA extraction from the plasma and Gel and clot activator tubes for ELISA assay of serum, and consent was taken from all participants before sampling as well as provided responses to the administered questionnaire. and the practical work.

The evaluation of BRCA2 and TP53 serum levels:

Evaluation of Tumor Protein (P53/ TP53) by using the ELISA kit from SUNLONG BIOTECH CO. (Cat. NO.SL1323Hu) as well as the evaluation of Human Breast Cancer Susceptibility Protein 2 (BRCA2) from INOVA BIOTECH CO. (Cat. NO. In-Hu4261), all utilized in accordance with the manufacturer's instructions provided in the accompanying catalogue of both kits. These ELISA kits used Sandwich-ELISA as the method of the assay.

Molecular study:

Total circulating tumor DNA extraction from plasma, was done by using MagicPure® Cell -Free DNA Kit (TransGen, biotech. EC201-01) strictly according to the steps detailed in the manufacturer's catalogue. After extracting DNA, the quantification for TP53 and *BRCA2* of ctDNA levels in the bloodstream were estimated by Quantitative Real-Time PCR (RT-qPCR) to amplify the desired gene segments and to ensure that our genes we determined and designed were working efficiently. The primers were synthesized and lyophilized by Alpha DNA Ltd. (Canada). Primers that are used for RT-qPCR are listed in table (1).

Table (1): Primer's sequence of TP53 and BRCA2 that used for RT-qPCR

Gene	sequence (5′→3′ direction)	primer size	product size	Tm
GAPDH	Forward: TGAGAAGTATGACAACAGCC Reverse: TCCTTCCACGATACCAAAG	20bp 19bp	164	60 °C
TP53	Forward: AAGGAAATTTGCGTGTGGAG Reverse: CCAGTGTGATGATGGTGAGG	20 bp	748 bp	56 °C

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BRCA2	Forward: GGCTTCAAAAAGCACTCCAG Reverse: GAGGGAACTTGGCCTCCTAC	20 bp	684 bp	54 °C

CtDNA concentration and purity assessment:

The One-c Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) was used to evaluate the concentration and purity of extracted DNA in order to determine the quality of samples for subsequent analysis in RT-qPCR. The samples ranged in DNA concentration from 2.0-6.0 ng/µl, while the absorbance of the samples was measured at two distinct wavelengths to determine DNA purity (260 and 280nm). The presence of an A260/A280 ratio of around 1.8 suggested that the DNA sample was purectDNA purity (260 and 280nm). The presence of an A260/A280 ratio of around 2.0 suggested that the ctDNA sample was pure.

Results and Discussion

The statistical analysis of the current study detected a high significant difference in the age between the studied groups with P-value = 0.001. The mean ages and the standard error for both patients and healthy people as follows: (49.60±36.48), (1.611±2.242) respectively, as shown in Table (2). This suggests that age plays a potential role in the incidence of breast cancer, as the patients were slightly older than the control group. This finding aligns with McPherson [13] research that indicates age is a significant risk factor for breast cancer, particularly in women over 40, where the risk increases progressively.

Table (2): Comparison of the age's mean of cases and control groups

Age properties	Breast cancer	Control	P-value
Age range	25-70	20-70	
Mean	49.60	36.48	
Standard deviation	9.531	11.854	0.001*
Standard error	1.611	2.242	
Number	35	50	

As for the statistical analysis of the family history detected no significant difference with P-value = 0.2 NS between the groups, as shown in Table (3). 77.1 % of the breast cancer patients had no family history with the disease and 22.9% of the patients had. In simpler terms, the history (whether positive or negative) is not significantly different across these groups, as the p-value is greater than the common threshold of 0.05 for statistical significance. In this study, family history did not play a decisive role in the development of the disease for most participants, while international research consistently highlights the significant role of family history as a major risk factor for the development of breast cancer, as proved by Liu [14] that could be due the environment and life style factors [15].

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Table (3): Comparison of the family history between patient and control groups

Family History		Breast cancer	Control	P-value
Negative	**Count**	27	38	0.2 NS
	%	77.1 %	76.0 %	
Positive	**Count**	8	12	
	%	22.9 %	24.0 %	
Totally	**Count**	35	50	
	%	100.0 %	100.0 %	

As shown in Table (4), the factor smoking indicated no significant difference between the groups with P-value = 8.8, concerning smoking history as all the participated subjects whether it was patient or healthy control are not smokers. Since the P-value is much greater than 0.05, it suggests that smoking is not significantly different across the groups (Breast Cancer and Control). Such as the previous risk factor, smoking did not play a decisive role in the development of the disease for most participants, but that does not deny the significant role of smoking in causing breast cancer worldwide according to Luo J.[16] This attributed to the fact that Iraqi women have lower prevalence of smoking compared to other communities.

Table (4): Comparison of smoking risk between patient and control groups

Smoking		Breast cancer	Control	P-value
No	**Count**	35	50	8.8 NS
	%	100.0 %	100.0 %	
Yes	**Count**	0	0	
	%	0.0 %	0.0 %	
Totally	**Count**	35	50	
	%	100.0 %	100.0 %	

As for in Table (5), when we compared serum BRCA2 levels between breast cancer patients and a control group. The mean serum BRCA2 levels were (327.02) in the breast cancer group and (511.85) in the control group. The P-value is 0.001, which was highly significant difference (since it was less than 0.05) between the serum BRCA2 levels with standard error (20.73), (17.69) in breast cancer patients and the control group respectively. This shows individuals with breast cancer have lower levels of the produced protein from the gene, which indicates that mutations or altered regulation of the *BRCA2* gene in these patients might lead to decreased protein production or non-function protein which it could hinder the ability of cells to repair damaged DNA and contributing to oncogenesis in breast tissue, supported by the study of Maia [17].

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Table (5): Comparison between patients and control groups in serum BRACA2.

Serum BRCA2	Breast cancer	Control	P-value
Mean	327.02	511.85	0.001*
Standard deviation	97.722	75.062	
Standard error	20.73	17.69	
Number	35	50	

In Table (6) when we compared serum TP53 levels between breast cancer patients and a control group showed that the mean was (198.41) in the breast cancer group and (389.57) in the control group. The P-value was 0.001, which indicated statistical significance difference (since it is less than 0.05) with standard error for both breast cancer group and the control group, (7.950), (24.097) respectively. This suggests that lower TP53 protein levels are associated with breast cancer pathology, because it acts as a transcription factor that responds to cellular stress, particularly DNA damage, by halting the cell cycle or inducing apoptosis to prevent the propagation of damaged DNA and the development of cancer [18,19].

Table (6): Comparison between patients and control groups in serum TP53.

Serum TP53	Breast cancer	Control	P-value
Mean	198.41	389.57	0.001*
Standard deviation	42.070	99.357	
Standard error	7.950	24.097	
Number	35	50	

It compared in Table (7), the level of ctDNA fragment for *BRCA2* gene between breast cancer patients and a control group using Ct values and other calculations. Mean Ct values: The breast cancer group had a lower mean Ct value for *BRCA2* (23.067) compared to the control group (24.749). This suggested high level of ctDNA for *BRCA2* gene in the breast cancer group since a lower Ct value indicating higher DNA concentration. The calculated ΔCt values showed a larger difference in the control group (4.417) compared to the breast cancer group (3.130). The 2^-ΔCt values showed that the relative level of ctDNA for *BRCA2* gene in the breast cancer group was about 2.44 times higher than in the control group (experimental/control = 0.1142/0.0468). The P-value was 0.001, indicated a highly statistically significant difference in *BRCA2* DNA concentration between both groups. The elevation of ctDNA levels in breast cancer patient attributed to the aggressiveness of the tumor or a large tumor burden, according to studies done by Arimura [20] and Huang [21].

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Table (7): Comparison between patients and control groups in BRACA2 DNA concertation level.

groups	Means Ct of BRACA2	Means Ct of GAPDH	ΔCt (Means Ct of BRACA2)	$2^{-\Delta Ct}$	experimental group / Control group	DNA concertation
Breast Ca.	23.067	19.936	3.130	0.1142	0.1142/0.0468	2.440
Control	24.749	20.331	4.417	0.0468	0.0468/0.0468	1.000

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Breast Ca.	6.8542	3.1074	0.6942	0.001**
Control	1.1468	0.9002	0.15004	

As for Table (8) when we compared level of ctDNA for *TP53* gene between breast cancer patients and a control group. Mean Ct values: The breast cancer group showed a higher Ct value for *TP53* (23.344) compared to the control group (21.351), indicating low level of ctDNA for *TP53* gene in the breast cancer group since a higher Ct value reflects lower DNA concentration. The calculated ΔCt showed a larger difference in the breast cancer group (3.407) compared to the control group (1.0198). The 2^-ΔCt values reflect that the relative level of *TP53* gene is about 0.191 times that of the control group (experimental/control = 0.094/0.4931), meaning significantly low levels *TP53* gene in the blood of the breast cancer group. The P-value is 0.001, indicated a highly statistically significant difference between the two groups. The low levels of TP53 gene in blood of breast cancer group is sometimes common due to the large burden of the tumor and the reduction of tumor cell death because of the non-functional TP53 protein [20,22].

Table (8): Comparison between patients and control groups in TP53 DNA concertation level.

groups	Means Ct of TP53	Means Ct of GAPDH	ΔCt (Means Ct of TP53)	$2^{-\Delta Ct}$	experimental group / Control group	DNA concertation
Breast Ca.	23.344	19.936	3.407	0.094	0.094/0.4931	0.191
Control	21.351	20.3318	1.0198	0.4931	0.4931/0.4931	1.00

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Breast Ca.	0.4208 b	0.2819	0.06456	0.001**
Control	1.248 a	0.5811	0.0968	

Conclusions

The analysis of the of ctDNA fragment level for *TP53* and *BRCA2* genes in breast cancer, given its use as circulating tumor DNA, provides critical insights into tumor biology and progression. Lower levels of ctDNA for *TP53* gene, led to poor production of its p53 protein which indicates impaired tumor suppression, contributing to cancer growth. On the other hand, elevated levels of ctDNA for *BRCA2* gene, showed lower production of its own protein suggesting that the detected gene is neither stable nor functioning properly. Despite its typical association with tumor suppression, it reflects complex tumor dynamics in breast cancer patients.

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Author's Declaration

- We hereby confirm that all the Tables in the manuscript are original and have been created by us.
- The study protocol was approved by the Medical Ethics Committee of Al-amal National Hospital for Cancer Treatment ethical review committee (No. 2383/3/2 dated 23/10/2023). All members gave composed informed assent after checking on the review depiction. at [Al-Nahrain University/College of Biotechnology]. This approval underscores our commitment to ethical research practices and the well-being of our participants.

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