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Evaluation of CTLA-4 Gene Expression and CD46 Protein Levels as Potential Immunological Biomarkers for Early Detection of Breast Cancer in Iraqi Women

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Abstract:

Background: Breast cancer is the most prevalent type of cancer affecting women and stands as a primary contributor to cancer-related mortality. Timely identification is crucial for improving therapeutic results. **Objective**: This study examines the functions of *CTLA-4* and CD46 in the preliminary detection of breast cancer in Iraqi women. **Materials and methods**: A case-control study was conducted involving 70 women diagnosed with malignant breast cancer ,25 benign breast tumor and 25 healthy controls. Blood specimens were collected, and immunological parameters, including CD46 levels, were measured using the ELISA technique. in addition molecular study to determine expression levels of *CTLA-4* were quantified using quantitative real-time PCR (qPCR) and Statistical analyses were performed with SPSS version 24, and ANOVA test analysis was used to determine differences between research results from three groups. **Results**: The results show that 38.99% of malignant tumor cases were in the 50–59 age group, while 50% of benign tumor cases were under the age of 30–39. Furthermore, 80% of benign tumor patients were obese, compared to only 22.54% of malignant tumor patients and The results showed a significant increase in CD46 protein concentration with the severity of the disease, with the average concentration in the malignant group reaching 160.02 ± 0.61, compared to 135.43 ± 1.27 in the benign group, and 121.21 ± 0.12** in the healthy group. The probability value ($P = 0.0001$) indicates that these differences are highly statistically significant ($P \leq 0.01$), indicating a strong relationship between high CD46 and the progression of the disease from benign to malignant. In this study, *CTLA-4* gene expression was analyzed using RT-qPCR in samples from malignant and benign cases, using *GAPDH* as the reference gene. The results showed that *CTLA-4* gene expression was 2.21-fold higher in malignant cases compared to benign cases, suggesting that the gene is overexpressed in malignant tumors, which could play a role in suppressing the immune response and tumor progression.

تقييم التعبير الجيني لـ CTLA-4 ومستويات بروتين CD46 كمؤشرات مناعية محتملة للكشف المبكر عن سرطان الثدي لدى النساء العراقيات

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مستخلص:

يُعدّ سرطان الثدي أكثر أنواع السرطانات شيوعًا بين النساء، ويمثّل سببًا رئيسيًا للوفاة المرتبطة بالسرطان. ويُعد الكشف المبكر خطوة مهمة لتحسين النتائج العلاجية. تهدف هذه الدراسة إلى تقييم وظائف CTLA-4 و CD46 ودورهما كمؤشرات مناعية محتملة للكشف المبكر عن سرطان الثدي لدى النساء العراقيات. شملت الدراسة 70 مريضة مشخصة بسرطان الثدي الخبيث، و25 حالة ورم حميد، و25 امرأة سليمة كمجموعة سيطرة. جُمعت عينات الدم، وتم قياس المعايير المناعية بما في ذلك مستويات CD46 باستخدام تقنية ELISA، إضافةً إلى دراسة التعبير الجيني لـ CTLA-4 باستخدام تقنية RT-qPCR. أظهرت النتائج ارتفاعًا معنويًا في مستويات CD46 وتعبير CTLA-4 لدى مريضات الأورام الخبيثة مقارنةً بالمجموعات الأخرى، مما يشير إلى إمكانية اعتماد هذه المؤشرات الحيوية التي تساعد في التمييز بين الأورام الحميدة والخبيثة وتحسين التشخيص المبكر .

Introduction

Breast cancer represents the most frequently occurring malignant tumor among women worldwide. Women diagnosed with breast cancer comprise approximately 36% of all oncology patients. According to GLOBOCAN (2018), about 2.089 million women were diagnosed with breast cancer worldwide. It is estimated that around 2.089 million women received a diagnosis of breast cancer[1, 2]. Breast cancer comprises a variety of different malignancies that occur in the mammary glands[3]. Carcinomas represent the predominant form of breast cancer, whereas sarcomas, such as phyllodes tumors and angiosarcomas, are infrequently encountered. The primary risk factor associated with breast cancer is being female, as it occurs approximately 100 times more frequently in women compared to men. Medical professionals believe that early treatment significantly improves recovery chances and reduces morbidity and mortality risks. Therefore, specialists recommend screening strategies for early diagnosis[4]. Despite advancements in medical treatments, this condition accounts for approximately 685,000 deaths annually, representing 16% of all cancer-related fatalities among women[5]. Recent developments have occurred in immunotherapy have shown new approaches for breast cancer treatment, primarily via modulating immune cell activities within the tumor microenvironment (TME). Tumours have the ability to circumvent immune surveillance which promote their growth and alter the tumour microenvironment to inhibit immune cell function[6].

Cytotoxic T Lymphocyte-Associated Antigen-4 (CTLA-4), is one of immunecheckpoint protein that downregulates immune responses. These immune checkpoints play a vital role in comprehending how tumors manage to evade the immune system and represent significant targets for cancer immunotherapy[7]. CTLA-4 exerts a negative regulation on the functionality of T effector cells by outcompeting the co-stimulatory receptors CD28 and CD80, as well as by binding to their common ligand, B7-1. This action leads to an inhibition of T-cell activation through mechanisms that involve enhanced avidity and affinity[8].

The complement system is vital to innate immunity, significantly contribution to acute inflammation and continuing to play a role in chronic inflammation. It also aids in cellular regeneration and growth, including , bone marrow engraftment ,bone and cartilage development, liver regeneration and neurogenesis. Additionally, the activated complement system has a dual role in the tumor microenvironment[9].

The complement regulatory protein CD46, referred to as membrane cofactor protein (MCP), It is categorized as a type 1 membrane protein that protects autologous cells from complement-dependent cytotoxicity (CDC) by inactivating C3b and C4b[10]. CD46 is increasingly recognized as a significant factor in malignant transformation and cancer immunotherapy[11]. It has recently been discovered that CD46 is crucial for tumor growth and metastasis. New research suggests that the excessive expression of CD46 in solid tumors, including breast, bladder cancers, colorecta and ovarian, may shield malignant cells from the damaging effects of the complement system[11, 12].

Aim of the Study

1-To assess the role of CTLA-4 gene expression and CD46 protein concentration as potential biomarkers for the early detection of breast cancer among Iraqi women.

2-To determine the relationship between their levels and disease progression, evaluating their possible involvement in immune regulation and tumor development.

Study Design

This observational case-control study encompasses 120 women divided into three groups: 70 Iraqi women aged 23–72 years who were newly diagnosed with malignant breast cancer without a history of receiving any treatment like chemotherapy or radiotherapy and 25 women diagnosed with benign breast tumor . Samples were collected at Baquba Teaching Hospital and Al-Amal National Hospital for Oncology Treatment in Iraq, from January 2025 to June 2025.

Blood samples were obtained from these women after performing several diagnostic procedures, comprising clinical examination, ultrasound, mammogram, and multiple laboratory tests. A biopsy was conducted to confirm the diagnosis of breast cancer. In addition a control group of 25 healthy women matched (22–70 years) was included in the study. They did not have any tumor, fibrosis, mass, or inflammation in the breast without any surgicalintervention or nipple secretions, and they were confirmed by conducting a clinical examination, ultrasound, and mammography to ensure that there was no tumor in the breast or other problems. The clinicopathological characteristics included tumor grade and hormonal status as well as HER2 status. Verbal consent was obtained from all participants to ensure that they were fully informed of the study objectives and benefits.Exclusion criteria were patients with other cancer or metastasis, treated patients with chemotherapy, radiation, hormonal or other anti-cancer drugs, presence of any other autoimmune or chronic disease, taking any biological agents and recent blood transfusions (during the last 6 months).

Ethical approval

This study was conducted based on the ethical standards stipulated in the Declaration of Helsinki. Before collected the sample the information of patient oral acceptance and information was obtained and written , after the review and approval of the study protocol and subject's information by the local ethics committee on the base of the document number 101 in 13/4/2025) to get this agreement .

Sampling

Six milliliters of venous blood were collected and distributed into two parts. A volume of two milliliters of blood was introduced into a tube containing Ethylene Diamine Tetra acetic Acid (EDTA). and then take 250 whole blood to 500ul trizol , while four milliliters of blood were placed in a dry clean gel tube. The blood, in the gel tube, The sample was permitted to coagulate for a duration of 15 minutes at ambient temperature, Afterward, the mixture was subjected to centrifugation at a speed of 2000 revolutions per minute (rpm) for a duration of 10 minutes in order to isolate the serum. The serum was divided among four sterile, carefully sealed Eppendorf tubes and kept in a deep freezer at -80°C until it was analyzed.

Methods

ELISA Test

Determination of complement regulatory protein CD46 in patient serum was performed using a sandwich enzyme-linked immunosorbent assay (ELISA) kit designed for in vitro quantitative measurement of human CD46.

Molecular Study

The molecular analysis included the Determine of the Expression levels of CTLA-4 were quantified using qPCR.

Extraction of RNA, cDNA synthesis, and Real-Time PCR

TRIzol (Life Technologies) were used for isolation of total RNA, while cDNA was prepared from RNA through Easy Script® First-Strand cDNA Synthesis SuperMix. The second section it’s done by selected the cDNA sample from control and patient . The detection of Quantity determination based on fluorescent power of Syber- Green- The following primer sequences were used for semi-quantitative and Real-time PCR CTLA-4, sequences for primer were obtained from Invitrogen. The sequence of CTLA-4 and GAPDH primers used in this study can be seen in Table 1 .

Table 1. Primers used in real-time PCR

Name	Sequence	Product size	Reference
CTLA-4F	CTGTGCGGCAACCTACATGAT	155bp	Newly designed based on NM_ 005214.5
CTLA4-R	GGTACATGAGCTCCACCTTGC		
GAPDH-F	ATCACCATCTTCCAGGAGCGA	157bp	Newly designed based on NM_002046.7
GAPDH-R	CAGAGGGGGCAGAGATGATGA		

The Power SYBR Green PCR mix (ThermoFisher Scientific) was utilized for real-time RT-PCR on the Step One Plus Real-Time PCR System. The thermal cycling conditions were as follows in Table 2. GAPDH was used as the internal control gene. ΔCt values were computed by subtracting GAPDH Ct from PD-L1 Ct. Relative expression levels were calculated using the 2^-ΔΔCt method and using the benign group as a reference [13].

Table 2: The thermal cycling conditions

Cycle Step	Temperature	Time	Cycles
Initial Denaturation	95°C	60 seconds	1
Denaturation	95°C	15 seconds	45
Extension	60°C	30 seconds (+plate read)	
Melt Curve	60-95°C	40 minutes	1

Statistical Analysis:

The Statistical Packages of Social Sciences (SPSS-26) (IBM® USA) was used to analyze and interpret data and determine the impact of various factors on study parameters expressed as frequency and percentages. The Chi-Square test of independence was used to and SD-Least Significant Difference test facilitated a meaningful comparison of these percentages, enabling a detailed examination of the relationships between the variables. The independent samples t test was used to determine the difference between continuous data which were expressed as Mean±SD. The level of significance was set as p≤0.05.

Results

Levels of CD46 in studied groups

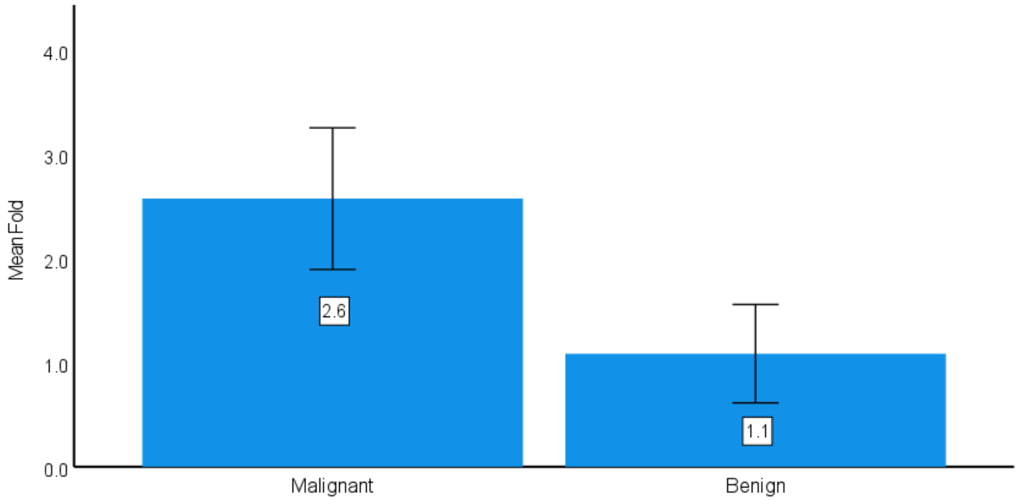
The results in Table 1 compares the concentration of CD46 (Complement Regulatory Protein) between the three study groups (healthy, benign tumors, and malignant tumors). There were highly statistically significant differences (P = 0.0001**) between these groups. The mean CD46 concentration in the healthy group was 121.21±0.12 pg/ml, In the benign tumor group, the mean increased to 135.43±1.27 pg/ml, a significant difference from the healthy group. In contrast, the malignant tumor group had the highest mean CD46 concentration, 160.02±0.61 pg/ml, a significant and statistically significant increase compared to the other two groups.

Table 3 : Comparison between difference groups/ Type in CD46 conc.

Group/ Type	Mean ±SE of CD46 conc.
Healthy	121.21±0.12 c
Benign	135.43±1.27 b
Malignant	160.02±0.61 a
L.S.D.	**
P-value	0.0001
Means having with the different letters in same column differed significantly. ** (P≤0.01).	

Molecular study

The results of current study revealed that the mean gene expression of CTLA-4 in malignant cases was 2.6, compared to 1.1 in benign cases, with clear differences indicating overexpression in malignant tumors, reflecting a potential role of the gene in disease progression or suppression of the immune response. Figure 1



Error Bars: 95% CI

Error Bars: +/- 2 SE

Figure 1: The comparison of mean fold change of CTLA-4 between malignant and benign cases.

The results of this study indicate that CTLA-4 gene expression differs significantly between malignant and benign tumors, as shown in Table 4. The ΔCT value—the difference between CTLA-4 and the reference gene GAPDH—was lower in malignant tumors (0.096) compared to benign tumors (1.24), indicating higher CTLA-4 expression in malignant cases. The relative expression, calculated as 2^-ΔCT, was also higher in malignant tumors (0.935) versus benign tumors (0.423). This corresponds to an approximate 2.21-fold increase in CTLA-4 expression in malignant tumors.

Table 4: The overall fold change of CTLA-4 of malignant versus benign cases.

Group	Meant CT CTLA-4	Mean CT GAPDH	Δ mean CT	$2^{\wedge-\Delta CT}$	Fold
Malignant	18.12066667	18.02466667	0.096	0.935623	2.21
Benign	16.154	14.914	1.24	0.423373	1

Discussion

Breast cancer is one of the most prevalent malignant tumors impacting women. The development and occurrence of breast cancer arise from a variety of external and internal factors [14]. Unhealthy lifestyle choices, social-psychological influences and environmental factors are all related to its progression. Studies show that five to ten percentage of breast cancer cases can be as a result of family history and genetic change via mutation, While 20% to 30% of breast cancer cases can be linked to various factors that may be subject to modification[5].

The results of the current study showed statistically significant differences at the significance level at ($p<0.05$) between studied groups, CD46 was considerably upregulated in breast cancer tissues. The role of CD46 as a complement regulatory protein (CRP) protects cells from immune degradation mediated by the complement system [10]. Normally, CD46 is expressed at moderate levels to protect self-renewing cells from accidental damage. In tumors, cancer cells exhibit increased expression of CD46 as an immune escape mechanism, preventing complement activation and escaping immune destruction. The slight increase in CD46 expression in benign tumors compared to healthy cells may indicate an early protective response of cells to abnormal changes. The significant increase in malignant tumors confirms that cancer cells increasingly rely on this protein as a mechanism for survival and growth within a hostile immune environment [15, 16]. In alignment with our results, excessive expression of CD46 has been observed in breast cancer, ovarian cancer, prostate cancer, colon cancer and bladder cancer tissue vs. adjacent normal tissues [17-19]. Similar results have been observed in additional research concerning gynecological tumors, in which CD46 expression t was associated with poor prognosis in breast and ovarian cancer patients and provided as an independent risk factor for survival. CD46 expression may be a survival pathway for Breast cancer cells to escape from tumor-specific CDC18]].

CTLA4 serves as a checkpoint that primarily operates at the initial stages of the immune response, specifically Throughout the activation and priming of T-cells, and it amplifies the immunosuppressive functions of regulatory T-cells [20]. The CTLA4 protein is crucial in stimulating the antitumor response directed at cancer cells [21].

The validation of the aforementioned findings indicated that the expression of CTLA4 mRNA in Breast cancer tissues was significantly elevated compared to that in benign tumor and normal tissues ($P < 0.01$). In line with our thesis, previous research has reported on the investigation of the association between CTLA4 expression profiles and the clinical outcomes of breast cancer [22]. Moreover, additional studies have provided supporting evidence that reinforces the credibility of our findings, reporting consistent associations between CTLA4 expression profiles and the clinical outcomes of breast cancer which have shown a correlation between the expression levels of CTLA4 and lower survival rates in patients suffering from melanoma, colorectal cancer and renal cell carcinoma. Additionally, individuals with breast cancer mutations demonstrate a significantly higher expression of this particular biomarker[23]. In addition other Previous studies have shown that cancer cells express CTLA4 and PD-L1which hinder the immune system's capacity to recognize and destroy tumor cells. Additionally, antibodies that inhibit CTLA4 and PD-L1 can enhance the antitumor response, resulting in a decrease in tumor size [24]

A research study has indicated that the expression of CTLA4 correlates with the presence of multiple lesions, an increase in tumor size, a more advanced stage of lymph node involvement, lymphovascular invasion and skin infiltration in breast cancer[25] in addition to other factors such as age, PAM50 subtypes in patients ,progesterone receptor (PR) status and estrogen receptor (ER) status with BRCA [22] the mechanism of CTLA4 in breast cancer progression is unclear CTLA4 serves as an essential checkpoint that plays a significant role during the early phases of the immune response, particularly in the activation and priming processes of T-cells. Additionally, it enhances the immunosuppressive capabilities of regulatory T-cells (Tregs) [20]. Identifying genes that exhibit abnormal expression patterns in tumors is essential for developing personalized treatment approaches, which could lead to improved therapeutic outcomes [26].

In summary our findings suggest a prognostic role for CTLA4 in BRCA, consistent with previous studies that have reported similar associations. This alignment with existing research not only reinforces the credibility of our results but also contributes to a better understanding of CTLA4's impact, thereby paving the way for further investigations .

Conclusion

The elevation of CD46 protein levels and CTLA-4 overexpression in malignant cases indicates their potential roles in tumor progression through immune evasion. These findings emphasize their value as biomarkers for distinguishing malignant from benign conditions and highlight their promise as future therapeutic targets to improve cancer prognosis and management.

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