Trends in Immunotherapy (2023) Volume 7 Issue 2
doi: 10.24294/ti.v7.i2.2935

ORIGINAL RESEARCH ARTICLE

Estimation of gene expression level of CDH1 as a predisposing factor for metastasis in localized and locally advanced and metastatic breast cancer Iraqi patients

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ABSTRACT

Breast cancer (BC) is a prevalent malignancy among women, ranking as the second most commonly diagnosed cancer globally. Notably, a substantial proportion of breast cancer-related fatalities, up to 90 percent, are attributed to the development of distant organ metastases. While Cadherin1 (CDH1) has conventionally been considered a tumor-suppressor gene in cancer research, recent investigations have unequivocally revealed that both CDH1 and its encoded E-cadherin exhibit oncogenic characteristics. The primary focus of this case-control study is to ascertain the involvement of the CDH1 gene in a specific subset of Iraqi female patients. A total of ninety patients sought diagnosis and treatment at the Oncology Teaching Hospital in Medical City and the Oncology Unit at Al-Yarmouk Teaching Hospital in Baghdad. In addition, we included 30 apparently healthy individuals as blood control subjects and another 30 women with benign breast tumors as tissue control subjects for the study. In the initial phase of the study, we conducted a serological analysis using enzyme-linked immunosorbent assay (ELISA) to detect the concentration of E-cadherin in serum samples from two groups of BC patients. The group with locally advanced and metastatic BC exhibited a significantly higher E-cadherin concentration (963.4 ± 89.8 pg/mL) compared to the group with localized BC. In the second part of the research, qRT-PCR was performed to analyze the expression of the CDH1 gene across all sample types. CDH1 was shown to have the greatest fold expression (2.550 ± 0.164) in cases with locally progressed and metastatic BC. Compared to the seemingly healthy control group, the fold expression for localized BC was 1.456 ± 0.055, and for malignant tissue it was 1.886 ± 0.08621. In conclusion, this study provides compelling evidence supporting CDH1 as an oncogene in BC. The significance of CDH1 in BC tumorigenesis underscores its potential for the development of novel detection biomarkers and targeted therapeutic approaches for BC treatment. Notably, CDH1 exhibited elevated expression levels in BC tissues and demonstrated an association with an unfavorable distant metastasis-free survival outcome.

Keywords: CDH1; breast cancer; oncogenic characteristics; metastasis; gene expression; E-cadherin

ARTICLE INFO

Received: 27 September 2023
Accepted: 7 November 2023
Available online: 6 December 2023

1. Introduction

Cancer is a disease that results from the abnormal growth of cells due to genetic mutations in DNA[1]. Breast cancer (BC) is one of the most common malignant tumors and the second leading cause of cancer in women in the world and in Iraq it is considered the first among the population cancer and cause cancer related female death[2]. Breast cancer is a prevalent form of malignancy that contributes to female mortality on a global scale. BC is a genetically diverse illness, resulting in significant variations in diagnostic and clinical outcomes. Consequently, the availability of standard clinicopathological tools for prognostic or diagnostic purposes in breast cancer is likely to be limited[3]. Breast cancer is a malignant tumor arising from epithelial

cells of glandular lactiferous ducts or terminal duct lobular units (TDLUs) of the breast which is affected by the oxidative stress and the defensive mechanisms against it[4]. After moving out of the ducts or lobules, cancer cells can metastasize through the blood or lymphatic systems to distant organs preferentially to the bones lungs, liver, or brain[5]. Metastatic cells migrate and colonize in a multistep cascade with molecular events that are directed by gene mutations and altered expressions. The distinct steps of metastasis include; detachment and migration away from the primary tumor site, invading neighboring tissues and penetrating through the basement membrane, entering the lymphatic or blood vessels, surviving forming anoikis, exiting the blood or lymphatic vessels at a distant organ, form micrometastatic nodule, adaptation and reprogramming the surrounding stroma, and form macrometastases[6]. The invasion and spread of cancer cells are two of the most notable characteristics of malignant tumors. Recent studies suggest that the epithelial-mesenchymal transition (EMT) has been linked to this significant occurrence. It is linked to the absence of the epithelial brow and the presence of mesenchymal facial hair[7]. Adherens junctions and desmosomes play critical roles in maintaining cell and tissue integrity by mediating strong adhesion between adjacent epithelial cells[8].

The CDH1 gene, located on chromosome 16q22.1, encodes the 120-kDa E-cadherin protein, a member of the cadherin family responsible for calcium-dependent cell adhesion and tissue organization[9]. E-cadherin interacts with catenins to achieve this function and has five extracellular domains with calcium-binding sites critical for adhesion stabilization[10,11]. E-cadherin plays a key role in maintaining neural stem cells' pluripotent state and embryonic self-renewal[12], as well as in forming and maintaining polarized epithelial tissues through adherens junctions[13]. However, it is frequently lost during cancer due to the epithelial-mesenchymal transition (EMT)[14].

E-cadherin functions as a vital cell-to-cell adhesion molecule and forms the E-cad/β-catenin/α-catenin complex that interacts with the actin cytoskeleton, reinforcing cell polarity, communication, and epithelial tissue integrity. Disruptions in E-cadherin function lead to changes in intercellular junctions, tumor invasion, and cell migration, promoting metastasis. EMT, characterized by reduced E-cadherin expression, is a crucial process in morphogenesis and occurs in both cancer progression and developmental processes like gastrulation, neural crest development, and placental formation. It's suggested that epithelial cancers may require mesenchymal traits for metastasis and invasion due to the importance of EMT in embryogenesis[15].

CDH1 is frequently identified as a gene with tumor suppressor properties within the domain of cancer investigation. The phenomenon of down-regulation or loss of E-cadherin (E-cad), which is encoded by the CDH1 gene, has been extensively studied due to its significant contribution to the facilitation of invasive and metastatic behavior in malignant tumors. Nevertheless, current research has brought to light more aspects of CDH1 and its encoded E-cad, thereby exposing its carcinogenic characteristics. An example of this can be seen in the CDH1 oncogene, which has been demonstrated to stimulate the process of self-renewal in lung cancer stem-like cells[16]. Furthermore, subgroups of prostate cancer cells expressing E-cad+ display traits commonly associated with cancer stem cells. Notably, the flexibility in the expression of E-cad plays a significant role in the process of cell invasion[17].

The observation of increased expression of E-cadherin in SKBR3 cells in BC has been found to accelerate the production of mammospheres. In addition, a research investigation has provided insights into the observation that although E-cad is categorized as a transmembrane molecule, its extracellular configuration can undergo cleavage and subsequently be released into the circulatory system as a soluble variant known as soluble E-cadherin (sE-cad)[18]. Several studies have extensively investigated the potential of sE-cad as a diagnostic and prognostic biomarker for malignancy, as it has been observed to be significantly increased in the serum of patients with malignant tumors[19]. It is worth mentioning that the concentration of sE-cad in the serum of individuals with lung cancer was seen to be markedly elevated compared to that of individuals in the control group. Moreover, patients with distant metastases exhibited an even more pronounced escalation in sE-cad levels. A study conducted on breast cancer patients revealed a significant association between elevated

levels of serum sE-cad and many indicators of disease severity, including TNM stage, tumor grade, and lymph node metastasis[20].

These findings underscore the controversial nature of CDH1's role as either a tumor suppressor or pro-oncogene in malignant tumors, which remains to be fully elucidated.

In this report, we studied the expression of the CDH1 gene in the serum and fresh tissue of breast cancer patients. This study provides evidence for CDH1 as an oncogene in BC because the results revealed an upregulation of CDH1 expression, which increased with the progression of cancer. Additionally, the study demonstrated elevated levels of E-cadherin (E-cad) in patients with locally advanced and metastatic BC. These findings suggest that CDH1 might possess oncogenic characteristics and play a crucial role in BC tumorigenesis. Moreover, sE-cad in serum can be used as valid noninvasive markers for BC diagnosis. These insights offer new perspectives for the development of detection biomarkers and targeted therapies for BC.

2. Materials and methods

2.1. Samples collection

The purpose of this case-control study, which ran from March 2022 to January 2023, was to look at a large group of women spanning the ages of 20 and 75. Participants were drawn from the Oncology Unit at Al-Yarmouk Teaching Hospital in Baghdad and the Oncology Teaching Hospital in the Medical City. A total of 150 participants were split evenly between two groups for the study:

The first set of blood samples included 60 women who had been diagnosed with breast cancer. Thirty samples came from individuals with locally-confined BC, while another thirty came from women with more advanced forms of the disease. In addition, 30 control samples were taken from healthy women. This brings the total number of samples to 90.

The 30 samples in the second tissue group, they were taken during biopsies performed with Tru-cut needles and those removed during mastectomy procedures for breast cancer. In addition, we gathered 30 control samples representing a wide range of diseases, including fibroadenoma, accessory breast, mastitis, lipoma, duct ectasia, benign breast masses, and mastectomy/quadrectomy due to hemorrhagic cysts/capsules. We also provided mammoplasty patients with normal breast tissues for comparison; this in-depth analysis of a wide range of samples contributes greatly to our knowledge of BC and has important implications for both clinical practice and future research.

2.2. Gene expression techniques

In this study, two techniques were employed: polymerase chain reaction (PCR) and the enzyme-linked immunosorbent assay (ELISA). The detailed procedures are outlined as the following:

2.2.1. PCR technique

In this section, both patient and control samples underwent an examination of gene expression for the CDH1 gene using qRT-PCR, employing the relative quantitation method. Gene expression levels were standardized by comparing them to a reference housekeeping gene (TBP). The quantification of CDH1 gene expression was achieved through the $2^{-\Delta\Delta CT}$ method.

Samples collection and preparation

- Blood samples

From each participant five milliliters (mL) of venous blood sample were taken (all patients and healthy control women), using disposable syringes, these 5 mL of the collected samples were placed in gel tubes (serum separation tube SST), left for about 30 min to coagulate at room temperature, then centrifuged at 5000 rounds per minute (rpm) for 10 min in order to obtain serum. The separated sera were divided into two tubes. In the

first Eppendorf tube, 0.4 mL (400 µL) of serum was added to 0.6 mL (600 µL) of TRIzol™ Reagent, the lysate was homogenized by pipetting up and down several times and stored at −20 °C for analysis gene expression of CDH1 gene.

- Fresh tissue samples

Tissue samples were taken and added to 0.6 mL (600 µL) of TRIzol™ Reagent, the lysate was homogenized by pipetting up and down several times and stored at −20 °C for analysis gene expression of CDH1 gene.

Total RNA extraction with TRIzol

Total RNA, including mRNAs from the samples, was extracted using the TRIzol™ Reagent following the protocol provided by the manufacturer (Thermo Fisher/USA).

RNA quantitation by qubit 4.0

Total RNA was quantified using the Qubit® RNA HS Assay Kits, which offer a non-traditional yet precise method, yielding a wide range of RNA concentrations, ranging from low (4.7–46.1 ng/µL) to high concentrations. Importantly, there were no significant variations observed in the total RNA concentrations between the tumor and control samples. Nevertheless, slight variations in RNA purity were noted within the same groups.

Complementary DNA (cDNA) synthesis for CDH1

To synthesize complementary DNA (cDNA) for CDH1, the total RNA sample underwent reverse transcription using the ProtoScript® miRNA First-strand cDNA synthesis SuperMix kit. This process was carried out in a 20 µL reaction volume, following the manufacturer's provided guidelines. A 20 µL quantity of total RNA was used for reverse transcription, as detailed in **Table 1**. The efficacy of cDNA concentration was evaluated by measuring the efficiency of the subsequent qRT-PCR. All steps were executed with precision, resulting in a successful reverse transcription process with optimal yields.

Table 1. Reverse transcription PCR reaction components and volumes.

Material	Volume (µL)
RNA	5 µL
Protoscript reaction mix	10 µL
MuLV enzyme	1 µL
Primer	1 µL
Nuclease free water	To 20 µL

The primers

The primer sequences for the CDH1 gene were newly designed using Geneious Prime software and synthesized by Macrogen (South Korea). They were stored in a lyophilized state until they were ready for use.

Quantitative reverse transcriptase PCR (qRT-PCR)

The quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) method relies on the detection of fluorescent light to quantify the amount of specific gene complementary DNA (cDNA). This process involved extracting total RNA from samples and performing reverse transcription using the high-capacity cDNA Kit following the provided kit instructions.

To normalize the mRNA levels of CDH1, we amplified the mRNA levels of the internal control gene TBP small nuclear. The qRT-PCR procedure was conducted using the smart cycler real-time PCR system from Bioer/Japan. Gene expression levels and fold changes were determined by measuring the threshold cycle (Ct) using components from the Wizbio pure™ (SYBR) qPCR kits, along with the reverse transcription PCR

reaction components and volumes detailed in **Table 1**.

For accuracy, each reaction was duplicated, and we included negative controls, such as a non-template control (NTC), a non-amplification control (NAC), and a non-primer control (NPC). Additionally, the total RNA underwent reverse transcription using the ProtoScript® II First Strand cDNA Synthesis Kit. For qRT-PCR analysis of mRNA levels, we employed SYBR-Green Reagents. The qPCR reaction setup and run, including the cycling protocol, were programmed based on the thermal profile and are illustrated in **Tables 2** and **3**, respectively.

Table 2. Components used in qRT-PCR for CDH1 and TBP expression experiments.

Components	Volume (µL)	Concentration
Master mix syper green	10	
Forward primer	0.5	10 pmol
Reverse primer	0.5	10 pmol
CDNA	6	
Nuclease-free water (N.F.W)	3	
Total	20	

Table 3. Thermal profile for gene expression of CDH1.

Cycle step	Temperature	Time	Cycles
Initial denaturation	95 °C	8 sec	1
Denaturation **Extension**	95 °C 60 °C	15 sec 30 sec (+ plate read)	50
Melt curve	60–95 °C	40 min	1

Gene expression calculation and statistical analysis

Fold changes in the quantified expression of mature RNAs were determined using the related cycle threshold ($2^{-\Delta\Delta Ct}$) method, which was initially introduced by Livak and Schmittgen in 2001[21]. The data that was accessible underwent analysis using GraphPad Prism version 9 software.

2.2.2. ELISA test

The concentration of E-cadherin was assessed in the sera of patients with localized breast cancer, locally advanced breast cancer, and metastatic breast cancer, as well as in control samples. This assessment was performed using the quantitative Sandwich-ELISA technique, employing ELISA kits manufactured by Sunlong (China).

3. Results and discussions

Demographical distribution of samples

A. Age distribution:

All female participants in the blood group exhibited an age range between 24 and 75 years. The age range for the localized BC group was between 24 and 75 years, while it ranged from 25 to 75 years for the locally advanced and metastatic breast cancer group and 20 to 67 for the healthy women control group. The mean age of females with BC was significantly higher ($P < 0.05$) compared to the control groups; values of 50.7 ± 2.0 for localized breast cancer patients were obtained as shown in **Table 4**.

Similarly, the mean age of females with BC was significantly higher ($P < 0.05$) compared to the control groups; hence, values of 50 ± 1.6 versus 38.7 ± 2.1 for the locally advanced and metastatic breast cancer group were estimated as shown in **Table 5**.

Table 4. The correlations between age of localized breast cancer patients' group and control group.

Groups	No.	Age mean ± S.E.	t-test	*P*-value
Patients	32	50.7 ± 2.0	4.04	< 0.05
Control	32	38.7 ± 2.1		

Note: *P*-value < 0.05 = significant.

Table 5. Correlations between the ages of locally advanced and metastatic breast cancer patients and the control group.

Groups	No.	Age mean ± S.E.	t-test	*P*-value
Patients	38	50 ± 1.6	4.167	< 0.05
Control	32	38.7 ± 2.1		

Note: *P*-value < 0.05 = significant.

The findings of this study highlight the existence of breast cancer risk among individuals of various age groups, particularly throughout the middle stages of women's lives. This discovery underscores the importance of implementing proactive screening and early detection strategies for breast cancer. There exists a significant association between age and the onset of cancer. As humans progress through the aging process, they experience notable alterations at the cellular and tissue levels, ultimately leading to the development of several age-related ailments that have the potential to curtail overall life span. Age is commonly acknowledged as the primary demographic risk factor for various chronic diseases, such as cancer[22,23], when considering the larger perspective.

B. The BMI:

The Body Mass Index (BMI) was calculated by measuring the weight and height of each individual. BMI serves as an index that adjusts weight in relation to stature, calculated by dividing weight in kilograms by height in meters squared. BMI is a valuable diagnostic tool for assessing both obesity and protein-energy malnutrition. The results pertaining to body weight indicated that a majority of individuals with localized breast cancer were either obese or overweight when compared to the control group. The mean Body Mass Index (BMI) of females with localized breast cancer was significantly higher ($P < 0.05$) in comparison to the control group, with values of 26.2 ± 0.9 versus 22.5 ± 0.4, as presented in **Table 6**.

Table 6. Correlations between the Body Mass Index of localized breast cancer patients and the control group.

Groups	No.	BMI mean ± S.E.	t-test	*P*-value
Patients	32	26.2 ± 0.9	3.633	< 0.05
Control	32	22.5 ± 0.4		

Note: *P*-value < 0.05 = significant.

Obesity is linked to elevated morbidity and mortality rates in numerous chronic diseases, including BC. Research has demonstrated that obesity, along with the chronic inflammation it induces, promotes the growth and metastasis of breast cancer. Several factors, including leptin, adiponectin, estrogen, and various pro-inflammatory cytokines, play integral roles in the development of obesity-driven breast cancer by activating multiple oncogenic and pro-inflammatory pathways[24,25].

C. Menopause:

Women who experience menopause after the age of 55 face an elevated risk of ovarian, breast, and uterine cancers. This risk is further heightened if a woman began menstruating before the age of 12. The extended exposure to estrogen over a woman's lifetime increases the risk of developing breast cancer. The risk of breast cancer development is amplified in both pre-and post-menopausal patients who had early onset of menarche and late menopause, likely owing to the prolonged hormonal exposure[26].

Hormonal contraceptives, containing progesterone and estrogen, have been implicated in uncontrolled breast tissue growth, potentially leading to breast cancer. Exposure to high levels of estrogen stands as one of the factors that can heighten the incidence of breast cancer risk, either causing permanent damage to breast tissue or triggering the presence of cancer cells within the ducts and lobules. The incidence of breast cancer is increased in individuals using menopausal hormone therapy[27]. The current study's results revealed a significantly higher frequency of postmenopausal status among females with localized breast cancer, with 56% being postmenopausal patients, 38% perimenopausal patients, and 6% premenopausal patients, as depicted in **Figure 1**.

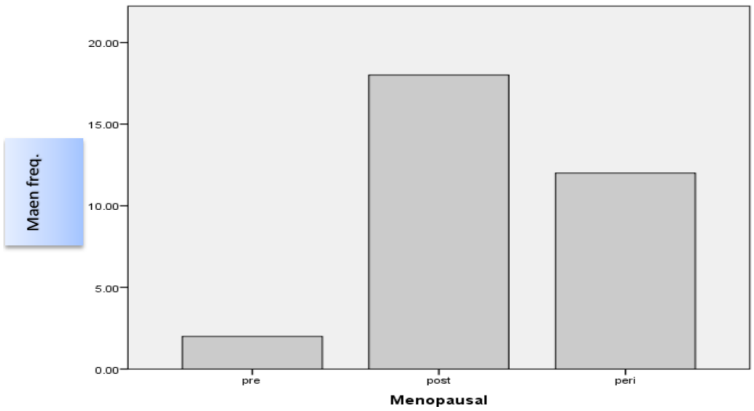

Figure 1. Menopausal distribution of localized breast cancer patients.

D. Side of breast cancer:

Both sides of the breast share the same genetic and environmental risk factors for the development of breast cancer. However, differences in anatomical structures, such as tissue composition, blood vessel supplies, and lymphatic drainage, can exist between the two sides of the breast. These structural distinctions may contribute to variations in the occurrence of breast cancer on each side. Over time, factors like the relatively larger size of the left breast, early detection of tumors in right-handed individuals, and more frequent breastfeeding from the right breast may influence the likelihood of breast cancer occurrence[28].

In addition, it has been observed that left-sided breast tumors typically display a more proliferative genomic profile, exhibit weaker responses to neoadjuvant chemotherapy, and demonstrate slightly inferior long-term results in comparison to right-sided breast cancer[29]. The findings of the study revealed a notable discrepancy in the average incidence of breast cancer between the left and right sides. Within the localized breast cancer cohort, a significant majority of patients, specifically 84%, presented with left-sided breast cancer. Conversely, a minority of patients, comprising 16%, exhibited right-sided breast cancer, as visually depicted in **Figure 2**. According to the data shown in **Figure 3**, the majority of patients in the malignant breast cancer tissue group (75%) exhibited left-sided breast cancer, while the remaining 25% were diagnosed with right-sided breast cancer.

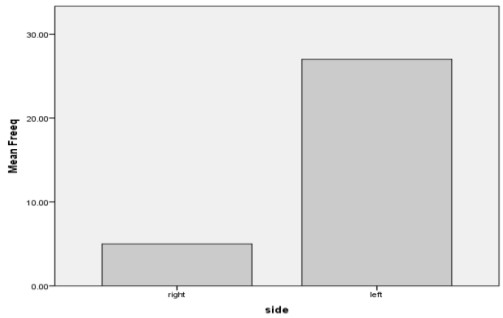

Figure 2. Distribution of breast cancer sides in localized breast cancer patients.

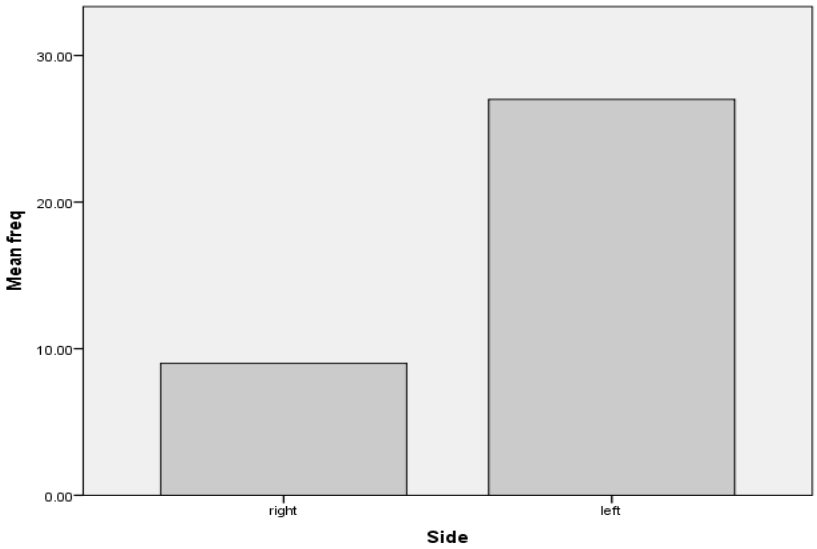

Figure 3. Distribution of breast cancer sides in malignant tissue of breast cancer patients.

E. Marital status:

The results show a significant increase ($P < 0.05$) in the number of married women in the localized breast cancer group compared to unmarried women. Specifically, 27 patients (84%) were married, while 5 patients (16%) were not married, as shown in **Table 7**.

In a study conducted by Weltz and Port[30], they reported that childbearing reduces the risk of breast cancer, with greater protection associated with early first births and a larger number of births. Additionally, breastfeeding likely has a protective effect. The most probable explanation for this is the hormonal changes that occur during pregnancy and lactation, as well as the physical changes in mammary epithelial cells, which tend to differentiate and thereby delay ovulation. It's worth noting that there is an inverse relationship between the length of the breastfeeding period (more than 6 months) and the risk of breast cancer[31,32].

Table 7. Distribution of marital status in the localized breast cancer patients group.

Marge state	**No. (%)**	**Chi square (x^2)**	***P*-value**
Yes	27 (84%)	15.125	< 0.05
No	5 (16%)		

Note: *P*-value < 0.05 = significant.

F. Family history distribution:

The investigation of family history is of paramount importance, as the development of breast cancer is influenced by various inherited factors, including dominant autosomal mutations such as BRCA1 and BRCA2[33]. Women with a family history of breast cancer, particularly if it involves a first-degree relative (mother, sister, or daughter), face a significantly elevated risk—two or more times greater—of developing breast cancer themselves. Furthermore, certain hereditary cancer syndromes heighten the risk of cancer in specific families, often characterized by a high incidence of cancer among family members, typically occurring at an early age[34]. The results, as presented in **Table 8**, reveal that localized breast cancer patients without a family history of breast cancer accounted for a higher percentage, at 60%, in comparison to patients with a family history, who comprised 40%. Notably, there were no statistically significant differences between these two groups ($P > 0.05$).

Table 8. Distribution of the sample study according to family history in the localized breast cancer patients group.

Family history	No. (%)	Chi square (x^2)	*P*-value
Yes	13 (40%)	1.125	> 0.05
No	19 (60%)		

Note: *P*-value > 0.05 = non-significant.

The results, as presented in **Table 9** demonstrate that patients with locally advanced and metastatic breast cancer who lack a family history of breast cancer accounted for a higher percentage, at 53%, in comparison to patients with a family history, who comprised 47%. Importantly, there were no statistically significant differences between these two groups ($P > 0.05$).

Table 9. Distribution of the sample study according to family history in the locally advanced and metastatic breast cancer patients group.

Family history	No. (%)	Chi square (x^2)	*P*-value
Yes	20 (53%)	0.105	> 0.05
No	18 (47%)		

Note: *P*-value > 0.05 = non-significant.

The findings regarding the impact of family history align with recent studies, which reported a similar trend. The study by Mboungou Malanda et al.[35] noted that only 11 cases, accounting for 7.33%, had a family history of breast cancer. Likewise, another study by Gautam et al.[36] found that 10.4% of patients had a family history of breast cancer, while the majority, 89.6%, had no family history of breast cancer. These consistent results across multiple studies underscore the significance of family history as a contributing factor in breast cancer.

G. Hormone receptors distribution:

The detection of estrogen receptor (ER) and progesterone receptor (PR) status plays a crucial role in the prognosis and therapeutic decision-making for BC cases. ER/PR positivity in breast cancer patients has been associated with improved survival outcomes, particularly when adjuvant hormonal therapy is administered to reduce recurrence rates and lower mortality during treatment. Consequently, ER/PR expression status is considered both a good prognostic factor and a predictor of responsiveness to endocrine therapy[37].

The results, as presented in **Table 10**, reveal that in the localized BC group, patients with luminal A and luminal B subtypes of breast cancer had the highest percentages, at 63% and 28%, respectively, compared to other receptor types. Triple-negative breast cancer had the lowest percentage, at 9%, with statistically significant differences observed among these subtypes. In the locally advanced and metastatic BC group, most patients had either luminal A (42%) or triple-negative (39%) subtypes, while Her2 Enriched and luminal B subtypes had the lowest percentages, at 11% and 8%, respectively, with significant differences between these subtypes ($P < 0.05$), as shown in **Table 11**.

It's worth noting that changes in hormone receptor status, such as ER and PR, between primary tumors and metastatic sites can have a significant impact on therapy management and patient survival. Patients whose hormone receptor status changed from negative to positive tended to have a longer survival rate compared to women with stable hormone receptor-negative disease. However, in multivariable models, the conversion of estrogen receptor (ER) status from positive to negative was associated with a worse survival rate[38].

Table 10. Distribution of sample study according to hormone receptors in the localized breast cancer patients group.

Receptors	No. (%)	Chi square (x^2)	*P*-value
luminal A	20 (63%)	13.938	< 0.05
luminal B	9 (28%)		
Triple negative	3 (9%)		

Note: *P*-value < 0.05 = significant.

Table 11. Distribution of sample study according to hormone receptors in the locally advanced and metastatic breast cancer patients group.

Receptors	No. 38 (%)	Chi square (x^2)	*P*-value
luminal A	16 (42%)	15.263	< 0.05
luminal B	3 (8%)		
Triple negative	15 (39%)		
Her2 Enriched	4 (11%)		

Note: *P*-value < 0.05 = significant.

H. Hormone receptors distribution:

The assessment of estrogen receptor (ER) and progesterone receptor (PR) status continues to be an effective method for evaluating prognosis and selecting treatment choices for patients of BC. The presence of estrogen and progesterone receptors (ER/PR) in breast cancer has been correlated with improved survival outcomes, particularly when adjuvant hormone therapy is offered to patients with ER/PR-positive tumors. This approach aims to reduce recurrence rates during the treatment period and decrease mortality, thus establishing ER/PR expression status as a favorable prognostic factor and a predictive indicator for the response to endocrine therapy[36].

The results, as presented in **Table 12**, reveal that in the localized BC group, patients with luminal A and luminal B subtypes of breast cancer had the highest percentages, at 63% and 28%, respectively, compared to other receptor types. Conversely, the triple-negative subtype exhibited the lowest percentage, at 9%, with statistically significant differences observed among these subtypes. In the locally advanced and metastatic BC group, the majority of patients had either luminal A (42%) or triple-negative (39%) subtypes, while Her2 Enriched and luminal B subtypes had the lowest percentages, at 11% and 8%, respectively, with significant differences between these subtypes ($P < 0.05$), as shown in **Table 13**.

Table 12. Distribution of sample study according to hormone receptors in the localized breast cancer patients group.

Receptors	No. (%)	Chi square (x^2)	*P*-value
luminal A	20 (63%)	13.938	< 0.05
luminal B	9 (28%)		
Triple negative	3 (9%)		

Note: *P*-value < 0.05 = significant.

Table 13. Distribution of sample study according to hormone receptors in the locally advanced and metastatic breast cancer patients group.

Receptors	No. 38 (%)	Chi square (x^2)	*P*-value
luminal A	16 (42%)	15.263	< 0.05
luminal B	3 (8%)		
Triple negative	15 (39%)		
Her2 Enriched	4 (11%)		

Note: *P*-value < 0.05 = significant.

The findings concerning the molecular subtypes of breast cancer in your study align with previous research. It is in accordance with studies that have reported a higher percentage of luminal-A subtype, which accounts for more than 50% of invasive breast cancer cases[39,40]. Furthermore, the results are consistent with recent Iraqi studies indicating that hormone receptor-positive cases constitute a significant proportion, with 70% being positive compared to the negative cases[41,42]. These concordant results further validate the prevalence of these subtypes in breast cancer and their significance in clinical research.

The findings from a previous study[43] suggest that there is a markedly higher incidence of advanced stages (III and IV) in individuals with HER2 Enriched subtypes of breast cancer. Conversely, hormone receptor-positive breast cancer expressions are often linked to an earlier stage of presentation. In contrast, HER2+ breast cancer is associated with poorly differentiated tumors and advanced stages. These observations underscore the importance of molecular subtyping in understanding the clinical presentation and characteristics of breast cancer, which can guide treatment strategies and prognosis assessment.

I. Distribution of breast cancer women according to stages of disease:

In the localized BC patients group, the results revealed that BC women in stage IIA constituted the highest percentage, accounting for 47%, compared to other stages. Stage I and stage 0 had lower percentages of 28% and 25%, respectively, with no significant differences among the various stages, as indicated in **Table 14**.

In contrast, among locally advanced and metastatic BC patients, the results showed that BC women in stage III represented the highest percentage, with 47%, compared to other stages. Stage IIB and stage IV exhibited lower percentages of 13% and 40%, respectively, with significant differences observed among the stages, as presented in **Table 15**. These findings underscore the variation in stage distribution among different groups of breast cancer patients and highlight the importance of stage classification in diagnosis and treatment planning.

Table 14. Distribution of sample study according to stages of disease in the localized breast cancer patients group.

Stage	**No. 32 (%)**	**Chi square (x^2)**	***P*-value**
0	8 (25%)	2.68	> 0.05
I	9 (28%)		
IIA	15 (47%)		

Note: *P*-value > 0.05 = non-significant.

Table 15. Distribution of sample study according to stages of disease in the locally advanced and metastatic breast cancer patients group.

Stage	**No. 38 (%)**	**Chi square (x^2)**	***P*-value**
IIB	5 (13%)	7.316	< 0.05
III	18 (47%)		
IV	15 (40%)		

Note: *P*-value < 0.05 = significant.

The findings in your study, particularly the higher occurrence of stages II and III in breast cancer patients, are in line with previous Iraqi studies. These studies have similarly reported that higher stages, specifically stages II and III, are prevalent[36].

J. Histological appearance:

In the context of breast cancer types arising from the inner lining epithelium of the ducts or lobules responsible for supplying milk, classification based on histological appearance is of particular relevance. invasive ductal carcinoma (IDC) is the predominant form of invasive breast cancer. It originates in a milk duct of the breast and extends into the surrounding breast fatty tissue through the lymphatic and circulatory systems.

This finding is consistent with numerous Iraqi studies, which have likewise identified IDC as the most prevalent histological type[44]. In your study, IDC accounted for the highest percentage, at 65.6%, among patients. On the other hand, ductal carcinoma in situ (DCIS), invasive lobular carcinoma (ILC), and lobular carcinoma in situ (LCIS) exhibited lower percentages, at 15.6%, 9.4%, and 9.4%, respectively, with significant differences observed among the various histological types in the localized BC patients group, as presented in **Table 16**.

In the locally advanced and metastatic breast cancer group, the results indicated that IDC was the most prevalent histological type, accounting for 84% of cases, in contrast to other types. ILC and mixed histological types showed lower percentages, at 8% each, with significant differences observed among patients, as shown in **Table 17**. Moreover, in tissue samples from breast cancer patients, the results revealed that IDC was the dominant histological type, constituting 97% of cases, with ILC representing 3%, as indicated in **Table 18**, and these differences were statistically significant.

Table 16. Distribution of sample study according to histological appearance in the localized breast cancer patients group.

Histological	**No. (%)**	**Chi square (x^2)**	***P*-value**
IDC	21 (65.6%)	28.50	< 0.05
DCIS	5 (15.6%)		
ILC	3 (9.4%)		
LCIS	3 (9.4%)		

Note: *P*-value < 0.05 = significant.

Table 17. Distribution of sample study according to histological appearance in the locally advanced and metastatic breast cancer patients group.

Histological	**No. 38 (%)**	**Chi square (x^2)**	***P*-value**
IDC	32 (84%)	44.263	< 0.05
ILC	3 (8%)		
Mixed	3 (8%)		

Note: *P*-value < 0.05 = significant.

Table 18. Distribution of sample study according to histological appearance in the tissue breast cancer patients group.

Histological	**No. 36 (%)**	**Chi square (x^2)**	***P*-value**
ILC	1 (3%)	32.111	< 0.05
IDC	35 (97%)		

Note: *P*-value < 0.05 = significant.

K. Grade of disease:

Cancer grading provides an indication of how abnormal cancer cells appear under a microscope when compared to healthy cells, with lower grades indicating well-differentiated cells and vice versa. In the case of BC, the grade is a valuable tool for assessing the tumor's level of spread and aggressiveness.

In our study, the results indicate that women with grade III BC constituted the highest percentage, at 45%, compared to other grades. In contrast, grade I had the lowest percentage, at 19%, with no significant differences observed among the grades of malignant breast cancer tissue, as shown in **Table 19**.

The consistency of your study's results with previous Iraqi studies is noteworthy. These studies have also reported a high percentage of histological grade III in breast cancer cases, indicating a low degree of differentiation[45]. Furthermore, another Iraqi study has highlighted that a significant proportion of patients presented with stage III and grade III breast cancer, followed by those with stage and grade II[46].

Table 19. Distribution of sample study according to grade of disease in the tissue breast cancer patients group.

Grade	No. 36 (%)	Chi square (x^2)	*P*-value
I	7 (19%)	3.50	P > 0.05
II	13 (36%)		
III	16 (45%)		

Note: $P > 0.05$ = non-significant.

1) Expression of CDH1:

In this section, we present the analysis of CDH1 expression data normalized using TBP as a reference gene. Our aim was to detect the amplification plots for both CDH1 and TBP, enabling the determination of the threshold cycle (CT) values for each gene, as depicted in **Figures 4** and **5**, respectively.

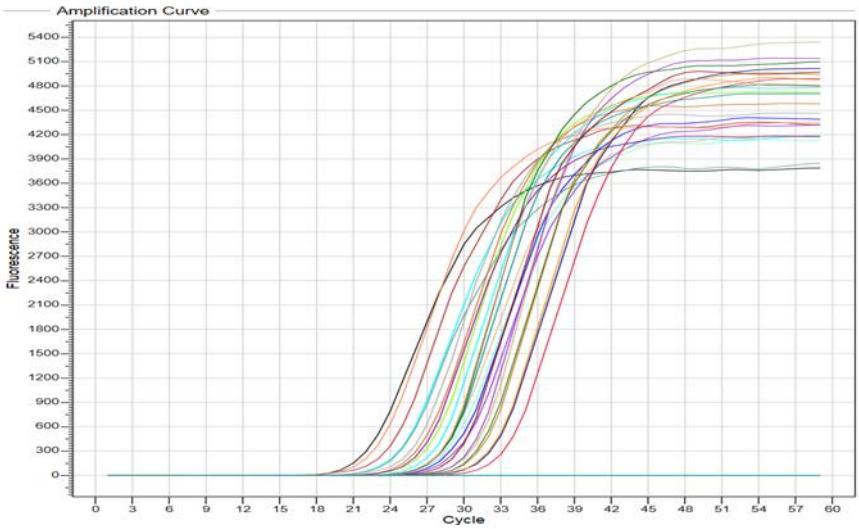

Figure 4. The amplification plots acquired through real-time PCR for CDH1 gene expression.

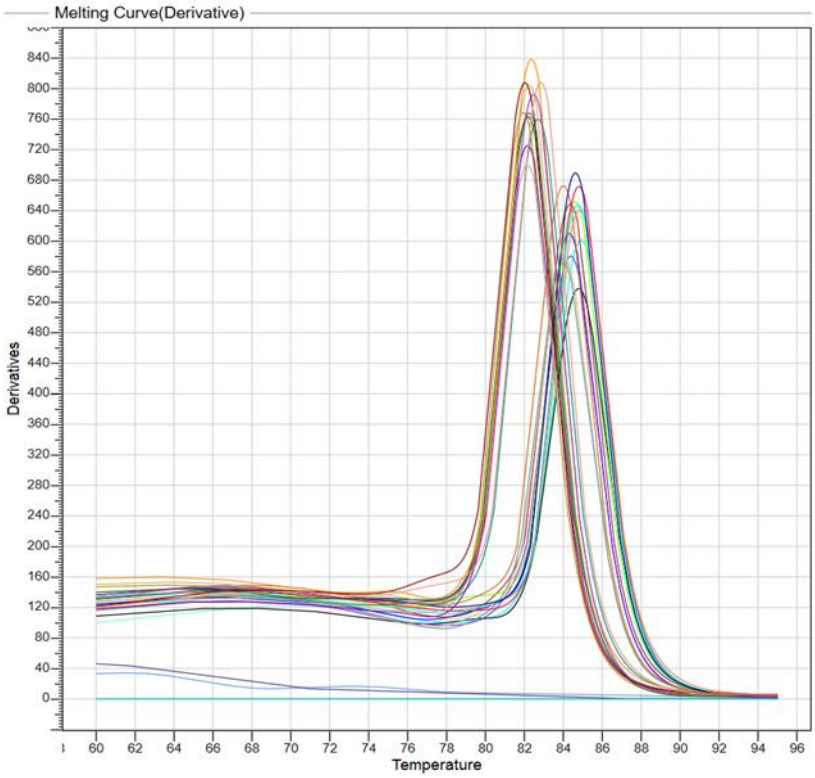

Figure 5. The CDH1 gene expression melting curve.

We observed a notable increase in CDH1 expression levels among patients with locally advanced and metastatic breast cancer when compared to those with localized breast cancer and the malignant breast cancer tissue group, as illustrated in **Figure 3**. The fold change in CDH1 expression was markedly elevated in patients with locally advanced and metastatic breast cancer, exhibiting a value of (2.550 ± 0.164). In patients with localized breast cancer, the relative CDH1 expression increased by (1.456 ± 0.055)-fold. Furthermore, a significant difference was observed in the malignant breast cancer tissue group compared to both the localized breast cancer group and the control group, with a fold change of (1.886 ± 0.08621), as displayed in **Figure 6**.

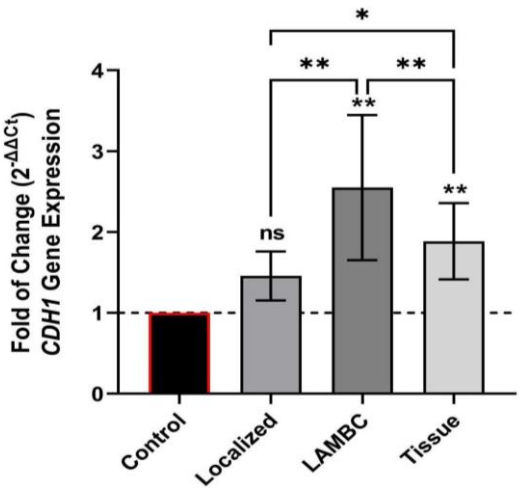

Figure 6. Fold of change of CDH1 gene expression.
*LAMBC = locally advanced and metastatic breast cancer.

The presented results concerning CDH1 expression levels underscore its potential functional significance in BC metastasis. An upregulation of CDH1 was observed across all three BC groups, with the most remarkable increase seen in patients with locally advanced and metastatic BC compared to the other groups. These findings are in alignment with Ye et al.'s study from 2020[47], which highlighted CDH1 and its encoded E-cadherin's oncogenic properties. Notably, the CDH1 oncogene has been implicated in promoting self-renewal in lung cancer stem-like cells. Prostate cancer cells with E-cadherin-positive subsets have exhibited attributes associated with cancer stem cells, and the plasticity of E-cadherin expression has been observed during cell invasion[48].

In the context of BC, Manuel Iglesias et al. in 2013[49] demonstrated that increased E-cadherin (E-cad) expression in SKBR3 cells enhances mammosphere formation. Furthermore, Padmanaban et al.[20] provided evidence that E-cadherin plays a role in metastasis in both murine and human models of luminal and basal-like BC. Notably, both mRNA and protein levels of CDH1 (the gene encoding E-cadherin) have been found to be elevated in BC tissues when compared to their normal counterparts. Importantly, elevated CDH1 expression is positively correlated with advanced stages of BC, increased metastatic potential, stemness characteristics, and an unfavorable patient prognosis, as highlighted by Xi et al. in 2022[12].

The up-regulation of E-cadherin can be linked to the termination of tumor cell invasion, functioning as a tumor suppressor[50]. Specifically, in gastric carcinoma, faulty E-cadherin mechanisms strongly correlate with cancer metastasis. Patients with somatic E-cadherin alterations exhibit the poorest prognosis and the lowest overall survival probability[51]. Given that the majority of solid human tumors originate from epithelial cells, adhesion molecules at epithelial cell junctions and cell signaling pathways are of significant interest. Numerous studies have characterized E-cadherin's role as a tumor suppressor[52]. However, recent research has revealed that in late-stage cancers, E-cadherin might also facilitate cell migration, tumor progression, and invasion[53]. The process of metastatic colonization, known as mesenchymal-to-epithelial transition (MET), involves the re-expression of E-cadherin[19].

Furthermore, the function of newly identified miRNA in breast cancer pathogenesis has been outlined, exemplifying the role of CDH1 in regulating the cell cycle and apoptosis[13]. E-cadherin has also been demonstrated to promote metastasis in various models of invasive ductal carcinomas[20]. CDH1's activity influences the cell cycle and apoptosis pathway, emphasizing its pivotal role as a central molecule in the epithelial-to-mesenchymal transition (EMT) process. The impact of CDH1 overexpression on BAX and PTEN aligns with its tumor suppressor function. The correlation between CDH1 and differentiating tumor grade in BC suggests its potential as a marker for tumor progression. Predicted downstream targets associated with cancer underscore the CDH1 miRNA's relevance in the realm of tumor biology[13]. In the context of drug targets, CDH1 is intricately linked to the cell adhesion network, thus making it connected to critical factors in drug resistance, as elucidated by Ku et al. in 2022[18]. It's worth noting that CDH1 expression has been detected in both invasive lobular carcinomas (ILCs) and invasive ductal carcinomas (IDCs), as reported by Ribatti et al.[54].

CDH1 expression levels in breast cancer exhibit a significant increase when compared to normal tissue specimens, and this elevated expression is associated with adverse outcomes in distant metastasis-free survival (DMFS). This observation is substantiated by the results presented in **Table 20**, which categorizes the fold change of CDH1 expression within the localized breast cancer patient group based on the disease stage. Specifically, the fold changes of CDH1 expression in various stages are as follows: stage 0 (1.07), stage I (1.30), and stage IIA (1.70). Notably, CDH1 expression levels display an inverse correlation with the degree of malignancy, and a substantial difference in expression levels is observed between stage 0 and stage IIA.

Table 20. Fold change of CDH1 expression in localized breast cancer patients group according to stages of disease.

Genes	**Folding**		
	Stage 0	**Stage I**	**Stage IIA**
CDH1	1.07^{b}	1.30^{a}	1.70^{a}

Note: LSD at 0.05 probability.

While examining CDH1 expression, we observed up-regulation in different stages of breast cancer. Specifically, the fold changes in CDH1 expression were as follows: stage IIB (1.72), stage III (2.11), stage IV recurrence (2.68), and stage IV de novo (4.20). Notably, CDH1 expression levels displayed an inverse correlation with a degree of malignancy. Our analysis revealed that there is a significant difference in CDH1 expression between stage IIB and stage IV as detailed in **Table 21**.

Here, our study indicates significant findings, where a positive association between elevated serum sE-cadherin levels and various clinical parameters, including TNM stage, tumor grade, and lymph node metastases in breast cancer are observed[55]. These findings underscore the complexity of CDH1's role in malignant tumors, with ongoing debates and a lack of a comprehensive understanding. It could be suggested that the overexpression of CDH1 is positively correlated with the presence of stemness in breast cancer. The CDH1 gene's role is significant in both cancer progression and as a risk factor and prognostic indicator for adverse outcomes in breast cancer[12].

Table 21. Fold change of CDH1 expression in locally advanced and metastatic breast cancer patients group according to stages of disease.

Genes	**Folding**			
	Stage III	**Stage IIB**	**Stage IV recurrence**	**Stage IV DeNovo**
CDH1	2.11^{b}	1.72^{b}	2.68^{a}	4.20^{c}

Note: LSD at 0.05 probability.

In malignant breast cancer tissue, our results demonstrate up-regulation of CDH1 expression, which is

associated with increased protein folding. We observed that the fold changes in CDH1 expression in grade I was 1.22, in grade II was 1.66, and in grade III was 2.31. Importantly, CDH1 expression levels exhibit an inverse correlation with malignancy. Our data also revealed a significant difference in CDH1 expression between grade I and grade III, as illustrated in **Table 22**. CDH1 up-regulation plays a crucial role in promoting the mesenchyme-to-epithelial transition (MET) during the colonization phase of metastasis. Additionally, our findings suggest that CDH1 may have a promoting effect on stem cell self-renewal[22].

The findings in your study are consistent with the observations made by Burandt et al.[56], who noted that high levels of E-cadherin were more frequently observed in malignant soft tissue tumors than in benign ones and were associated with high-grade tumors.

Table 22. Fold change of CDH1 expression in malignant tissue of breast cancer women according to grade of disease.

Genes	**Folding**		
	Grade I	**Grade II**	**Grade III**
CDH1	1.22^{bc}	1.66^{b}	2.31^{b}

Note: LSD at 0.05 probability.

2) The serum level of E-cadherin:

Serum E-cadherin levels were estimated and compared between the healthy control and patient groups, as outlined in **Table 4**. The findings demonstrated that E-cadherin concentration exhibited an elevation in the locally advanced and metastatic BC patient group when contrasted with both the localized BC group and the apparently healthy control group ($P = 0.00014$), as depicted in **Table 23**. The results further indicated that the mean E-cadherin levels were notably higher in the locally advanced and metastatic group (963.4 ± 89.8 pg/mL) compared to the localized BC patient group (539.77 ± 52.88 pg/mL), in comparison to the controls (318.21 ± 31.28 pg/mL), with statistically significant differences observed among the groups.

E-cadherin (E-cad) is a transmembrane molecule with an extracellular structure that can undergo cleavage, leading to its release into the bloodstream as soluble E-cadherin (sE-cad). This soluble form, sE-cad, is significantly elevated in the serum of individuals with malignant tumors and is considered a potential diagnostic and prognostic biomarker for malignancy[56]. This outcome aligns with a study that identified a positive correlation between CDH1 overexpression and various aspects of BC progression, including stage, metastatic behavior, stemness traits, and unfavorable patient prognosis[12]. Similarly, elevated serum levels of sE-cad have been observed to positively correlate with TNM stage, lymph node metastasis, and tumor grade in BC[57]. In the context of lung cancer, serum sE-cad levels are remarkably higher in patients compared to control subjects, with a particularly pronounced increase in patients with distant metastasis[58].

Table 23. Distribution of study samples based on E-cadherin concentration in the serum of patients with localized, locally advanced, and metastatic BC groups, and control group.

Groups	**E-cadherin pg/mL (mean + SD)**
Control	C (318.21 ± 31.28)
Localized BC	B (539.77 ± 52.88)
Locally advanced and metastatic BC	A (936.4 ± 89.8)
P-value	0.00014
Sign.	Significant

Repetto et al.[59] conducted a study demonstrating elevated levels of E-cadherin protein expression in various experimental systems. This elevation, in conjunction with members of the HER family, contributed to the enhancement of signaling pathways, including MAPK, PI3K/Akt/mTOR, and IAP. The study also highlighted the pro-oncogenic functions of E-cadherin in both HER2+ and triple-negative breast cancer

(TNBC) cell lines. In vitro experiments revealed that E-cadherin collaborated with the EGF ligand to stimulate breast cancer migration, proliferation, and invasion.

4. Conclusion

In summary, this study provides compelling evidence supporting CDH1 as an oncogene in BC. The significance of CDH1 in BC tumorigenesis underscores its potential for the development of novel detection biomarkers and targeted therapeutic approaches for BC treatment. Notably, CDH1 exhibited elevated expression levels in BC tissues and demonstrated an association with unfavorable distant metastasis-free survival outcomes. The co-expression of genes alongside CDH1 was linked to the regulation of epithelial-to-mesenchymal transition (EMT) and cell adhesion processes, both of which are pivotal in driving tumor metastasis. Additionally, the presence of soluble E-cadherin (sE-cad) in serum emerged as a promising noninvasive diagnostic marker for BC. Given these findings, CDH1 emerges as a potential target for BC diagnosis, prognosis, and treatment. Future research endeavors will focus on exploring CDH1's clinical implications. The identification of CDH1's epigenetic and structural changes within diagnostic or pre-operative biopsies could yield valuable insights for enhancing patient management, particularly in predicting BC prognosis and metastatic patterns. An intriguing new discovery is that CDH1's oncogenic activity might be driven by its role in promoting self-renewal among cancer stem cells, akin to its function in normal stem cells. This opens up exciting avenues for further investigation into the mechanisms underlying CDH1's role in BC.

Author contributions

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

Acknowledgments

The authors like to express their thanks to Baghdad University for partially supporting this work.

Ethics statement

The clinical trial strictly adhered to the principles outlined in the Declaration of Helsinki and the standards established by the International Conference on Harmonisation for Good Clinical Practice. The study methodology underwent thorough evaluation and received approval from the University of Baghdad's Research Ethics Committee under reference number iGBEC/1246A.

Conflict of interest

The authors declare no conflict of interest.

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