VER
Vascular & Endovascular Review

The Role of CD44 and CD24 as a Marker in Breast Cancer Metastasis

Shireen Ahmed Dzayee[1]

[1]Department of Physiology, Microbiology and Genetics, College of Medicine, Hawler Medical University, Erbil, Kurdistan Region, Iraq
Email ID : shireenahmed455@gmail.com

ABSTRACT

Background: CD44 and CD24 are important factors in stimulating metastasis, treatment failure and recurrence in breast cancer. The aim of this research is to explore the manifestation of these CD44 and CD24 in females diagnosed with breast cancer. Methods and Materials: In this descriptive-analytical study, 40 patients with breast cancer after mastectomy and 40 healthy people were included as a control group in this study in medical care at Rizgary teaching hospital of Erbil were included in the study in the period from November 2021 to October 2022, the patients were divided into two groups. including 40 patients and 40 healthy people. After completing the consent form, clinical information about all the samples was taken. EDTA blood samples were gathered from patients and healthy individual, and RNA was obtained using the RNX-Plus kit (Sinaclon, Iran). Following the extraction of total RNA and the creation of complementary DNA, we assessed relative gene expression using a quantitative real-time PCR technique. Serum was also prepared for ELISA analysis, levels of CD44 and CD24 in serum were measured using ELISA kits(eBioscience, USA) according to manufacturer instructions Finally, the results was examined by statistical analysis. Results: The results of examining the changes in the expression of CD44 and CD24 showed that the expression of CD44 and CD24 increased significantly in patients compared in healthy women ($P \leq 0.001$), Conversely, the gene expression was demonstrated to increase further with an extended cultivation time. Furthermore, there was a distinct rise in gene expression observed in breast tumors undergoing metastasis. The results showed that the plasma of the patients contained 34.3 and 3.15 respectively of CD44 and CD24. The results of serum level of CD44 and CD24 increased significantly in patients compared in healthy women ,The mean ± SD of CD44 in the control group was 177.35 ± 31.55 (ng/ml) and in the patient group was 725.22 ± 289.998 (ng/ml) and The mean ± SD of CD24 in the control group was 98.21 ± 12.36 (ng/ml) and in the patient group was 504.25 ± 96.357 (ng/ml), and this difference in gene expression between the two groups was statistically significant ($P \leq 0.001$) . Conclusions: The findings from the present research illustrate the substantial role that CD44 and CD24 play in regulating the biological processes of cancer cells. CD44 and CD24 in particular were found to be substantially expressed in the patients' samples.

KEYWORDS: Breast cancer, CD44, CD24, metastasis, ELISA

How to Cite: Shireen Ahmed Dzayee (2025) The Role of CD44 and CD24 as a Marker in Breast Cancer Metastasis., Vascular and Endovascular Review, Vol.8, No.17s, 298-306.

INTRODUCTION

With the greatest incidence rate among women worldwide, breast cancer is the most common kind of malignancy. The problems brought on by metastasis are mostly to blame for death from breast cancer.[1,2] The initial tumors are generally larger and affect adjacent tissues. They can be removed or eliminated through surgery or radiation therapy. However, when the tumor extends beyond physiological boundaries, these mentioned methods are either ineffective or contribute to hastening death.[3,4]

The five-year survival rate for breast cancer decreases from 100% when it is localized to less than 25% when it has spread. Hence, the most pivotal milestone in cancer therapy would be the prevention of metastasis.[5,6,7] Metastasis encompasses a complex series of stages, including the detachment of cancer stem cells from the primary tumor, the breakdown of the basement membrane and extracellular matrix via proteases, penetration into nearby blood vessels, circulation through the bloodstream, anchoring at a vessel site, extravasation, and, ultimately, the development of a secondary tumor. [7,8] In addition to identifying the disease mechanism, identifying markers that can help with rapid disease diagnosis would be very useful. Various factors are involved in the development of cancer, and genetic background is one of the factors contributing to the development of cancers in humans.

CD44 As a hyaluronic acid receptor, is one of the most common surface markers expressed in most cancer cells. CD24 is also a surface marker that is a heat-stable antigen that is expressed in many types of tumors. However, their expression and prognostic value in cancer cells are debatable.[10] CD44 plays an important role in the process of metastasis because it facilitates cell attachment to blood vessels and trans endothelial migration, helps to maintain basic characteristics in tumor cells through stimulation of key signaling pathways, and causes resistance. to drugs increased through[11] Research supports the role of cancer stem cells (CSCs) and their associated markers, including molecules CD44 and CD24 in malignancies. As a non-kinase cell surface transmembrane glycoprotein that is highly expressed in CSCs and often undergoes alternative transplantation to support cancer progression, it also affects treatment outcomes.[13,14] Breast cancer ranks as the most prevalent cancer in the female

population, underscoring its paramount significance in research and study.[12] The molecules CD44 and CD24 possess the capacity to direct intracellular signals related to growth and mobility, and they are implicated in various forms of cancer, including breast carcinoma.[13, 14] In cases of prostate cancer and neuroblastoma, the CD44 molecule has been recognized as a gene with the ability to suppress metastasis.[15] Even though recent evidence suggests a potential involvement in prostate cancer growth, the function of the CD44 molecule in breast cancer remains ambiguous and subject to controversy. In laboratory settings, this molecule acts as a mediator for both pro-tumor and anti-tumor signals and can potentially induce metastasis inhibition or progression in an in vivo environment through the activation of caspase-3, leading to apoptosis .[16, 17]

The main aim of this study was to investigate the expression level of CD44 and CD24 genes in breast cancer and its role in the development and spread of cancer in patients with breast cancer.

MATERIAL AND METHODS

In this descriptive-analytical study, 40 patients with breast cancer after mastectomy and 40 healthy people were included as a control group in this study, and the patients were divided into two groups. including 40 patients and 40 healthy people. This study took place in the city of Erbil between the years November 2021 to October 2022. Patients and healthy people were selected from those who visited Rizgari Teaching Hospital and collecting information was done on the patients. All Patients completed the fully informed consent form. In addition to demographic characteristics, blood samples were taken from the patientsand healthy control . The study entry criteria included confirmation of cancer by a pathologist and clinical information. Patients with benign tumors or undiagnosed tumor types were excluded from the study. Additionally excluded were participants who had undergone chemotherapy or had a history of tumor recurrence.

Blood sample

Blood samples were taken from patients and healthy control and divided into two parts. The first part was placed in an EDTA-test measurement for hematology tests and the second part was placed in a non-coagulant test tube at room temperature to allow clotting (for 15 minutes) then the sera were separated by centrifugation (3,000 rpm for 10 minutes). Serum was stored at -80°C until used for quantification of sCD44, sCD22 levels by enzyme-linked Immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, USA).

Blood processing

From the samples, EDTA blood samples were taken, and the plasma was quickly processed within two hours after collection. The first blood tube drawn after venipuncture was not used to collect plasma since it might have been contaminated with epithelial cells as a result of the original skin puncture. After that, the blood was centrifuged at 1300 g for 20 minutes at 10°C. The plasma-containing supernatant from this process was put into ultracentrifuge tubes. To remove cell debris and other particles, a further high-speed centrifugation was carried out at 15,500 g for 10 minutes at 10°C. When not in use, the plasma was separated into cryogenic vials, quickly frozen with liquid nitrogen, and kept at -80°C.

Gene expression using the Real-time PCR method

The Real-time quantitative PCR method was employed to examine the gene expression levels of CD44 and CD24. Cellular RNA extraction was carried out using the RNX-Plus kit (Sinaclon, Iran). In summary, the RNA extraction process involved the following steps: The cellular pellet was exposed to one milliliter of Plus-RNX solution, followed by a 5 to 10-second vortexing, and subsequently, it was allowed to incubate at room temperature for five minutes. Following the addition of 200 microliters of chloroform, the mixture was incubated for five minutes at 4°C on ice. To help separate the phases, the mixture was centrifuged at 12,000 rpm for 15 minutes at 4°C. The top blue solution, which is the aqueous phase, was carefully transferred into a fresh microcentrifuge tube, and isopropanol in an equal amount was added. Following a 15-minute incubation on ice, the mixture underwent centrifugation at 12,000 rpm for 15 minutes at 4°C. Subsequently, 1 milliliter of 75% ethanol was poured onto the pellet, and it was subjected to centrifugation at 7,500 rpm for 8 minutes at 4°C. The resulting pellet was air-dried at room temperature, and then 50 microliters of RNase-free water were added to dissolve the RNA pellet. The RNA pellet was completely dissolved in the mixture after 10 minutes of incubation at 60-55°C, which was followed by storage at 75°C until later usage. The extracted RNA integrity and purity were confirmed through agarose gel electrophoresis, spectrophotometry at wavelengths of 260 and 280 nanometers, as well as PCR validation using a reference gene. Adhering to the guidance provided by the manufacturer of the Prime ScriptTM RT reagent kit (Takara, Japan), we utilized one microgram of total RNA to generate a single copy of cDNA within a reaction volume of 20 microliters. For this purpose, 2 micrograms of total RNA, along with 4 microliters of x replication buffer, 0.5 microliters of reverse transcription enzyme, and 0.5 microliters of each forward and reverse primer (10 picomoles), were mixed in the final volume of 20 microliters and incubated at 37°C for 15 minutes. The reaction tubes were then quickly heated to 85°C for 5 seconds to deactivate the reverse transcriptase enzyme. The reaction product that was produced was stored at -20°C until it was required for use.

The Real-time PCR reaction was performed to amplify the CD44 and CD24 genes, using 2 microliters of cDNA product, specific primers for each gene, Takara SYBR Green Real-Time Master Mix kit, and the StepOnePlus Real-Time PCR instrument. The temperature parameters comprised an initial activation phase at 95 degrees Celsius for 30 seconds, followed by 40 cycles of

denaturation at 95 degrees Celsius for 5 seconds, and annealing/extension at 60 degrees Celsius for 34 seconds (Table 1). A melting curve for the genes was generated following the Real-time PCR experiment, and the PCR products were verified by agarose gel electrophoresis to guarantee the specificity of the results. The gene expression levels were quantitatively analyzed and determined using the relative quantification method and the $2^{-\Delta\Delta CT}$ formula. Specific primers were designed using the 7 OLIGO software and ordered from Macrogen (South Korea). The primer sequences are provided in Table 1. The experiments were repeated three times (Table 2).

Table .1 Temperature cycle and time spent in technique Real Time PCR

Phase	Temperature	Duration of cycles
Initial activation	95° c	20 minutes
Denaturation	95° c	5 seconds
Extension	60° c	34 seconds

Table .2 Sequence and characteristics of primers used in the study

Gene expression	Sequence primer	Product length PCR (bp)
Patients		
CD44	Forward: GCATCAATGGTTCAGCTCCAC Reverse: GGGATGCTGGCGTAGATGTC	165
CD24	Forward: ACGTCCACTTCGTCAAGCTCAT Reverse: TCAACCACGCTGTTGCTGTA	163
Healthy women		
CD44	Forward: CCAACTATGCTTCAGCTGCAC Reverse: GCGATCCTGCCGTAGATTTC	165
CD24	Forward: ACGTCACTGCTTCAGCTTCAC Reverse: GACATGCTGCCGTAGAAGTC	163

Statistical analysis

We employed GraphPad Prism software, version 9, to analyze and present the generated data. Statistical analysis was conducted using the t-test, with significance determined at the 0.05 level. After performing the PCR reaction and amplifying the target genes, the ct values of the samples were entered into Excel software, and $2^{-\Delta\Delta ct}$ was calculated to plot the gene expression graph. A significance level of less than 0.05 was considered

RESULT

The demographic characteristics of the samples under study showed that the mean age of the patients was 46.40 ± 9.21 years and mean age of the healthy women 45.22 ± 8.36 years. The mean age of menarche in the patients was 13.45 ± 1.24 years and mean age of menarche in the healthy women 13.01 ± 1.22years, the mean age of menopause in the patients was 47.42 ± 3.85 years and healthy women 48.55 ± 7.12 years. The mean age of first pregnancy in the patients was 20.55 ± 5.36 years and healthy women 21.14 ± 4.33 years, the mean BMI in the patients was 28.74 ± 2.14 kg/m^2 and healthy women 26.17 ± 7.44 kg/m^2. 12 (30%) of the patients and 6 (15%) of the healthy women had a family history of breast cancer. Tumor histology in the patients showed that 32 (80%) had invasive ductal carcinoma, 6 (15%) had invasive lobular carcinoma, and 2 (5%) had inflammatory cancer (Table 3).

Table.3: Patient demographics and clinical characteristics

Variable		patients Mean ± SD	Healthy women Mean ± SD	P-value
Age		46.40 ± 9.21	45.22 ± 8.36	0.422
Menarche Age		13.45 ± 1.24	13.01 ± 1.22	0.144
Menopause age		47.42 ± 3.85	48.55 ± 7.12	0.613
Age of first pregnancy		20.55 ± 5.36	21.14 ± 4.33	0.364
BMI		28.74 ± 2.14	26.17 ± 7.44	0.09
Family history		12 (30%)	6 (15%)	0.812
Histology	Invasive ductal carcinoma	32 (80%)		
	Invasive lobular carcinoma	6 (15%)		
	Inflammatory cancer	2 (5%)		

In this current study, we utilized the fluorescent dye SYBR Green to probe alterations in the expression of the CD44 and CD24 genes. A separate melting curve analysis was conducted using the PCR machine for the CD44 and CD24 genes. This analysis aimed to assess the specificity of the primers and the SYBR Green fluorescence, verify the amplification of specific fragments, and confirm the absence of non-specific fragments in the PCR results. Additionally, the PCR products were subjected to electrophoresis on a 1% agarose gel.

The outcomes of the melting curve analysis affirmed the effective binding of the primers and verified the specificity of the PCR product for the target genes. Moreover, the agarose gel electrophoresis displayed a singular band, indicating the absence of amplification of non-specific products within the PCR product (Figure 1). These findings collectively demonstrate the validity and reliability of our experimental methods in detecting the desired gene expression changes.

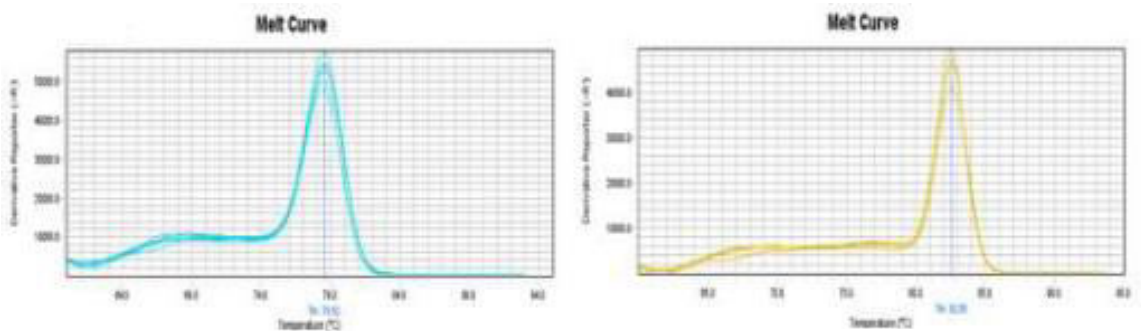

Figure 1. Gene melting curve diagram CD44, CD22

The finding of the present study revealed a noteworthy rise in the expression of CD44 and CD24 genes in breast cancer cells compared to healthy cells after one and three days of culturing ($P \leq 0.001$). The increase in gene expression was relatively modest on the first day of culture. However, on the third day, a more pronounced increase was observed, indicating a time-dependent escalation in gene expression. These finding are depicted in Figure 2, which visually represents the gradual upregulation of CD44 and CD24 gene expression over the course of the culture period in patients.

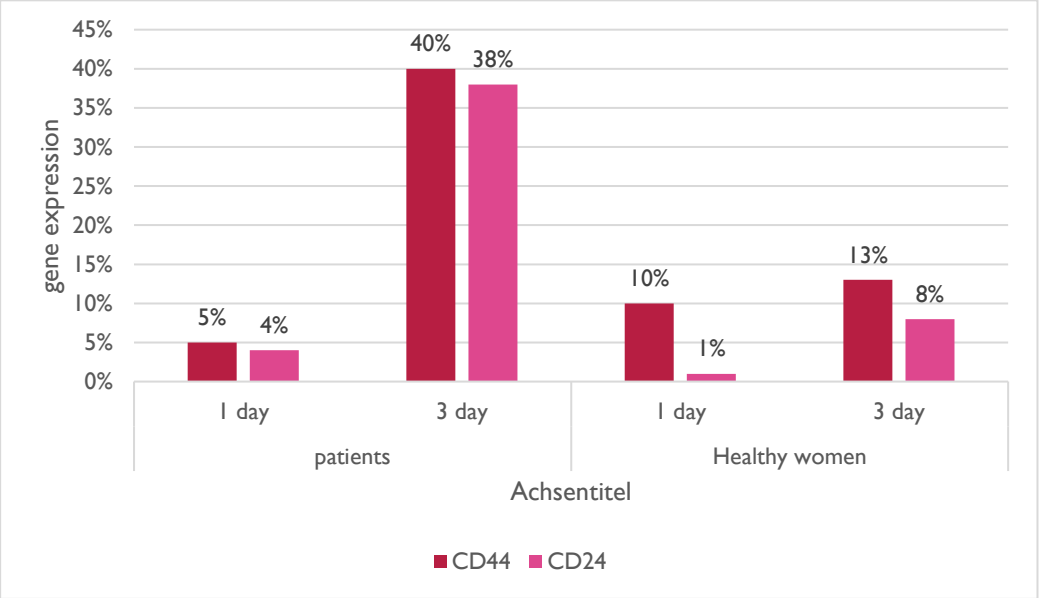

Figure 2. changes in CD44 and CD24 gene transcription in the first- and third-days following culture in patients and healthy women ($P \leq 0.001$)

The assessment of the levels of CD44 and CD24 in the plasma of the patients who underwent examination revealed a considerable rise in CD44 and CD24 in the patients' plasma. The findings revealed that the patients' plasma had a CD44 level of 34.3 and a CD24 level of 3.15 (Figure 3). According to the results, the levels of plasma CD44 in patients is higher than CD24

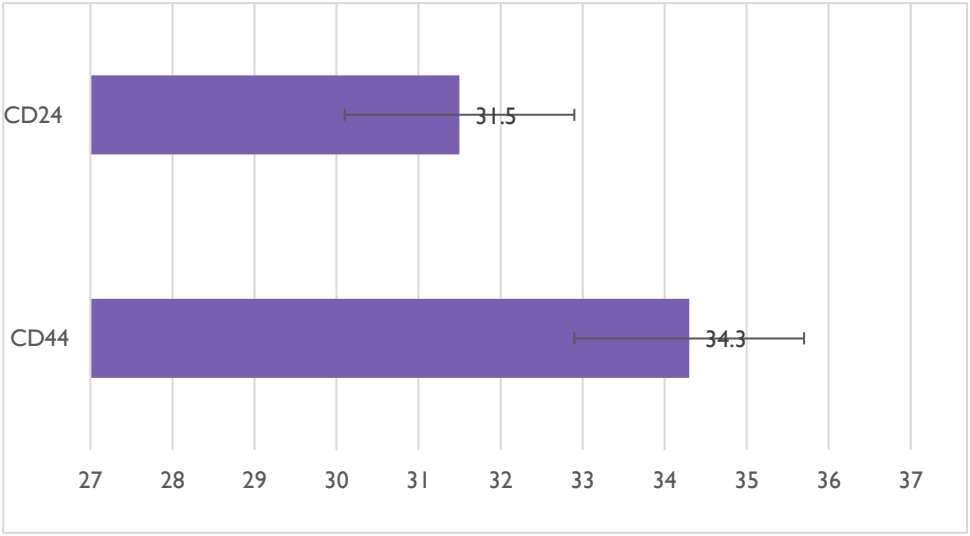

Figure 3. Soluble CD44 and CD24 in patient's (plasma)

The results of confirmation of qRT-PCR products on agarose gel are shown in Figure 4.

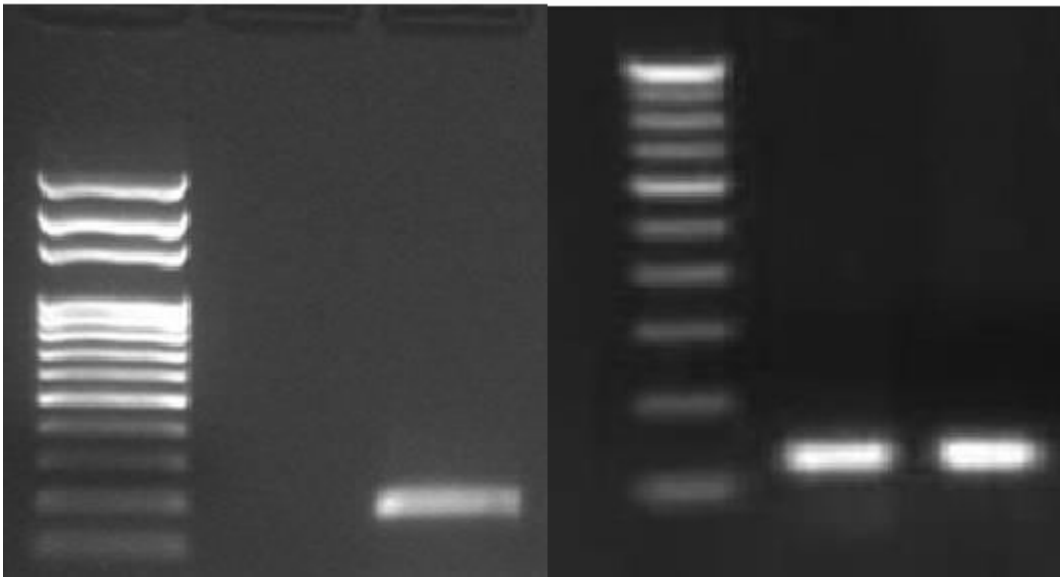

Figure 4. Confirmation of qRT-PCR products on agarose gel

The Serum value of CD24 and CD44 is shown in table number (4). The mean ± SD of CD44 in the control group was 177.35 ± 31.55 (ng/ml) and in the patient group was 725.22 ± 289.998 (ng/ml), and this difference in serum between the two groups was statistically significant (P≤0.001). The mean ± SD of CD24 in the control group was 98.21 ± 12.36 (ng/ml) and in the patient group was 504.25 ± 96.357 (ng/ml), and this difference in serum between the two groups was statistically significant (P≤0.001).
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Table.4 Differences in serum biomarkers in breast cancer patients and control groups

Biomarker	**Control group (n=40)**	**Patients group (n=40)**
CD44 (ng/ml)		
Rang	122.85 – 286.30	161.69 – 1639.23
Mean ± SD	177.35 ± 31.55	725.22 ± 289.998
	P-value 0.001*	
CD24 (ng/ml)		
Rang	90.66 – 195.58	96.88 – 953.149
Mean ± SD	98.21 ± 12.36	504.25 ± 96.357
	P-value 0.001	

*P vale t-test

DISCUSSION

The finding of the present study revealed a notable and statistically significant increase in the expression of both CD44 and CD24 genes. This finding suggests that the altered expression of these genes may have an impact on the mechanisms and progression of cancer in affected individuals.

One of the important biological markers in cancer is gene expression markers. Nowadays, based on gene expression markers,

methods have been developed to investigate the gene expression pattern in cancer cells.[18,19] CD44 and CD24, have shown great potential as biological markers for detecting and potentially treating cancer in the near future.[20] The expression levels of CD44 and CD24, undergo alterations in cancer, which can be clinically useful for diagnosis.[21,22]

CD44 and CD24 play a crucial role in facilitating communication and adhesion between neighboring cells and between cells and the surrounding extracellular matrix. These molecules can exert influence over intracellular signaling pathways that regulate both cell growth and mobility, in addition to their function in cell adhesion. As a result, CD44 and CD24 have been associated with a range of malignancies, encompassing neuroblastoma, ovarian, endometrial, colorectal, and breast cancers.[23,24]

Identification of new biomarkers makes cancer diagnosis and treatment possible. We investigated the expression of CD44 and CD24 markers. In accordance with the hypothesis concerning cancer stem cells, these cells represent a specific subset of cancer cells characterized by unique features such as pluripotency and self-renewal. Recent findings suggest that these cells contribute to the initiation, advancement, and metastasis of cancer cells.[25] Research has shown that transcription factors related to embryonic stem cells regulate critical characteristics of stem cells, such as self-renewal and stemness preservation.[26,27] CD44 and CD24 markers play a vital role as factors in stem cells and are expressed in cancer stem cells.[28] It has been discovered that CD44 and CD24 regulate the expression of many genes, which has an effect on the development and proliferation of cancer cells, in conjunction with other embryonic stem cell factors.[29, 30]

The outcomes of this investigation demonstrated an elevation in the expression of CD44 and CD24 markers. This increase in marker expression was clearly observed in the results, consistent with findings from previous studies.[31,32,33]

The levels of soluble CD44 and CD24 in the patients' sera exhibited an elevation, which can serve as a diagnostic marker for the disease. Consistent with our findings, other studies have also demonstrated a similar increase in soluble CD44 and CD24, reinforcing their potential utility as diagnostic markers for the disease.[34,35]

In this study, the average age of patients and healthy people was more than 45 years. In the study conducted by N Riaz et al (2020) [36] in Pakistan, the average age of the studied people was more than 45 years, which is consistent with the results of the present study and this mean age obtained in Iran is consistent.[37]

Based on the results of the study, it was shown that the average age of menopause in patients was 47 years. In the study of Y Kong et al (2018)[38] in China, the average age of the menopausal patients examined was lower. In the present study, it was found that the lifestyle of people and cultural issues Social can be effective in this difference.

The participants' BMI was obtained in the study above 25 kg/m2, which in the systematic review study by RP Krishnan et al. (2023)[39] also showed that most breast cancer patients can be overweight and obese.

The histology results in this study showed that most of the patients had invasive ductal carcinoma, which was similar to the results of studies in Turkey [40] and America. [41] In general, CD44 and CD24 can serve as important tools in the early diagnosis and prediction of diseases, as well as significant targets for the design and development of new treatments. Screening for genes and proteins in breast cancer that have been identified as biological markers plays a crucial role in understanding the molecular mechanism of tumors.

CONCLUSION

The findings of the present investigation showed that the expression of CD44 and CD24 markers was noticeably greater in the patients who were subjected to examination. Furthermore, this gene upregulation is expected to further increase with longer cultivation periods. CD44 and CD24 are useful biomarkers that can be utilized in therapy, prediction, and even diagnosis. The investigation of CD44 and CD24 markers in this study has shown their potential application in clinical laboratories for breast cancer diagnosis. Moreover, the findings from this study can be utilized in clinical research and potential treatment approaches for breast cancer.

ACKNOWLEDGMENT

To everyone who helped with this study, we would like to convey our appreciation.

Ethical consideration

A fully informed consent form was obtained from all available participants, and in addition to age, pathological and clinical information of patients such as tumor histology was determined for each sample. This research has approved by Ethical committee of Hawler Medical University (1/12-15/10/2023).

Funding

None

Conflict interest

The authors assert that there are no conflicts of interest to disclose.

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