RESEARCH ARTICLE

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The Significant Role of *RUNX2* Expression in Women with Metastatic Breast Cancer

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Abstract

Objective: Breast cancer (BC) is one of the most common malignancies with second ranks among the causes of tumor-associated mortality in females. Since the overexpression of *RUNX2* gene were reported in the development of different malignancies. The present investigation was aimed to find its correlation with prognostic factors viz, tumor grade and stage in the breast cancer patients. **Methods:** This study, examined the expression level changes of *RUNX2* genes with the Real-time PCR and compared the results with various databases and previous research results, to understand the existence of differences between metastatic breast cancer patients' samples and non-metastatic BC samples of patients. **Results:** The prepared samples were divided into two target and control groups and different analyses after QPCR reaction were accomplished to understand the level of changes and significance. **Conclusion:** Results of this study suggest that an elevated *RUNX2* gene expression level could be a valuable independent predictor of tumor metastasis and survival prediction in invasive breast cancer patients. However, to reach more reliable results and better conclusions more samples, studies, and techniques are needed.

Keywords: Breast cancer- *RUNX2* gene- HER2 positive- metastasis

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Introduction

Breast cancer (BC) is one of the most common malignancies with second ranks among the causes of tumor-associated mortality in females [1, 2]. More than 80 percent of these patients who suffer from metastatic cancer die of their diagnosis [3]. Unfortunately, Bone is one of the first and most common sites for metastasis among BC patients which has impressed the survival and life quality of these patients seriously.

Nowadays, a limited number of therapies for bone metastatic breast cancer patients (BMBCP) are present, therefore it notes the key role of early diagnosis of metastasis in these patients especially BMBCP [4].

Depending on the clinical tumor subtype, therapeutic backbones include endocrine therapy, anti-HER2 targeting, and chemotherapy. In metastatic breast cancer, therapy goals are prolongation of survival and maintaining quality of life [5]. With increasing awareness of involved molecular pathways that cause breast cancer, new opportunities have emerged for tumor prediction and response to treatment, as well as evaluation of the tumor's aggressive potential [6].

Genetics and genomics changes assessment and study in metastasis breast cancer patients is a precious step to find the main driving changes in metastasis development as the majority of breast cancer patients die down after enduring a difficult period of illness and entering to metastasis phase of this disease that the most common site is bone [7]. Runx2 directly and indirectly by governing respective signaling pathways during bone metastasis of BC [8].

RUNX2 gene produces a crucial transcription factor that belongs to the Runx family consisting of Runx1, Runx2, and Runx3, *RUNX2* expression can be seen in osteoblast progenitors and osteoblasts which is crucial

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for osteoblast progenitors, and its differentiation to osteoblasts [9].

RUNX2 expression can be seen in chondrocytes as well [10]. Therefore, for growing skeletal, maturity of chondrocyte and differentiation of osteoblast RUNX2 expression is needed [11, 12]. The involvement of *RUNX2* in the development of different malignancies and conditions was reported [13, 14].

It is worth mentioning that Vishal and colleagues also examined the potential dysfunction and regulation of *RUNX2* in breast cancer-related bone metastasis. However, the regulatory mechanisms involved in the metastatic process and drug resistance in triple-negative breast cancer remain inadequately understood [15]. Overexpression of *RUNX2* has been associated with enhanced proliferation of overall survival (OS) cells in vitro, unfavorable clinical outcomes, and reduced effectiveness of chemotherapeutic treatments [16].

The Aim of this study was, examined the level of *RUNX2* gene expression to understand the differences between metastatic breast cancer patients and non-metastatic BC samples of affected patients.

Materials and Methods

Sampling

Fifty fresh HER2 positive breast tumor RNA purification and cDNA synthesis samples underwent metastatic symptoms as well as 50 non-metastatic biopsies received from Shohada Tajrish referral Hospital (Tehran, Iran) in Jun 2022. All data is saved for analysis and all of the breast tumors were tested by IHC staining in the pathology section.

All samples were classified into two metastatic and non-metastatic tumor groups. The GAPDH gene was used as an internal control for the accuracy of the experiment. 50 non-metastatic breast tumor samples that were tested through pathology tests were considered as a control group. Fifty metastatic breast cancer sample tissues were studied as a target group in this study.

Tumor cell lysis and homogenization

After moving outside of tumor samples, the supernatant was transferred into a 15 ml tube and centrifuged at 2,000 g for 5 minutes at 4 centigrade degrees. The supernatant was then discarded, and lysis buffer with 2-mercaptoethanol was added. A rotor-stator homogenizer was used to homogenize at room temperature. Then, the supernatant of each sample was transferred to a clean RNase-free tube for RNA purification for each sample related to both target and control groups.

Total RNA purification and cDNA Synthesis

RNA purification of breast tumor samples was performed using an Ambion pure RNA mini kit (Cat. nos.12183018A, 12183025). Fifty µg of each breast tumor sample was added to the proper micro-tube and one volume of 70% ethanol was added to each volume of cell homogenate. After mixing thoroughly and dispersing any visible precipitate that may form after adding ethanol, up to 700µl of any sample to the spin cartridge was transferred. Then centrifuged in 12000rfc for 15 seconds at room temperature.

Following that, the flow-through was discarded, and the spin cartridge into the same collection tube was reinserted. After 4 times repeating this step, using wash buffer I, centrifuging at 12,000 rfc for 15 seconds at room temperature, and placing the spin cartridge into a new collection tube, 15µl wash buffer II with ethanol was added to each spin cartridge.

Each spin cartridge was centrifuged in 12000rfc for 15 seconds at room temperature and after that, each flow-through was discarded. After one time repeating the previous step, drying the membrane with a bound RNA centrifuge in 12,000 rfc for 1-2 min at room temperature was done. Following that, the collection tubes were discarded and each spin cartridge was inserted into a recovery tube.

A proper volume of RNase-free water was added to the center of each spin cartridge and incubated at room temperature. Centrifuging of each spin cartridge in 12,000 rfc for 2 min at room temperature was done to elute RNA from the membrane into the recovery tube. Finally, all purified RNA was labeled and stored in -80 °C.

Reverse Transcription and Real-time PCR

0.5 µg of oligo dT and 16 µL RNase-free water were added to 5 µg of the whole RNA and incubated at 64 ℃ for 10 minutes. (Extracted RNA for each sample provided and stored in -80 °C).

The primers for SYBR Green real-time PCR were designed specifically for the *RUNX2* gene and the GAPDH gene reference gene, Table 1. The assays were repeated in their entirety for each measurement. Reverse Transcription is accomplished with the Superscript First-Strand Synthesis System for RT-PCR. The following procedure is based on Invitrogen's protocol (total RNA 5 µg, random hexamers (50 ng/µl) 3 µl, 10 mM dNTP mix 1 µl, DEPC H2O to 10 µl). Incubate the samples at 65°C for 5 min and then on ice for nearly 1-1.5 min.

Prepare reaction master mixture. For each reaction (10x RT buffer 2µl, 25 mM MgCl2 4 µl, 0.1 M DTT 2 µl, RNAase outing 1 µl). Add the mixture to the RNA/primer and then place it at room temperature for 3 min. Add 1 µl (50 units) of Superscript II RT to each tube, mix, and incubate at 25°C for 10 min. Incubate the tubes at 42°C for 50 min, heat inactivates at 70°C for 15 min and then chill on ice. Add 1 µl RNase H and incubate at 37°C for 20 min. Store the 1st strand cDNA at -20°C until use for Real-time PCR. Invitrogen™ AM1745، Invitrogen™ Ambion™ Message Sensor™ RT Kit was applied in this part.

Results

Breast cancer is one the most common cancer which is a heterogeneous condition with different pathological and cytological features. The pathology, cytogenetic, and molecular diagnosis are crucial steps to illustrate the prognosis and start therapy for these patients.

Using molecular biomarkers such as the *RUNX2* Gene can be helpful to prognosis more accurate which can also be useful for the metastasis potential prediction as

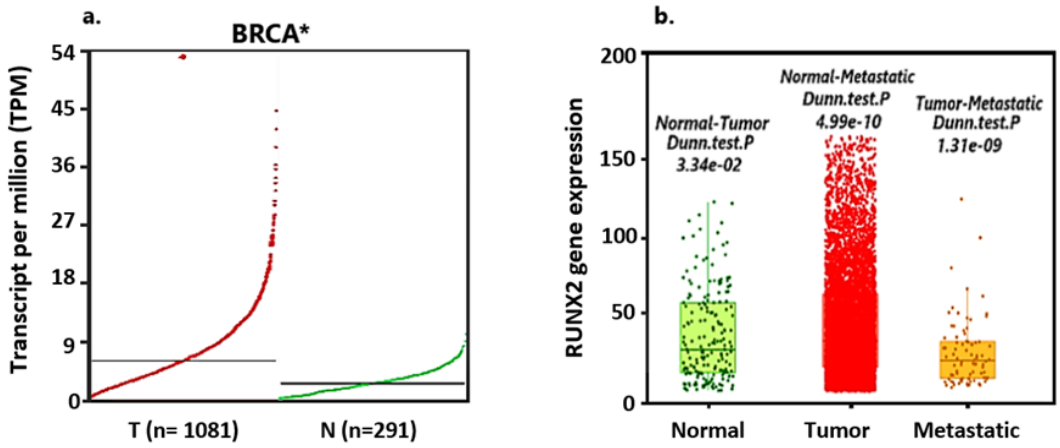

Figure 1. a. Analysis of the levels of expression for the *RUNX2* gene on the *GEPIA2* has been used to understand the changes between normal breast tissues and tumor breast cancer tissues. b. expression levels alteration has been compared among normal, tumor, and metastatic breast tissues in TNM-plot. *BRCA**; Breast carcinoma.

well as prediction of overall survival based on *RUNX2* gene expression levels which considering all aspects and comparing with other available data and researches, applying some reliable existing data in several prominent knowledge bases is one of the best ways to be more precise about selecting proper target gene.

In such cases, we have accomplished various analyses in GEPIA2 and TNM-Plot to evaluate the *RUNX2* mRNA levels of expression in tumor and normal cells GEPIA2, Figure 1a, and assess the expression level of this gene in normal, tumor, and metastatic tumor cells (TNM-plot), figure 1b and Table 2, in other previous firmed research which their results have been indicated in Figure 1, a and b.

Moreover, to understand the importance and correlation of the *RUNX2* gene expression levels with overall survival the Kaplan Meyer plotting has been used and its results are indicated below. In Figures 2a and b results related to the microarray data and in Figures 3 and 4 results related to RNA-seq data were analyzed.

Q-PCR results

Quantitative real-time PCR techniques can be applied for solid tumor profiling and can be considered a strong and cost-effective molecular method in cancer diagnostic and research approaches. The Q-PCR reaction, Figure 3, was performed on both metastatic and non-metastatic tumor samples as aforementioned, and the raw data were analyzed with Rest, GraphPad-prism9, and SPSS. 20 software, t-test, and Pearson chi-square analysis. Our study outputs revealed that the *RUNX2* gene was significantly up-regulated in metastatic tumor samples, and sound to be considered as a promising marker for bone metastasis tracing in breast cancer patients, especially the advanced cases.

REST Analyses results

RUNX2 gene was up-regulated in the target group that contains MBC tumors in comparison to the control group (NMBC samples) by a mean factor of 3.576 the standard error range was (S.E. range is 0.876 - 17.098).

The *RUNX2* expression in the MBC group was different from the control group with P(H1)=0.000. Table 3. The relative expression comparison graph for the *RUNX2* gene and its fold change analysis is shown in Figure 4. The information related to the values is introduced in Table 4.

Table 1. Primer Design with Gene Runner and NCBI BLAST for *GAPDH* and *RUNX2* Genes. * Homo sapiens with failure rate

Genes	Forward primer 1	Reverse primer 1	product length
Homo sapiens *RUNX2*, mRNA	TCTGGCCTTCCACTCTCAGT	GACTGGCGGGGTGTAAGTAA	161
Homo sapiens *GAPDH*, mRNA	GAAGGTGAAGGTCGGAGTCA	TGACAAGCTTCCCGTTCTCA	200

Table 2. Information Related to TNM-Plot Analysis is Shown in This Table

Kruskal.Wallis.p.			Fc.tumor.norm.		Fc.meta.tumor.	
3.05E-09			0.32		0.4	
	Min	Q1	Med	Q3	Max	Upper whisker	N
Normal	2	22	57	123	1765	234	242
Tumor	0	24	51	100	1816	214	7569
Metastatic	2	10	26.5	44	174	92	82

Min, Minimum; Med, medium; Max, Maximum; N, Number; The significance of this analysis is also shown in Figure 2, b.

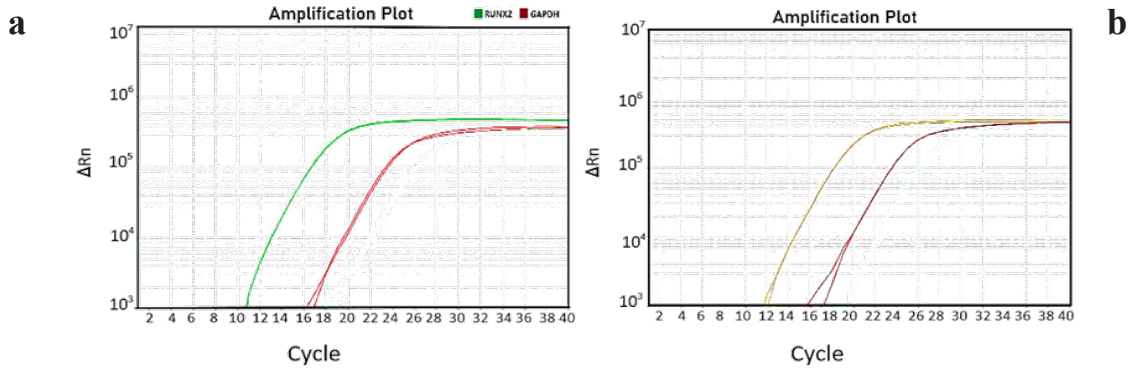

Figure 2. a. *RUNX2* amplification and *GAPDH* amplification graphs were shown in metastatic tumor samples. b. *RUNX2* amplification and *GAPDH* amplification graphs were shown also in non-metastatic tumor samples.

Graph Pad-Prism Analyses and T-test graphs

İnn this step, the significance of differences between levels of *RUNX2* mRNA in metastatic tumor and non-metastatic tumor samples is examined by Graph Pad Prism Analyses and T-test graphs. The observed result is provided in Table 5.

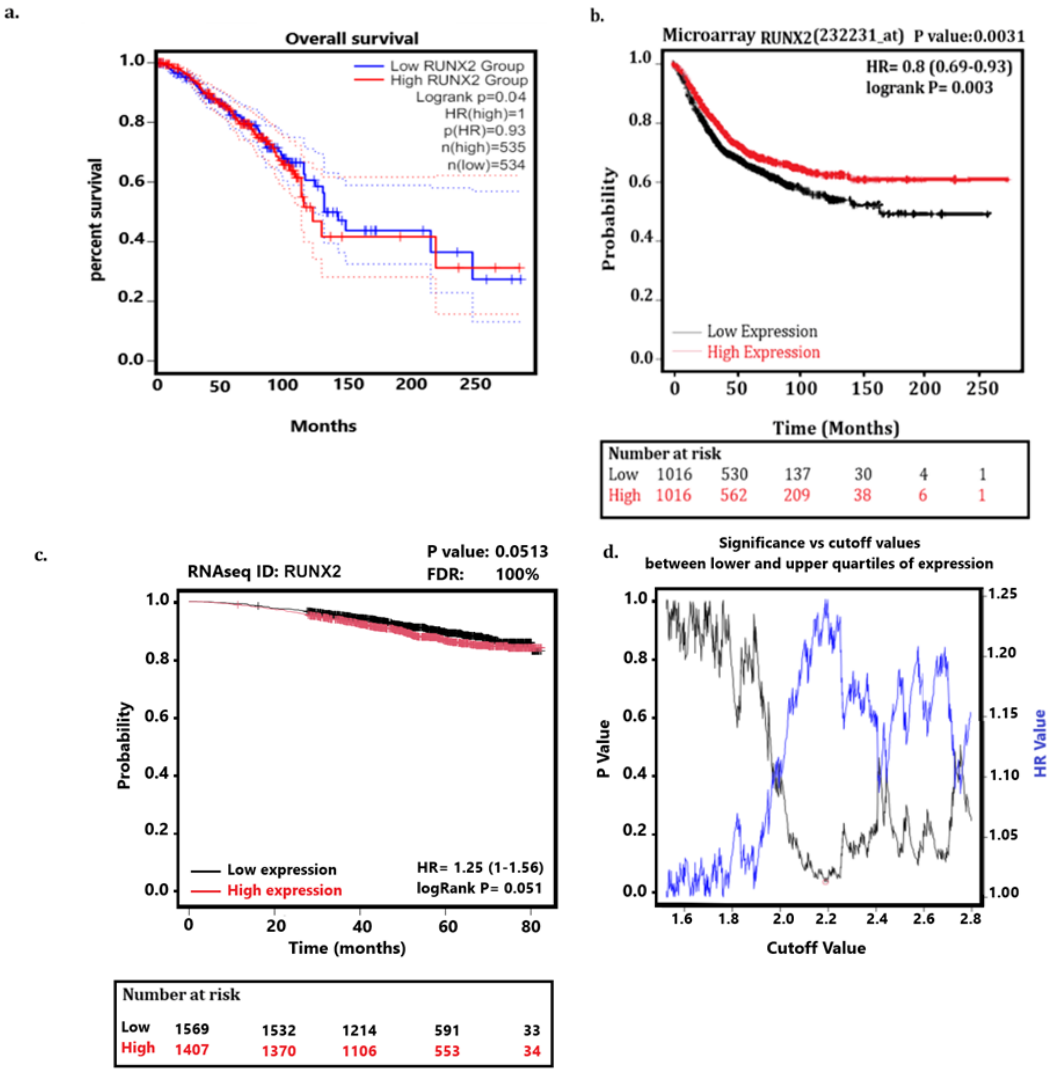

Figure 3. The Kaplan Meier Plotter is Capable of Assessing the Correlation between the Expression of the *RUNX2* Gene and Survival Available Samples from Breast Cancer Tumor Types. Applied statistical tools include Cox proportional hazards regression and the computation of the False Discovery Rate. a. The result of survival analysis based on *RUNX2* gene expression status accomplished by *GEPIA2*. b. The survival analysis based on microarray data results of *RUNX2* gene expression in *HER2*-overexpressed breast cancer accomplished by Kaplan Meier Plotter, P-value < 0.003, and c. RNA-Seq data results presented in Kaplan Meier, P-value< 0.05. d. The significance versus cutoff values between lower and upper quartiles of expression.

Table 3. *RUNX2* is UP-Regulated in the Sample Group (in Comparison to the Control Group) by a Mean Factor of 3.576 (S.E. range is 0.876 - 17.098).*= up-regulation, TRG: target, REF: reference

Relative Expression Results							
Parameter				Value			
Iterations			2000				
Gene	Type	Reaction	Efficiency	Expression	Std. Error	95% C.I.P H1	Result
RUNX2	TRG.	0.953	3.576	0.876 - 17.098	0.211 - 63.955	0	UP*
GAPDH	REF.	0.9724	1				

Table 4. Statistics Assessment of Results of Expression Values of Metastatic and Non-Metastatic Breast Cancer Cells

Number of values	50 met.	50 non-met
25% Percentile	0.08425	0.02060
Median	0.2104	0.05440
75% Percentile	0.4134	0.1250
Mean	0.3590	0.07344
Std. Deviation	0.4950	0.06611
Std. Error of Mean	0.07000	0.009350

ROC Analyses

All outputs were assessed by ROC plotter analysis, and the results of the metastatic samples were compared with non-metastatic samples for *RUNX2* mRNA expression to check specificity and sensitivity. The results shown in Table 6 and the related ROC curve can be seen in Figure 5.

Assessment of the correlation between the RUNX2 gene expression levels and tumor prognostic factors

In the present research, the correlation between the *RUNX2* gene expression level changes and prognostic factor contains tumor grade, Table 7 and Figure 6a, and tumor stage, table 8 and Figure 6b, and QPCR analysis indicated that this gene expression was remarkably correlated with both tumor grade and tumor stage. The methods used to compare CIs are noted below the Table 8.

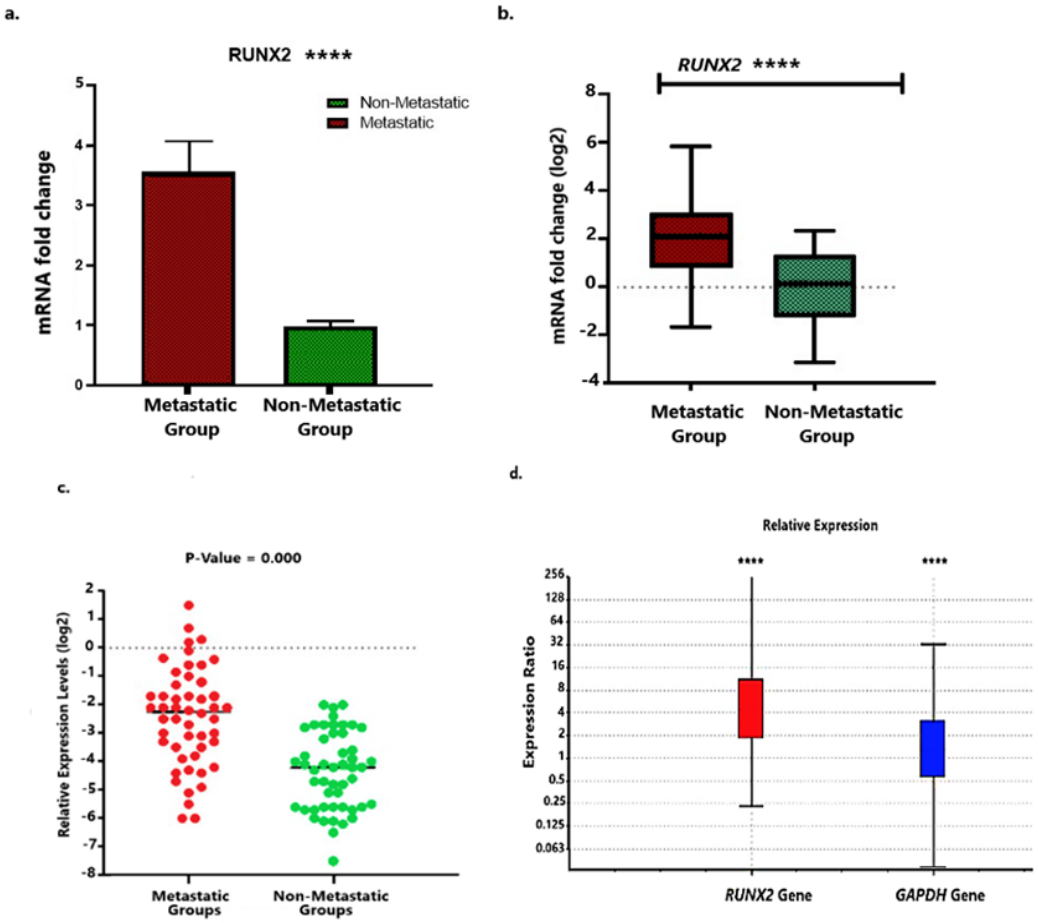

Figure 4. a. The comparison between the *RUNX2* expression levels in the metastatic group and non-metastatic group, P-value< 0.05 b. The levels of mRNA based on their fold changes between two groups, P-value < 0.05 c. Relative expression levels between metastatic and non-metastatic groups, P-value < 0.05 d. The expression ratio of *RUNX2* mRNA and *GAPDH* mRNA compared in metastatic breast tumor cells.

Table 5. Statistical Analysis Results for *RUNX2* Gene Expression Assessment.ddCt *: ΔΔCt

Data Analyzed		*RUNX2* Project
Column A		Metastatic Group
vs.		vs.
Column B		Non-Metastatic
P value and Statistical significance		
Test: chi-square, df		7.034, 1
Z		2.652
P value		<0.0080
P value summary		***
Significantly different (P < 0.05)?		Yes
One- or two-sided P value?		Two-sided
Sensitivity and specificity		
Sensitivity		99%
specificity		99%
Number of values	50 ((-ddCt* MBC))	50 ((ddCt NMBC))
Missing	0	0
Std. Deviation	1.739	1.365
Std. Error of Mean	0.043	0.0193

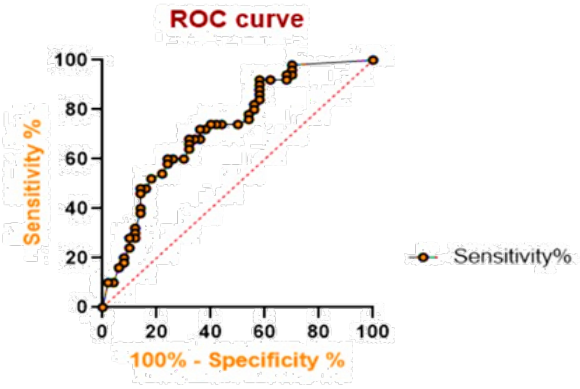

Figure 5. ROC Curve Analysis

Table 6. ROC Curve Analysis

Area under the ROC curve	
Area	0.8006
Std. Error	0.04366
95% confidence interval	0.7150 to 0.8862
P-Value	<0.0001
Data	
Controls ((ddct NMBC))	50
Targets ((-ddct MBC))	50

Discussion

One of the critical issues concerning various types of cancer, particularly breast cancer, is its complexity and heterogeneity [17]. Breast cancer is, in fact, a heterogeneous collection of molecular and cytological pathological manifestations. Accurate and precise diagnosis of the different molecular subtypes poses a significant challenge for researchers, as it is essential for providing accurate prognosis and determining the most appropriate treatment for the patient [18, 19].

Metastatic breast cancer cells acquire invasive characteristics from the tumor microenvironment through multiple mechanisms, including epithelial-mesenchymal transition (EMT) and epigenetic regulation [20, 21]. Therefore, understanding the nature and mechanisms of breast cancer metastasis can facilitate the development of metastasis-targeted therapies [22].

As aforementioned, this study explores the mechanisms that lead to metastasis and reviews current treatments aimed at improving early detection and prognosis for patients with metastatic breast cancer.

In the present study, analysis of *RUNX2* expression levels in two groups of breast tumor cells revealed a significant difference between metastatic and non-metastatic tumor samples, with a p-value of 0.0001

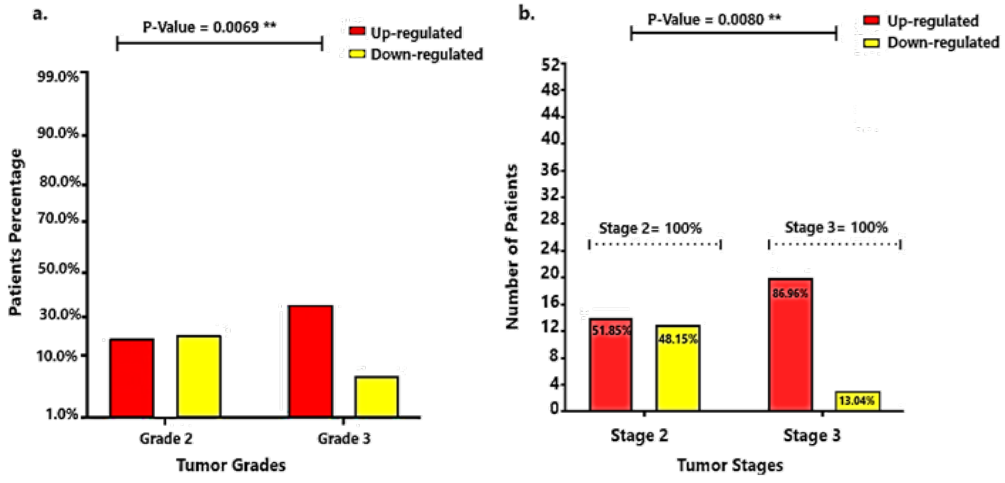

Figure 6. a. The introduced graph shows the percentage of the patients in both grades 2 and 3 and also shows the distribution of the high-level expressed *RUNX2* gene group in each grade. b. also in this figure the number of patients with stages 2 and 3 are exhibited and the distribution of highly expressed samples for the *RUNX2* gene in each stage is shown as well.

Table 7. *RUNX2* Gene Expression Level Changes Relationship and Tumor Grade Progression, Assessment, P.value < 0.0069. *Relative risk with Koopman asymptotic score, Attributed risk (P1-P2) with Newcombe/Wilson with CC, Odd ratio with Baptista-pike and Sensitivity, Specificity also with Wilson-Brown methods.

	Up-regulated	Down-regulated	Total
Data analysed			
Grade 2	11	12	23
Grade 3	23	4	27
Total	34	16	50
Percentage of raw total			
Grade 2	47.83%	52.17%	
Grade 3	85.19%	14.81%	
Percentage of column total			
Grade 2	32.035%	75.00%	
Grade 3	67.65%	25.00%	
Percentage of grand total			
Grade 2	22.00%	24.00%	
Grade 3	46.00%	8.00%	

Table 8. *RUNX2* Gene Expression Level Changes Relationship and Tumor Stage Development Assessment, P.value < 0.0080.

	Up-regulated	Down-regulated	Total
Data analysed			
Stage 2	14	13	27
Stage 3	20	3	23
Total	34	16	50
Percentage of raw total			
Stage 2	51.85%	48.15%	
Stage 3	86.96%	13.75%	
Percentage of column total			
Stage 2	41.18%	81.25%	
Stage 3	58.82%	18.75%	
Percentage of grand total			
Stage 2	28.00%	26.00%	
Stage 3	40.00%	6.00%	

and a standard error of 0.876. The findings indicated that *RUNX2* expression is markedly elevated in tumor cells (Mean Factor = 3.756), with approximately 3.5-fold higher expression in metastatic tumor cells compared to non-metastatic samples.

These results, supported by statistical analysis and relevant software, are consistent with numerous past studies regarding the increased expression of this gene in the aggressiveness of breast cancer and its progression to metastasis, particularly to bone.

Based on conducted studies, using markers to provide accurate prognoses has been widely discussed and investigated. For instance, research has shown that applying the HOXB13 marker is associated with a poor prognosis, as its overexpression induces tumor growth and increases its invasive potential in various types of tumors [23, 24].

Breast cancers frequently result in metastasis and frequently metastasize to the bone, where they induce osteoclasts to resorb bone and inhibit osteoblasts from producing new bone, resulting in osteolytic bone destruction [25]. Runt-related transcription factor 2 (Runx2) serves as a critical determinant of bone metastasis in breast cancer. Runx2 is recognized as a mediator of the activation of osteoclast activity and the inhibition of osteoblast differentiation by metastatic breast cancer cells [26].

In studies conducted by Daniel Mendoza et al., 2011 on human breast cancer cells, specifically the non-metastatic MCF7 cell line and the metastatic MDA-MB-231 cell line sourced from ATCC, a significant correlation was identified between the expression levels of the *RUNX2* gene and the progression of breast cancer cell metastasis. This study incorporated microarray analyses, validation of results through quantitative PCR (qPCR), and Western blotting. The laboratory findings of the present study are consistent with the results obtained by Mendoza et al., in 2011 [27].

Moreover, another study by George L. Barnes et al. in 2004, utilizing Western blot techniques, demonstrated that increased expression of the *RUNX2* gene facilitates a molecular phenotype that enables interactions between tumor cells and the bone microenvironment, leading to osteolytic disease. This finding provides evidence for the biological significance of the *RUNX2* gene product in promoting bone metastasis [28]. The results of their study are consistent with the findings of the present study and confirm the results obtained in this research.

In 2023, research conducted by Wang et al., published in the esteemed journal Frontiers in Oncology, revealed that elevated *RUNX2* expression plays a critical role not only in the progression of breast cancer metastasis but also in other cancer types. This study, which focused on gastric cancer cell lines (MKN-45 and AGS) as well as normal gastric epithelial mucous cells (GES-1), found that *RUNX2* is overexpressed, and knockdown of *RUNX2* significantly reduced proliferation, invasion, and migration of MKN-45 and AGS cells ($p < 0.05$) [29], and it indicates that not only is this gene important in breast tumor metastasis and invasiveness, but its elevated expression can also be examined in other types of cancers. The expression levels of *RUNX2* in the human gastric epithelial mucous cell line (GES-1) and gastric cancer cell lines (MKN-45 and AGS) were analyzed through q-RT-PCR and Western blotting. As depicted in Figure 1-4, both mRNA and protein levels of *RUNX2* are significantly elevated in MKN-45 and AGS cells, confirming the notable increase of *RUNX2* in gastric cancer, which parallels findings in breast cancer. This underscores the relevance of selecting *RUNX2* as a critical gene in the present study and other research endeavors.

In in vivo studies conducted by Huang, B. et al. in 2023, a positive correlation was demonstrated between *RUNX2* expression and bone degradation in mice bearing multiple myeloma. The results, with a p-value < 0.05, indicate that the inhibition of *RUNX2* may protect against bone destruction by maintaining the balance between osteoblast and osteoclast activity in multiple myeloma. This study involved 30 paraffin-embedded bone marrow samples and 15 fresh bone marrow samples from patients with multiple myeloma [30].

In another study – inconsistent with the findings of our study- by Yoshiaki Onodera in 2010, which involved the retrieval of patient records from 137 cases of invasive ductal carcinoma, significant associations were found between *RUNX2* expression and tumor stage ($p = 0.0004$), histological grade ($p = 0.046$), and HER2 status ($p = 0.002$). No significant correlation was observed between *RUNX2* expression and patient age ($p = 0.78$) or menopausal status ($p = 0.69$) [31].

Notably, research by Xiao-Qing et al. in 2016 demonstrated that increased expression of this gene promotes the aggressive behavior of breast cancer cells in metastatic processes, with bone identified as a key target for metastasis. They found a positive correlation between *RUNX2* expression and the risk of bone-specific metastasis in breast cancer patients with negative lymph nodes. Xiao-Qing and colleagues emphasized that *RUNX2* is a critical transcription factor for osteogenic lineage commitment, activating osteoblast-specific cis-acting elements, and is expressed in breast cancer cell lines with high metastatic potential to bone [32].

Furthermore, a significant association was observed between increased *RUNX2* expression and tumor stage in breast cancer patients ($p = 0.008$). The statistical analyses corroborate previous research findings in this area. Therefore, examining *RUNX2* expression levels could serve as a weak prognostic indicator, as noted in studies by Yang et al. [33], Leong et al. [34], and Gokulnath et al. [35]. Their results indicate that elevated and aberrant expression of *RUNX2* correlates significantly with poor prognosis in breast cancer, which also can confirm our results.

Additionally, the present study examined *RUNX2* expression across different tumor grades in breast cancer. *RUNX2* expression was notably higher in grade 3 tumors compared to grade 2 tumors. A strong correlation was observed between elevated *RUNX2* expression levels and increasing tumor grade ($p = 0.0067$), consistent with previous studies findings, and Yoshiaki Onodera and their colleagues obtained results as before mentioned (their results published in the Cancer Science Journal, in 2010, Ref; 32).

Given that prior research has established the role of *RUNX2* as a crucial transcription factor in osteogenic differentiation, its aberrant expression is critically involved in the aggressiveness and progression of breast carcinoma to bone [36]. Therefore, maintaining balanced *RUNX2* expression is vital in preventing the aggressiveness of breast tumor cells and tumor formation in other body regions, particularly in bones [37]. Studies have indicated that *RUNX2* expression also in a more aggressive breast cancer subgroup, especially in triple-negative cases, is associated with drug resistance and estrogen receptor signaling pathways [38].

Notably, increased and decreased *RUNX2* expression can lead to reduced osteoclast differentiation, facilitating the progression of bone metastasis and the formation of secondary tumors. Given the key role of *RUNX2* in the differentiation of bone cells, investigating this gene may serve as an appropriate indicator for examining hidden metastases in tumor cells and lymph nodes of patients who have not yet exhibited identifiable pathological changes. Future follow-up studies could further clarify the accuracy and sensitivity of this molecular assessment.

In conclusion, *RUNX2* is a crucial factor regulating various physiological and pathological conditions. Numerous studies have demonstrated a correlation between elevated *RUNX2* expression levels and poor prognosis across a range of cancers. For health care worker, it can be informative that increased *RUNX2* expression enhances the potential for breast cancer development and metastasis in patients with breast cancer.

Author Contribution Statement

AM: is the principal investigator in the study, MR: laboratory manipulation, MR and NM: Concept, Data acquisition, Data analysis, Manuscript preparation. AAK: Concept, Research. ZS, RG: Literature search. EPZ: Manuscript editing. SS and MM: Manuscript formatting

according to the Journal requirement. MT: contributed to drafting preliminary. All the authors contributed to the critical review, the approval of the final version and agreed to be responsible for all aspects of the work.

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Approval

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Ethical declaration

Shahid Beheshti University of Medical Sciences. Tehran Iran.

Conflicts of Interest

None.

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