The Transcription Factor ZEB-1 Conveys Chemoresistance in Breast Cancer

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Abstract:

Background: A transcription factor called zinc finger E-box binding homeobox 1 (ZEB-1) controls tissue-specific processes and cell differentiation. In numerous human cancer types, including breast, pancreatic, osteosarcoma, lung, liver, gastric, colon, and uterine cancers, aberrant expression of ZEB-1 has been reported. The aim of this study was to assess the prognostic role of immunohistochemical expression of ZEB-1 transcription factor in breast cancer.

Material and method: This retrospective study investigated the prognostic role of immunohistochemical expression of ZEB-1 in breast cancer patients. It included 63 cases of newly diagnosed breast cancer patients, presented and diagnosed at South Egypt Cancer Institute, Assiut University between January 2019 and December 2021. All patients were stained with anti ZEB-1 antibody. Patients followed up for at least 24 months.

Results: There was a significant association detected between high ZEB-1 expression and poor response to the first line chemotherapy, development of metastasis (P value <0.001) {42.2% of patients with high ZEB-1 expression progressed with newly developed distant metastasis after receiving adjuvant treatment } and disease progression especially disease progression with bone metastasis in the studied breast cancer cases (P value= 0.006 as six cases with high ZEB-1 expression progressed with newly developed bone metastasis after receiving adjuvant treatment }. There was a significant association detected between low ZEB-1 expression and increased disease free survival (P value= 0.001 as 92% of patients with low ZEB-1 expression were disease free during the whole period of follow up). There was a significant association detected between diagnosis with advanced tumor stage and decreased disease free survival (P value=0.023) and also with decreased overall survival (P value=0.042) of the studied breast cancer cases. There was a significant association detected between diagnosis with multicenteric tumor type &/or presence of Paget`s disease and decreased overall survival of the studied breast cancer cases with (P value =0.018) and (P value=0.014) respectively as patients with multicenteric tumor &/or patients with paget's disease shown to have decreased overall survival when compared with patients with unicenteric tumor &/or patients without paget's disease. However, there was no significant association found between high ZEB-1 expression and clinicopathological data, hormonal profile or overall survival (P value=0.009) of the studied breast cancer cases.

Conclusions: High ZEB-1 protein expression was a poor predictive marker of disease-free survival in breast cancer patients (P value=0.001). Also, there was a pivotal role of high ZEB-1 expression in disease progression and development of metastasis (P value<0.001) especially progression with bone metastasis among breast cancer patients (P value=0.006).

Keywords: Breast Cancer, Immunohistochemical Expression, ZEB-1 Transcription Factor.

Received: 3 April 2024
Accepted: 12 May 2024

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Background:

Worldwide, breast cancer is the most common cancer and leading cause of cancer-related death for women [1]. It is also the second most common cause of mortality for women in the United States, after lung cancer. In 2022, 43,250 women died from breast cancer, out of an estimated 287,580 new cases [2].

Global Cancer Observatory (GLOBOCAN) estimated that breast cancer is the most common cancer in Egypt and the second most common cause of cancer-specific mortality in Egypt after liver cancer [3].

Breast cancer continues to be the most common cause of cancer-related deaths among women in the developed world, despite advancements in early diagnosis and treatment [4].

Disease recurrence and disseminated malignant cells appear to be able to evade adjuvant treatments and remain dormant before reactivating and triggering disease relapse many years after diagnosis, which contributes to the common late recurrence of the disease [5].

ZEB-1 protein is involved in the differentiation of several tissues, such as neuronal, smooth muscle, and bone tissue. Numerous human malignancies, including those of the breast, pancreas, lung, liver, and colon cancer have been linked to abnormal ZEB-1 expression [6]. Furthermore, elevated ZEB-1 expression increased cancer resistance to chemotherapy and radiotherapy, suggesting that ZEB-1 markedly affect cancer prognosis in addition to its important role in the genesis and progression of cancers. New data also suggests that ZEB-1 plays a significant role in therapeutic resistance [7].

The aim of this work was to evaluate the prognostic role of immunohistochemical expression of ZEB-1 transcription factor in breast cancer.

Material and Method:

Study cohort

This is a retrospective study including 63 cases of newly diagnosed breast cancer patients, presented and diagnosed at South Egypt Cancer Institute, Assiut University between January 2019 and December 2021. All data were collected from the database registry at the institute. Inclusion criteria were patients with pathological diagnosis of breast cancer aged more than 18 years with available clinicopathological data and follow up data for at least 24 months. Exclusion criteria were having pathological diagnosis other than breast cancer or male breast cancer. This study was registered and approved by ethical committee of South Egypt Cancer Institute (Approval No: 590).

Histological review

All available hematoxylin & eosin and Immunohistochemistry stained slides from the study cohort were retrieved and reviewed by expert pathologist to confirm diagnosis.

Immunohistochemistry (IHC)

Five formalin fixed paraffin-embedded full-faced tissue sections were cut at 4 μm thick and mounted on coated positive charged purchased glass slides. The slides were heated in the oven for 2 hours at 95ºC Deparaffinization was then occurs by immersion in Xylene twice (10 minute for each) and rehydrated through graded alcohols (absolute, 90%, 80% and 70%; 10 seconds each) and then rinsed with distilled water. Heat induced epitope retrieval method was conducted, where tissue sections were immersed in an unsealed plastic container (Coplin jars) filled with sufficient amounts of antigen retrieval solution (Dako EnVision™ FLEX Target Retrieval Solution, Citrate buffer, low PH 6.1(50x) (Code DM829). The slides were then applied for microwave oven for 12 minutes (for three successive cycles, 4 minutes each). Slides were allowed to cool at room temperature. Slides were then washed 2-3 times with diluted phosphate buffered saline (PBS) using Dako EnVsion™ FLEX Wash Buffer (20x) (Code DM831). The slides were dried out except tissue section part. Antigen retrieval was done by using Dako EnVision™ FLEX Target Retrieval Solution, Citrate buffer, low PH 6.1(50x) (Code DM829). Blocking of endogenous peroxidase activity was performed using Dako EnVision™ FLEX peroxidase Blocking Reagent (Code SM801), applied and incubated for 5-10 minutes at room temperature. The primary antibody against ZEB-1 was diluted using ZEB-1 Rabbit pAb (Cat.NO: A16981) at concentration 1:300 and was added to the sections and incubated for 24 hours at -4C. The slides were then washed 2-3 times using PBS solution. After washing, the secondary antibody was applied for 20 min at room temperature using Dako EnVision™ FLEX HRP (Horseradish peroxidase) (Code SM802), then rinsed and washed with PBS 2 times. Diaminobenzidine (DAB solution) was applied to the slides for 5- 10 minutes using Dako EnVision™ FLEX DAB (Code DM827). Sections were then counter stained with haematoxylin and mounted with Dibutyl Phthalate Xylene (DPX).

IHC evaluation of ZEB-1:

ZEB-1 immunohistochemical expression was detected in the nucleus and cytoplasm of tumor cells in 63 cases of newly diagnosed breast cancer patients. Evaluation of ZEB-1 expression by immunohistochemistry was done on patients' blocks. Thirty five cases showed low ZEB-1 expression and twenty eight cases showed high ZEB-1 expression. H-score was achieved by semi-quantitative assessment of both the intensity (classified as absent (0), weak (+1), moderate (+2) and strong positive (+3)) and percentage of positive cells according to the following formula:

H-score = 1 x (% cells 1+) +2 x (% cells 2+) + 3 x (% cells 3+). The cut off value of H-score was calculated according to the median value of H-score which was 100. Cases were categorized as low H-score < 100, high H-score > or equal 100.

H-score for ZEB- 1 expression was calculated for the overall tumor of each case. For each case, the fields with higher percentage of stained tumor cells were used for the analysis. The positivity was identified in the nucleus and cytoplasm staining for ZEB-1.

The primary end point was assessment of the relation between ZEB-1 expression and prognosis of breast cancer patients. The secondary end point was assessment of the progression free survival (PFS) and

overall survival (OS) among breast cancer patients in relation to ZEB-1 immunohistochemical expression.

All patients were subject to: complete laboratory investigation (complete blood count, liver function test and renal function test), imaging study (Chest x-ray, abdominal ultrasound, bilateral sonomamography, CT or MRI according to stage), histopathological examination, ZEB-1 immunohistochemical expression evaluation, full histological data and hormonal receptors data.

Follow up was done for patients clinically and radiologically using Chest x-ray, abdominal ultrasound, bilateral sonomamography, CT or MRI according to stage.

Statistical analysis

Statistical analysis was done by SPSS v22 (IBM Inc., Chicago, IL, USA). Quantitative data were statistically described in terms of mean ± SD and median (range) when not normally distributed. Qualitative data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages) when appropriate. Comparison of quantitative variables was done using student t test. For comparing categorial data, Chi square ($\chi 2$) test was performed. Fisher Exact test was used instead when the expected frequency is less than 5. Kaplan-Meier's method with log rank test was used for calculation of disease free and OS analysis. Odds ratio (OR) with 95% Confidence Interval (CI) among the studied breast cancer cases. P-value is always 2 tailed set significant at 0.05 level.

Results and Discussion:

ZEB-1 immunohistochemical expression was evaluated in 63 cases of breast cancer patients and it was detected in the nucleus and cytoplasm of tumor cells. Twelve cases of them showed strong ZEB-1 expression, 26 cases showed moderate ZEB-1 expression, 13 cases showed weak ZEB-1 expression and 12 cases showed negative ZEB-1 expression. Figure 1.

Staining of ZEB-1 was detected in malignant epithelial cells and in stromal cells. However, assessment was done on epithelial malignant cells only. There was no significant association found between ZEB-1 expression and clinicopathological data of the studied breast cancer cases. Table 1

There was no significant association detected between ZEB-1 expression and hormonal profile of the studied breast cancer cases. Table 2

There was a significant association detected between high ZEB-1 expression and poor response to the first line chemotherapy, development of metastasis (P value <0.001) {42.2% of patients with high ZEB-1 expression progressed with newly developed distant metastasis after receiving adjuvant treatment } and disease progression especially disease progression with bone metastasis in the studied breast cancer cases (P value= 0.006 as six cases with high ZEB-1 expression progressed with newly developed bone metastasis }. Table 3

There was a significant association detected between low ZEB-1 expression and increased disease free survival (P value= 0.001 as 92% of patients with low ZEB-1 expression were disease free during the whole period of follow up), Patients with high ZEB-1 expression showed to have shorter disease free survival when compared with patients who have low ZEB-1 expression. Figure 2

Also, there was a significant association detected between diagnosis with advanced tumor stage and decreased disease free survival (P value=0.023). Patients diagnosed with early tumor stage have prolonged disease free survival when compared with patients diagnosed with advanced tumor stage. Figure 3

There was no significant association detected between ZEB1 expression and OS of the studied breast cancer cases (P value=0.09). Figure 4 There was a significant association between OS and tumor stage, type of tumor whether unicenteric or multicenteric and presence of Paget`s disease of the studied breast cancer cases. Patients diagnosed with early tumor stage have better OS when compared with patients diagnosed with advanced tumor stage (P value=0.042). Figure 5

Patients diagnosed with unicenteric tumor have better OS when compared with patients diagnosed with multicenteric tumor (P value=0.018). Figure 6

Patients diagnosed with Paget`s disease have worser OS when compared with patients who are not diagnosed with Paget`s disease (P value =0.014). Figure 7

Previous studies have reported that ZEB-1expression considered to be an indication of unfavorable clinical factors, such as larger tumor size, more lymph node metastasis and higher tumor stage, in breast cancer [6,8]. Another study showed that the ZEB-1 expression was related to patient age and menstrual status of the studied BC cases [9]. However, in the current study ZEB-1 expression was not related to the demographic and clinical details of the studied breast cancer cases. This difference could be contributed to difference in the inclusion criteria of the studied cases, as we included breast cancer cases admitted to our institution during the study period who received treatment protocol with adjuvant chemotherapy, while the patients included in Wu et al. study were locally advanced breast cancer and received neo-adjuvant therapy (NAT) only [9].

ZEB-1 was found to be associated with multiple chemoresistant genes, including ATM, CD4, and PIM3 which evidenced in a preclinical investigation done by Zhang [10]. Both in vitro and in vivo, ZEB-1 expression was linked to a chemoresistant tumor phenotype. ZEB-1 was also found to enhance radioresistance and has an important role in DNA damage response [7]. All of this molecular evidence points to ZEB-1 ability to reduce a tumor sensitivity to cytotoxic therapy, which may account for the fact that patients with high ZEB-1 expression levels are less

likely to respond to chemotherapy than those with low ZEB-1 expression levels [7].

Furthermore, it was found in this study that individuals with breast cancer with high ZEB-1 expression had a poor prognostic indicator of disease-free survival (DFS). In our study, elevated ZEB-1 expression was also a strong indicator of poor overall survival (OS) for patients with breast cancer, but it had not yet reached statistically significant results (P=0.090).

Our result was supported by Min, who reported that ZEB-1 protein expression was associated with poor survival in TNBC [11], Ang et al study which showed that ZEB-1 and ZEB-2 mRNA expression as well as protein expression were associated with poor survival in breast cancer [6], and the recent study of Wu et al. who reported that ZEB-1 expression was a significant indicator of poor survival in breast cancer patients [9]. The predictive role of ZEB-1 is also validated in other tumors, such as ovarian carcinoma [12], oral cavity squamous cell carcinoma [13] and hepatocellular carcinoma [14].

The current study demonstrated that ZEB-1 expression is involved in tumor metastasis. This finding was supported by the recent study of Zhang et al. who demonstrated that ZEB-1 expression has been identified as a key factor in the regulation of breast cancer differentiation and metastasis [10]. Similarly, Wu et al. stated that the aberrant expression of ZEB-1 is thought to be connected with tumorigenesis and poor prognosis in various tumors, especially in breast cancer [15].

Fu et al. recently demonstrated the high expression and activation of ZEB1 in the stroma was associated with increased ECM remodeling, immune cell infiltration, and angiogenesis through increasing VEGF and IL-6 expression and secretion into the surrounding stroma [16]. This finding highlighted the critical role of the ZEB/ p53 axis in stromal fibroblasts to promote mammary epithelial tumors.

In the current study we observed that the six BC cases who developed bone metastasis had high ZEB-1 expression. This finding demonstrated a pivotal role of ZEB-1 expression in development of bone metastasis among breast cancer patients.

Mohammadi Ghahhari et al. obtained a similar result. The author explained this observation by pointing out that ZEB-1 affects ER receptor-mediated transcription caused by estrogen signaling in breast cancer cells following the induction of EMT when the cells are still epithelial. About 70% of all cases of breast cancer are ER positive, and ER receptor is essential to the development of this type of cancer [18].

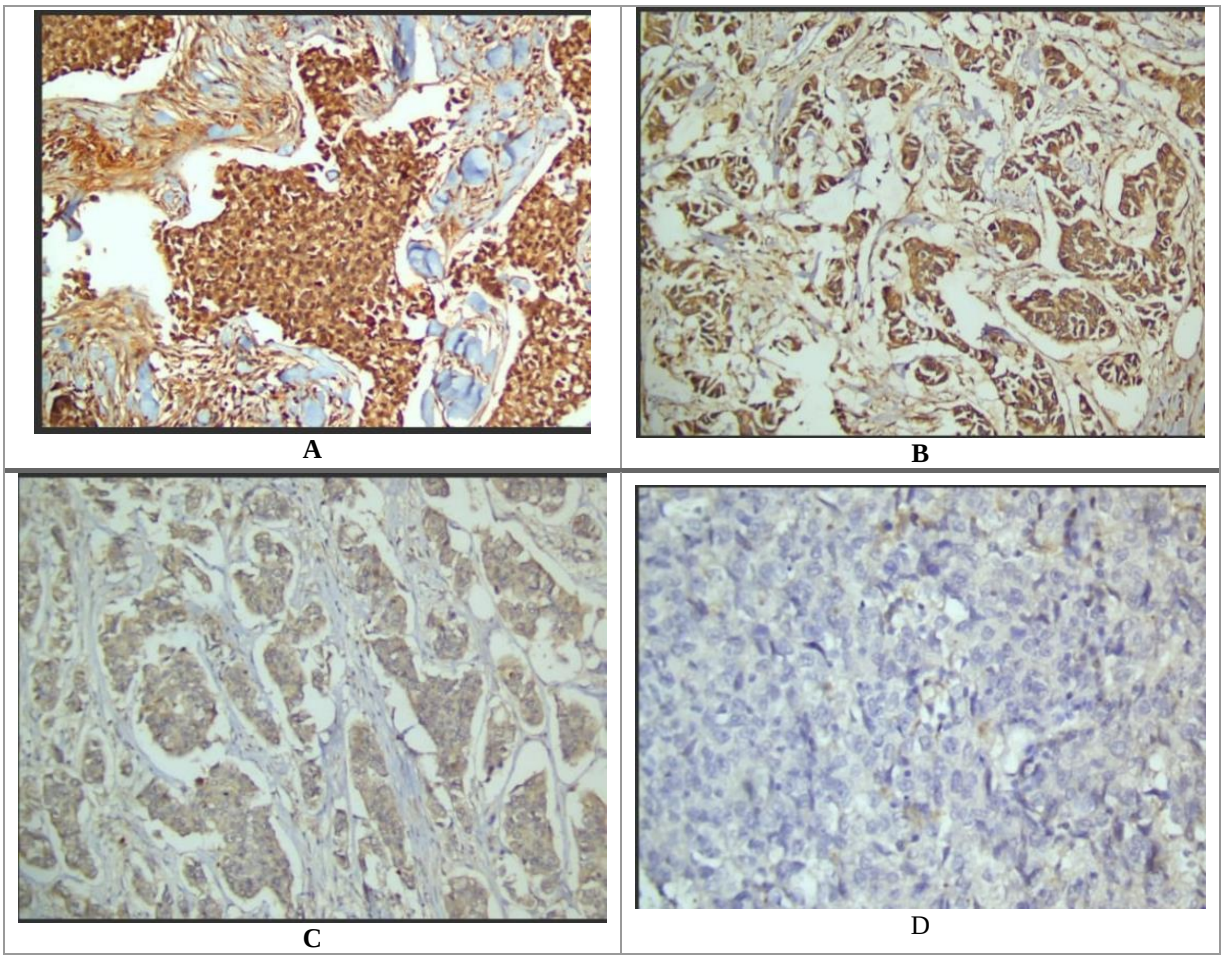

Figure (1): A case that shows (A) strong (B) moderate (C) weak (D) negative ZEB-1 expression

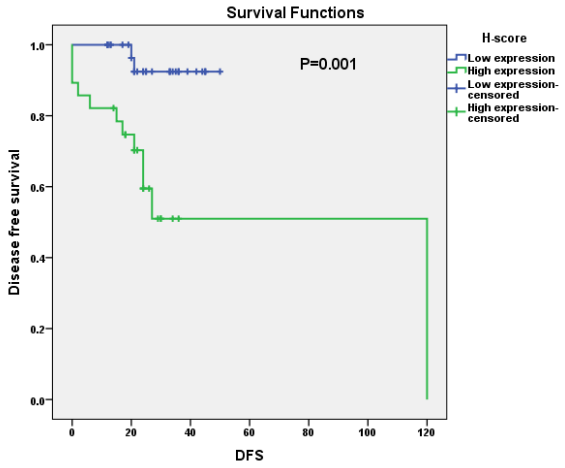

Figure (2): Kaplan Meier curve showing the disease free survival of the studied breast cancer cases according to the H-score expression.

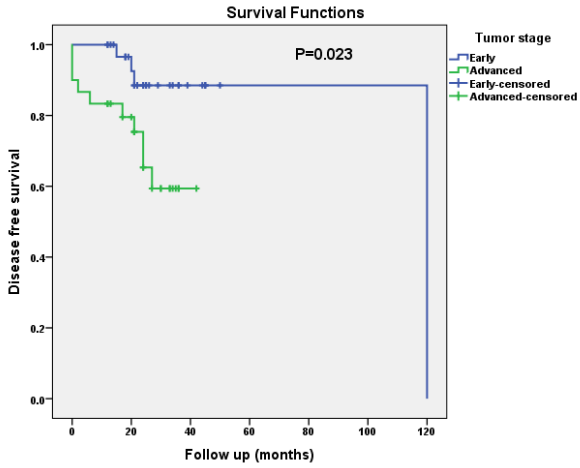

Figure: (3) Kaplan Meier curve showing the disease free survival of the studied breast cancer cases according to the tumor stage.

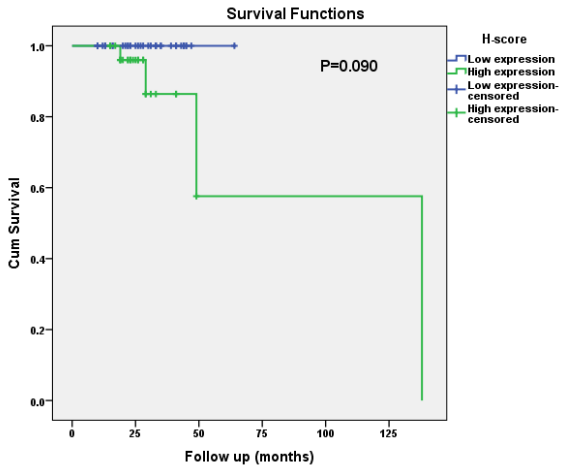

Figure (4): Kaplan Meier curve showing the overall survival of the studied breast cancer cases according to the H-score expression.

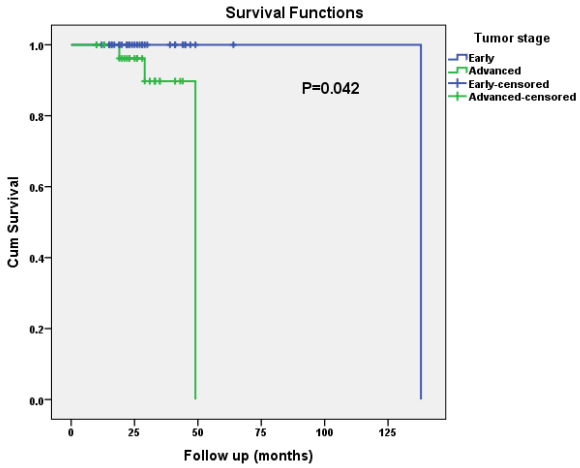

Figure (5): Kaplan Meier curve showing the overall survival of the studied breast cancer cases according to the tumor stage.

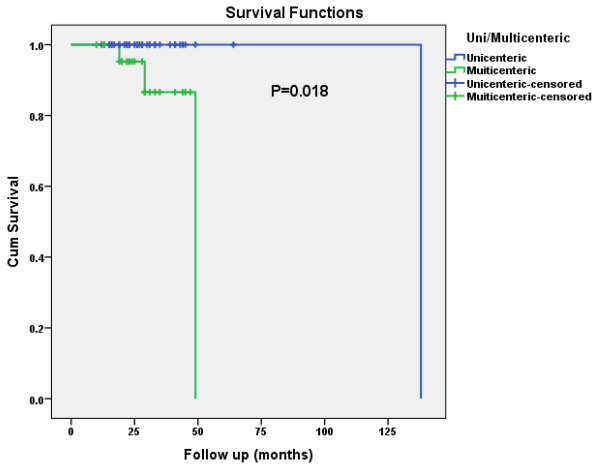

Figure (6): Kaplan Meier curve showing the overall survival of the studied breast cancer cases according to the tumor location (uni vs multicenteric).

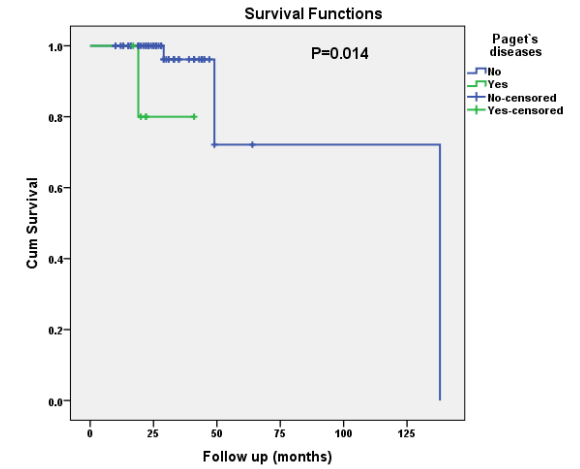

Figure (7) Kaplan Meier curve showing the overall survival of the studied breast cancer cases according to the presence of Paget disease.

Table 1: Association between ZEB-1 expression and clinicopathological data of the studied breast cancer cases

Clinicopathological data	Low expression (n=35)		High expression (n=28)		P value
Age (years)					
• Mean ± SD	53.69 ± 11.88		51.14 ± 12.14		0.139
• Median (range)	55 (27 – 77)		50 (30 – 71)		
• < 50 years	11	(31.4%)	14	(50.0%)	0.134
• ≥ 50 years	24	(68.6%)	14	(50.0%)	
Menopausal status					0.952
• Premenopausal	11	(31.4%)	9	(32.1%)	
• Postmenopausal	24	(68.6%)	19	(67.9%)	
Tumor stage					0.735
• Early	19	(54.3%)	14	(50.0%)	
• Advanced	16	(45.7%)	14	(50.0%)	
TNM staging (T)					0.117
• T1 + T2	33	(94.3%)	20	(80.0%)	
• T3 + T4	2	(5.7%)	5	(20.0%)	
TNM staging (N)					0.963
• Negative	11	(31.4%)	8	(32.0%)	
• Positive	24	(68.6%)	17	(68.0%)	
LVI					0.843
• No	8	(22.9%)	7	(25.0%)	
• Yes	27	(77.1%)	21	(75.0%)	
Tumor grade					0.321
• Grade 2	25	(71.4%)	23	(82.1%)	
• Grade 3	10	(28.6%)	5	(17.9%)	
DCIS					0.955
• No	16	(45.7%)	13	(46.4%)	
• Yes	19	(54.3%)	15	(53.6%)	
Lymphoplasmacytic infiltration					0.572
• No	20	(57.1%)	14	(50.0%)	
• Yes	15	(42.9%)	14	(50.0%)	
Perineural invasion					0.283
• No	21	(60.0%)	13	(46.4%)	
• Yes	14	(40.0%)	15	(53.6%)	
Necrosis					0.573
• No	24	(68.6%)	21	(75.0%)	
• Yes	11	(31.4%)	7	(25.0%)	
Uni or Multicenteric					0.223
• Unicenteric	24	(68.6%)	15	(53.6%)	
• Muiticenteric	11	(31.4%)	13	(46.4%)	
Paget`s diseases					0.080
• Absent	34	(97.1%)	23	(82.1%)	
• Present	1	(2.9%)	5	(17.9%)	

Table 2: Association between ZEB-1 expression and hormonal profile of the studied breast cancer cases

Hormonal profile	Low expression (n=35)		High expression (n=28)		P value
ER					0.092
• Negative	7	(20.0%)	11	(39.3%)	
• Positive	28	(80.0%)	17	(60.7%)	
PR					0.806
• Negative	11	(31.4%)	8	(28.6%)	
• Positive	24	(68.6%)	20	(71.4%)	
Her2neu					0.448
• Negative	30	(85.7%)	26	(92.9%)	
• Positive	5	(14.3%)	2	(7.1%)	
Triple negative BC					0.517
• Negative	30	(85.7%)	22	(78.6%)	
• Positive	5	(14.3%)	6	(21.4%)	
Molecular subtypes					0.943
• Luminal A	23	(65.7%)	17	(60.7%)	
• Luminal B	5	(14.3%)	4	(14.3%)	
• Her2neu overexpression	2	(5.7%)	1	(3.6%)	
• Triple negative	5	(14.3%)	6	(21.4%)	

Table 3: Association between ZEB-1 expression and response to therapy of the studied breast cancer cases

Lines of treatment	Low expression (n=35)		High expression (n=28)		P value
1st line					0.063
• Adjuvant	31	(88.6%)	19	(67.9%)	
• Neoadjuvant	4	(11.4%)	6	(21.4%)	
• Palliative	0	(0.0%)	3	(10.7%)	
Type of 1st line					1
• CTR	32	(91.4%)	26	(92.9%)	
• Hormonal	3	(8.6%)	2	(7.1%)	
RTH					0.892
• No	8	(22.9%)	6	(21.4%)	
• Yes	27	(77.1%)	22	(78.6%)	
Response to 1st line					**<0.001**
• Maintenance of CR after surgery	33	(94.3%)	16	(57.1%)	
• Progression	2	(5.7%)	12	(42.9%)	
Metastasis					**<0.001**
• No	33	(94.3%)	16	(57.1%)	
• Yes	2	(5.7%)	12	(42.9%)	
Recurrence					
• No	34	(97.1%)	26	(92.9%)	0.580
• Yes	1	(2.9%)	2	(7.1%)	
Bone					**0.006**
• No	35	(100.0%)	22	(78.6%)	
• Yes	0	(0.0%)	6	(21.4%)	
Brain					0.444
• No	35	(100.0%)	27	(96.4%)	
• Yes	0	(0.0%)	1	(3.6%)	
Lung					0.162
• No	34	(97.1%)	24	(85.7%)	
• Yes	1	(2.9%)	4	(14.3%)	
Liver					0.082
• No	35	(100.0%)	25	(89.3%)	
• Yes	0	(0.0%)	3	(10.7%)	
Peritoneal					0.194
• No	35	(100.0%)	26	(92.9%)	
• Peritoneal LN	0	(0.0%)	1	(3.6%)	
• Supraclavicular LN	0	(0.0%)	1	(3.6%)	

Tumors are often become more sensitive to endocrine therapy when the ER receptor is overexpressed. Activation of the ER receptor in response to estrogen promotes downstream signaling pathways, which results in EMT and ECM remodeling. By turning on the PI3K/AKT signaling pathway, estrogen promotes the growth of breast cancer in patients with ER-positive disease. Overall in all, ER receptor contributes significantly to the development of ER-positive breast cancer [17].

These results lead Mohammadi Ghahhari et al. to hypothesize that the tissue tropism of metastatic breast cancer cells towards bone may be modified by the functional interaction between ZEB-1 and ER receptor [18]. Therefore, through examining the transcriptional activities that are interdependent between ZEB1 and the ER receptor, researchers may reveal novel pathways through which ZEB-1 promotes the growth of tumors and the invasion of ER receptor-positive breast cancer cells.

Limitation: The study had a relatively small sample size and was conducted at a single center. As a result, we were unable to complete subgroup analysis in different subtypes of breast cancer. Because we rely on the patients' medical records to gather the necessary data, the retrospective study design offers a lower quality of evidence and is subject to recall bias. We thus rely on the accuracy of the data that has been recorded.

Conclusion:

High ZEB-1 protein expression was a poor predictive marker of disease-free survival in breast cancer patients (P value=0.001). Also, there was a pivotal role of high ZEB-1 expression in disease progression and development of metastasis (P value<0.001) especially progression with bone metastasis among breast cancer patients (P value=0.006).

List of abbreviations used:

ATM: Ataxia telangiectasia mutated gene
BC: Breast cancer
CD4: Cluster of differentiation 4
CI: Confidence Interval
DFS: Disease-free survival
ECM: Extracellular matrix
EMT: Epithelial to mesenchymal transition
ER: Estrogen receptor
IHC: Immunohistochemistry
IL-6: Interleuken-6
NAT: Neo-adjuvant treatment
OR: Odds ratio
OS: Overall survival
PFS: Progression-free survival
PIM3: Provirus-integrating Moloney site 3
PI3K/AKT: Phosphoinositide-3-kinase-protein kinase /AKT
TNBC: Triple negative breast cancer
P53: Tumor protein p53
VEGFR: Vascular endothelial growth factor receptor
ZEB-1: Zinc finger E-box binding homeobox 1

Conflict of Interest: None

Authors' contributions: All authors significantly contributed to the conception or design of the work, as well as to the collection, processing and interpretation of the data.

Acknowledgements: None

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