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Doublecortin like Kinase-1 is Overexpressed in Breast Cancer Tissues and Correlated with Epithelial-mesenchymal Transition Markers

Rasha S. Abo El Alaa[1], Samia A. Ebeid[1], Nadia A. Abd El Moneim[2], Sanaa A. El-Benhawy[3,*], Ahmed S. Ahmed[4]

[1]Applied Medical Chemistry Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[2]Cancer Management and Research Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[3]Radiation Sciences Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
[4]Experimental and Clinical Surgery Department, Medical Research Institute, Alexandria University, Alexandria, Egypt
*Corresponding author: dr_sanaa_ali13@yahoo.com

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Abstract **Background:** Much of the current literature support, the idea that epithelial to mesenchymal transition (EMT) is the key mechanism by which tumor cells gain invasive and metastatic ability, as EMT enables separation of individual cells from the primary tumor mass as well as promote migration. After undergoing EMT, thereby enabling access to hematogenous or lymphatic routes of dissemination, tumor cells can extravasate into secondary organs and establish micro-metastases. **Objective:** The main target of this work was to study the relation between expression of Doublecortin-like kinase-1 (DCLK-1) and epithelial to mesenchymal transition markers (E-cadherin, vimentin and transforming growth factor-beta (TGF-β)) in breast cancer patients and assess its role in cancer prognosis. **Materials and Methods:** This study included 60 breast cancer patients and 40 healthy females as control group. Tumor tissues and adjacent normal breast tissues were collected from patients with breast cancer. A single venous blood sample was collected concurrently from breast cancer patients (Before surgery) and from the control group. Tissue expression of DCLK-1, E-cadherin and vimentin were evaluated in breast cancer tissues and normal breast tissues by real-time reverse transcription polymerase chain reaction (RT-PCR). Serum level of TGF-β was assayed by enzyme linked-immunossorbant assay. **Results**: According to the results of the present study DCLK-1 is highly expressed in breast cancer tissues compared to normal breast tissues. Overexpression of DCLK-1 was significantly correlated with higher tissues Vimentin expression, increased serum TGF-β and lower tissues E-cadherin expression. Kaplan-Meier survival curves for breast cancer patients revealed that, patients with elevated tissue DCLK-1 and Vimentin expression and higher serum TGF-β were significantly associated with poor prognosis in primary breast cancer patients. However, patients with lower tissue E-Cadherin expression had shorter disease free survival time than patients with higher levels. **Conclusion:** DCLK-1 was significantly increased in breast cancer tissues in comparison to normal breast tissues and its overexpression was significantly correlated with EMT markers and poor prognosis in breast cancer patients. Further prospective studies using greater numbers of patients are required to confirm our findings.

Keywords: *breast cancer, DCLK-1, EMT markers*

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1. Introduction

Breast tumors are well known to be composed of phenotypically diverse groups of cells; however, it is unclear which of these cell types contribute to tumor development. [1] In contrast to the hypothesis that all cell populations have the capacity to become tumorigenic through accumulation of mutations, another hypothesis limits this ability to an elite group of cells that share classic features of cancer stem cells (CSCs). [2]

Doublecortin-like kinase 1 (DCLK-1) is a member of the protein kinase superfamily and the doublecortin family, and marks colon and pancreatic cancer stem cells. [3,4] DCLK-1 is a cancer stem cell marker overexpressed in many cancers, including colon, pancreas, [5] liver, kidney, [6] and esophageal cancer. [7] Studies showed that DCLK-1 expression is critical for cancer growth, epithelial to mesenchymal transition (EMT), metastasis, and cancer cell self-renewal. [8,9] The functional interdependence between EMT-associated transcription factors and enhanced self-renewal highlights the common mechanism involved in their regulation. Therefore, highlighting the regulatory

role of DCLK-1 supporting EMT and CSCs will enhance the understanding of drug targets and help to design novel and effective targeted therapies. [10]

Epithelial-mesenchymal transition has been considered to be one of the critical steps involved in cancer metastasis. [11] Core elements of EMT include reduction of cell-cell adherence via the transcriptional repression of cadherins, and functional loss of E-Cadherin is a well-known hallmark of EMT. Expression of epithelial intermediate filaments is typically reduced and the equivalent mesenchymal filament protein vimentin increased. [12]

Epithelial-mesenchymal transition can be triggered by a diverse set of stimuli that includes growth factor signaling, cytokines, and tumor-stromal cell interaction. [13] The most classical experimental model is the induction of EMT by transforming growth factor-beta (TGF-β) in epithelial cell culture. Upon TGF-β induction, the type II receptor (TGFR2) is activated and phosphorylates the type I receptor (TGFR1), thereby activating the Smad pathway and inducing EMT. [14] Recently, it has also been reported that TGF-β can induce the CSC phenotype and cause EMT in vitro by up-regulation of Oct-4, Nanog, N-cadherin, Vimentin, Slug and Snail, and down-regulation of E-cadherin. [15]

Understanding the molecular mechanisms responsible for EMT-mediated drug resistance and tumor metastasis will be essential for the discovery of new strategies to prevent EMT and restore the sensitivity of cancer cells to therapeutic treatment. [13]

The main target of this work was to investigate the relation between expression of Doublecortin-like kinase-1 and epithelial to mesenchymal transition markers (E-cadherin, vimentin and TGF-β) in breast cancer patients and assess its role in cancer prognosis.

2. Patients and Methods

This study included 60 female patients newly diagnosed with breast cancer as the patient group and 40 healthy females with normal mammography findings and no previous history of any kind of cancer as control group, matched for age and menopausal status with patients group. Patients were selected from those admitted to the Department of Cancer Management and Research, Medical Research Institute, Alexandria University. A written consent for participating in the study was taken according to the Declaration of Helsinki and approved by the Ethical Committee of the Medical Research Institute (IOROH: IORG 0008812). Metastatic patients at diagnosis and patients receiving chemotherapy or radiotherapy before surgery were excluded from this study.

Patients were subjected to preoperative evaluation that included history taking, clinical examination to detect the tumor site and the presence of enlarged axillary lymph nodes. Radiological investigations included mammogram, abdominal ultrasound and chest x-ray. Preoperative investigations also included fine needle aspiration cytology (FNAC) to diagnose the presence of malignancy. Patients were subjected to surgery (modified radical mastectomy or conservative surgery). Postoperative pathological evaluation of the tumor included type of tumor, grade, size of the tumor, numbers of axillary lymph nodes involved, and presence or absence of vascular invasion. Assessment of estrogen, progesterone receptors (ER, PR) and Her2/neu expression were also confirmed.

2.1. Tissue and Blood Samples Collection

Tumor tissue and adjacent normal breast tissue were collected from each patient with breast cancer and stored at -80°C until used. A total of 5 ml fasting venous blood sample was drawn from each control subject and within a week before surgery for breast cancer patients. Blood samples were allowed to clot for 30 minutes before centrifugation, centrifuged at 3000 rpm for 10 minutes to isolate sera. The serum was stored at -80°C until used. Serum levels of TGF-β were measured by enzyme linked immunosorbent assay (ELISA) according to the manufacturer's instructions (eBioscience, USA).

2.1.1. Real-time Reverse Transcription PCR

Total RNA was extracted from breast cancer tissues and adjacent normal breast tissues using a highly denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA (RNeasy Mini Kit, Qiagen, Germany). Total RNA reverse-transcription and amplification reactions taking place sequentially in the same tube in 25 µL final volume of reaction mix using one step Rotor Gene SYBR Green PCR Kit (Qiagen, Germany) according to manufacturer's protocol. All primers used in this study were purchased from Qiagen (QuantiTect Primer Assay). Expression of mRNA of DCLK-1, E-cadherin, vimentin and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) genes was determined by relative quantitation method using GAPDH as an endogenous control gene.

2.2. Statistical Analyses

Data were fed to the computer and analyzed using IBM SPSS software package version 20. (Armonk, NY: IBM Corp). The differences between groups were determined by the two sided chi-square test and Mann Whitney test. Correlations between quantitative variables were assessed using Spearman correlation coefficient. Kaplan-Meier curves were used to calculate disease free survival (DFS) using log-rank test. We defined DFS as the time between the date of diagnosis and the date of unfavorable outcome including local recurrence, distant metastasis or contralateral breast cancer. Significance of the obtained results was judged at the 5% level.

3. Results

3.1. Clinico-pathological Characteristics of Breast Cancer patients

Clinico-pathological characteristics for breast cancer patients were represented in Table 1.

3.2. Doublecortin like kinase-1, E-cadherin and Vimentin Genes Expression in Normal and Tumor Tissues

Statistical analysis of relative quantification of DCLK-1, E-cadherin and Vimentin genes in normal and breast cancer tissues were shown in Table 2 and illustrated in Figure 1 – Figure 3. As presented in Table 2, DCLK-1 expression was significantly higher in breast cancer tissues in comparison to normal breast tissues ($p<0.001$). The same is true for vimentin gene expression ($p=0.001$). Regarding relative quantification of E-cadherin gene, it was significantly lower in cancer tissues than that in adjacent normal tissues ($p=0.015$).

Table 1. Clinico-pathological characteristics of breast cancer patients

	Breast cancer patients (n=60)
Age Range Mean±SD	 30-87 56.63±13.36
Histological Grade	
II III	46 (76.6%) 14 (23.3%)
Clinical Stage	
I-II III	30 (50.0%) 30 (50.0%)
Tumor size	
≤5 >5	48 (80.0%) 12 (20.0%)
Axillary lymph node involvement	
Positive Negative	44 (73.3%) 16 (26.7%)
ER status	
Positive Negative	42 (70%) 18 (30%)
PR status	
Positive Negative	40 (66.7%) 20 (33.3%)
Her-2/neu expression	
Positive Negative	24 (40%) 36 (60%)
Vascular invasion	
Positive Negative	56 (93.3%) 4 (6.7%)

ER: Estrogen receptor status.
PR: Progesterone receptor status.
HER-2: Human epidermal growth factor receptor 2.

Table 2. Statistical analysis of relative quantification of Doublecortin like kinase-1, E-cadherin and Vimentin genes in normal and tumor tissues

Parameter	Adjacent normal breast tissues (n=60)	Breast cancer tissues (n=60)	P
DCLK-1			
Range	0.0 – 2.53	0.01 - 212.45	
Mean ± SE.	0.28 ± 0.06	4.29 ± 3.53	<0.001*
E-cadherin			
Range	0.09 - 85.04	0.0 - 17.75	
Mean ± SE.	11.92± 3.99	1.99 ± 0.47	0.015*
Vimentin			
Range	0.01 - 65.80	0.02 - 744.33	
Mean ± SE.	11.41± 3.65	60.41± 20.22	0.001*

p: p value for comparing between breast cancer tissues and adjacent normal breast tissues.
*: Statistically significant at $p \leq 0.05$.

3.3. Serum Transforming Growth Factor-β

Range and mean±SD of serum TGF-β (pg/ml) in control group and all breast cancer patients were shown in Table 3 and illustrated in Figure 4. As presented in Table 3, serum TGF-β concentration was significantly elevated in breast cancer patients in comparison to control group ($p<0.001$).

Table 3. Statistical analysis of serum TGF-β in control group and breast cancer patients

	Control group (n = 40)	Breast cancer patients group (n = 60)	p
TGF- β			
Range	276.82 – 876.05	452.06 – 3896.73	<0.001*
Mean ± SD.	491.34 ± 104.81	1376.85 ± 754.68	

p: p value for comparing between breast cancer patients and control group.
*: Statistically significant at $p \leq 0.05$.

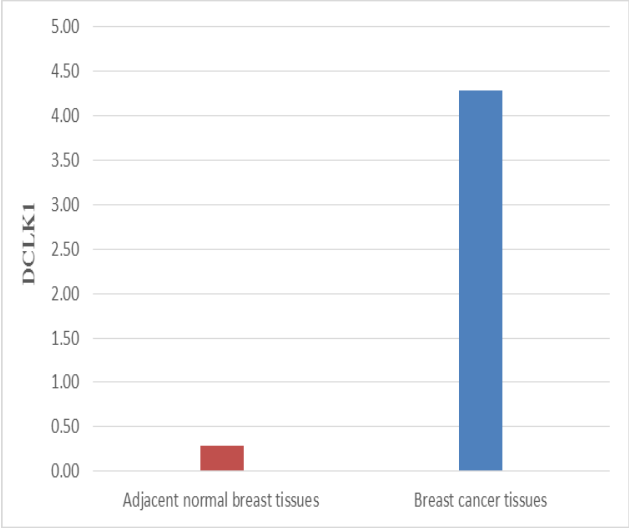

Figure 1. Bar chart illustrating relative quantification of DCLK-1 gene in normal and breast cancer tissues

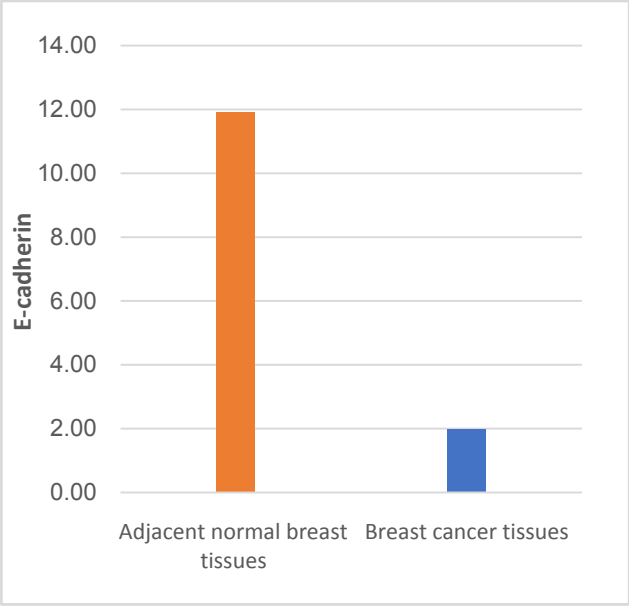

Figure 2. Bar chart illustrating relative quantification of E-cadherin gene in normal and breast cancer tissues

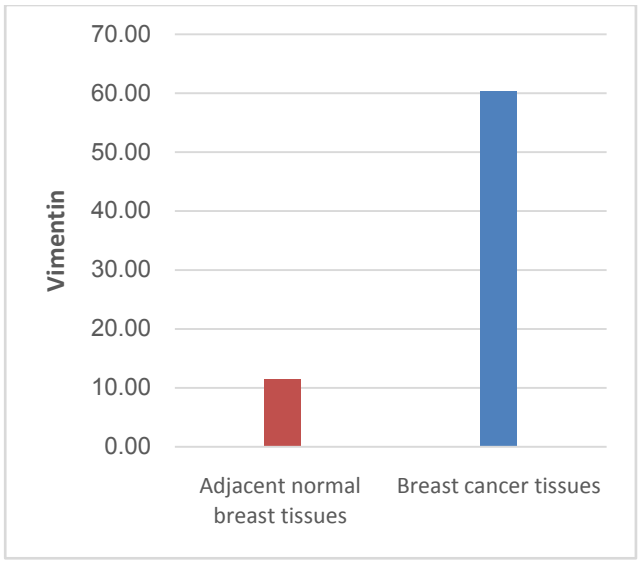

Figure 3. Bar chart illustrating relative quantification of Vimentin gene in normal and breast cancer tissues

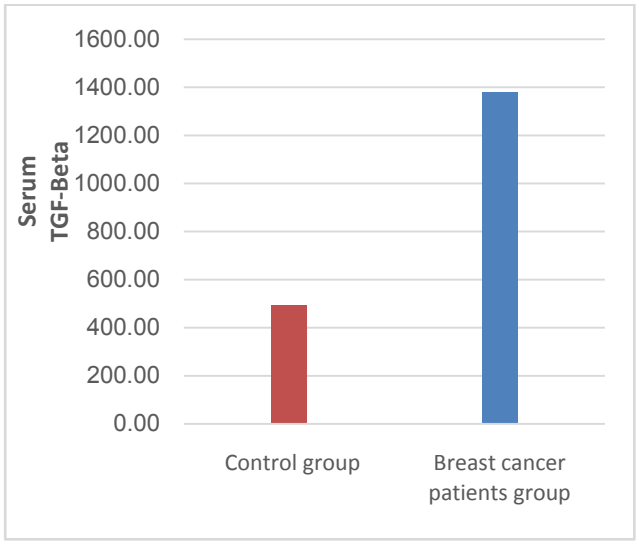

Figure 4. Bar chart illustrating serum TGF-β in control group and breast cancer patients

3.4. Correlation between Different Studied Parameters

According to Table 4, tissue DCLK-1 gene expression was significantly negatively correlated with tissue E-cadherin expression (p=0.014) and significantly positively correlated with tissue Vimentin expression and serum TGF-β (p=0.020 and 0.031 respectively), while there was insignificant correlation with histological grade, clinical stage, tumor size, axillary lymph node involvement, ER status, PR status, Her-2/neu expression and vascular invasion (p=0.822, 0.064, 0.184, 0.294, 0.090, 0.438, 0.802 and 0.378 respectively). Moreover, it was observed that, tissue E-cadherin expression was significantly negatively correlated with tissue Vimentin gene expression, histological grade, clinical stage, tumor size and axillary Lymph node involvement (p=0.007, 0.009, 0.010, 0.021, and 0.004 respectively), while there was insignificant correlation between this parameter and serum TGF-β, ER status, PR status, Her-2/neu expression and vascular invasion (p=0.056, 0.109, 0.892, 0.105 and 0.386 respectively).

With respect to tissue Vimentin, it was positively significantly correlated with histological grade, clinical stage, tumor size and axillary lymph node involvement (p= 0.027, 0.003, 0.033 and 0.019 respectively), while there was insignificant correlation between this parameter and TGF-β, ER status, PR status, Her-2/neu expression and vascular invasion (p= 0.272, 0.098, 0.363, 0.500 and 0.0 68 respectively). Furthermore, it was noticed that serum TGF-β was positively significantly correlated with clinical stage and tumor size (P= 0.001 and 0.006) while there was insignificant correlation between this parameter and histological grade , axillary lymph node involvement, PR status, ER status, Her-2/neu expression and vascular invasion (p= 0.208, 0.542, 0.678, 0.631, 0.399 and 0.051 respectively).

3.5. Prognostic Value of DCLK-1, E-cadherin, Vimentin and TGF-β in Breast Cancer Patients

To study the prognostic values of these four parameters Kaplan-Meier disease free survival (DFS) curves were constructed. As shown in Table 5 and Figure 5 - Figure 8. Kaplan-Meier survival curves revealed that, patients with elevated levels of tissue DCLK-1 and vimentin expression had shorter disease free survival time than patients with lower tissue levels. The same is true for serum TGF-β. However, patients with lower tissue E-Cadherin expression had shorter DFS time than patients with higher tissue levels.

Table 4. Correlations between different studied parameters

	DCLK-1		E-cadherin		Vimentin		Serum TGF-β	
	r_s	p	r_s	p	r_s	p	r_s	p
E-cadherin	-0.317*	0.014*						
Vimentin	0.299*	0.020*	-0.344*	0.007*				
TGF-β	0.278*	0.031*	-0.248	0.056	0.144	0.272		
Histological grade	0.030	0.822	-0.336*	0.009*	0.286*	0.027*	0.165	0.208
Clinical stage	0.241	0.064	-0.331*	0.010*	0.381*	0.003*	0.410*	0.001*
Tumor size	0.174	0.184	-0.297*	0.021*	0.276*	0.033*	0.354*	0.006*
Axillary lymph node	0.138	0.294	-0.370*	0.004*	0.303*	0.019*	0.080	0.542
ER status	0.221	0.090	-0.209	0.109	0.216	0.098	0.037	0.631
PR status	0.102	0.438	-0.018	0.892	-0.119	0.363	0.055	0.678
Her-2/neu expression	-0.033	0.802	0.212	0.105	-0.089	0.500	0.111	0.399
Vascular invasion	-0.116	0.378	0.114	0.386	0.218	0.0 68	-0.253	0.051

r_s: Spearman coefficient.
*: Statistically significant at $p \leq 0.05$.

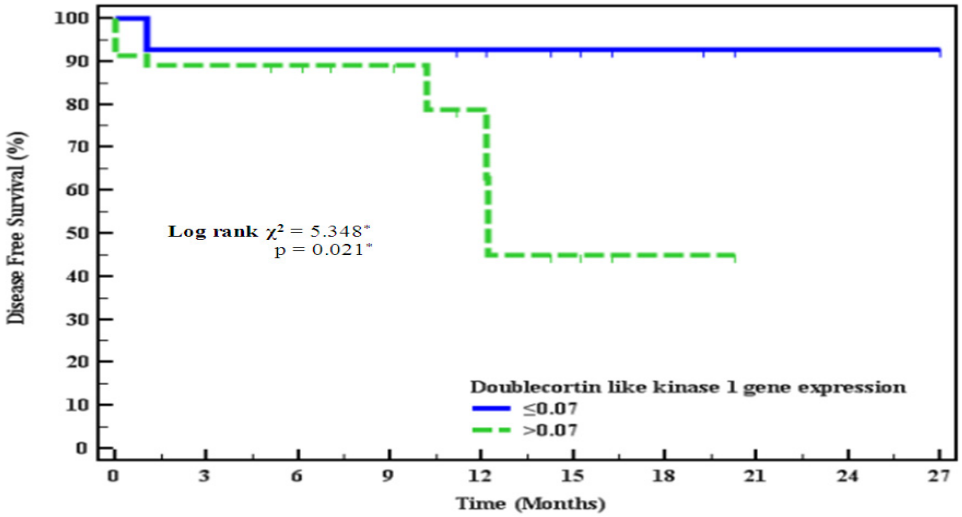

Figure 5. Kaplan-Meier disease free survival of tissue DCLK-1 gene expression in breast cancer patients

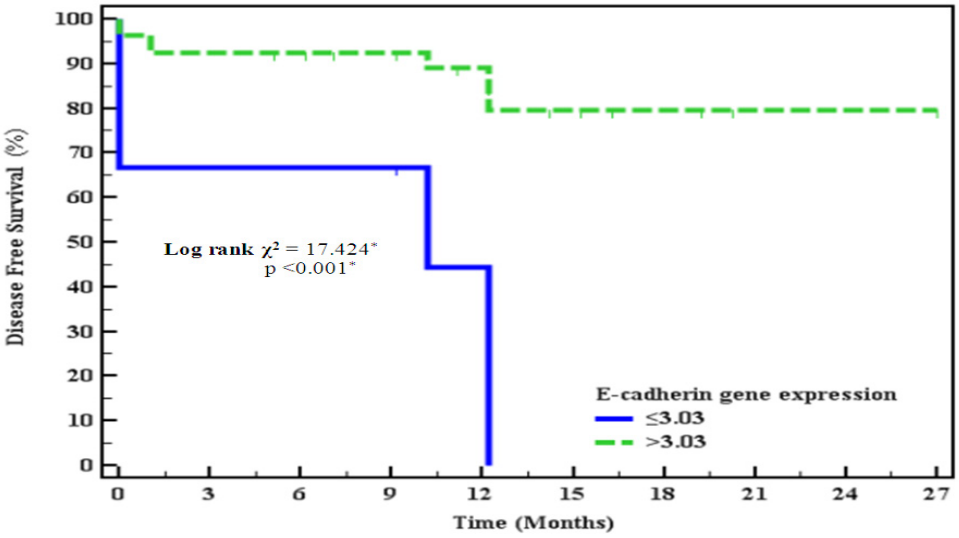

Figure 6. Kaplan-Meier Disease free survival of tissue E-cadherin gene expression in breast cancer patients

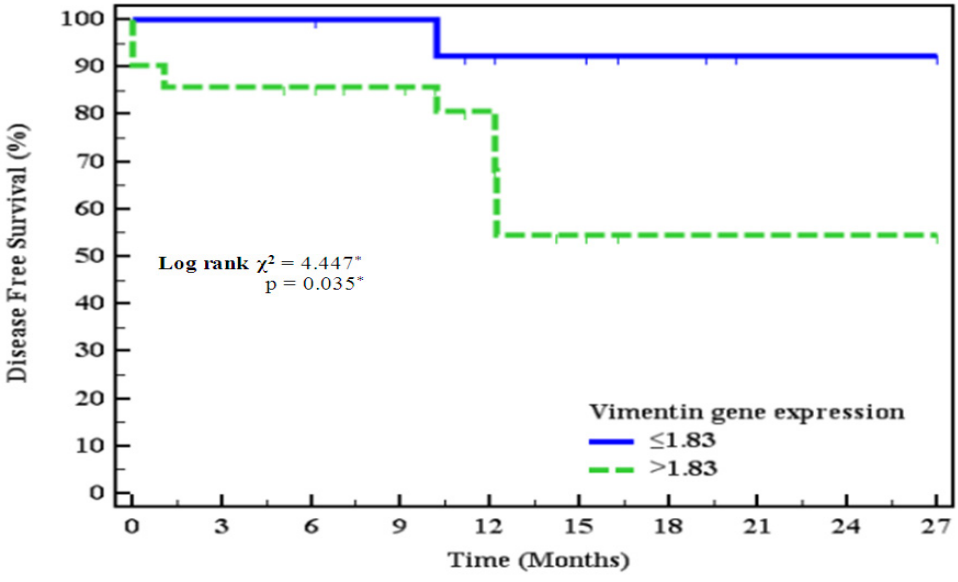

Figure 7. Kaplan-Meier Disease free survival of tissue Vimentin gene expression in breast cancer patients

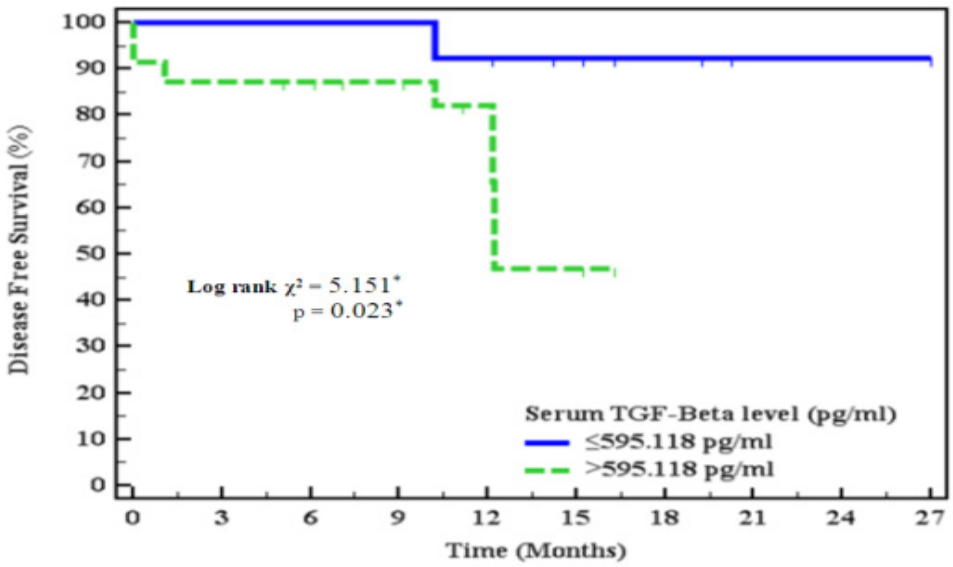

Figure 8. Kaplan-Meier disease free survival of serum TGF-β in breast cancer patients

Table 5. Test of significance of disease free survival of studied biomarkers in breast cancer patients.

	Average survival (Months)	Log rank	
		χ^2	P
DCLK-1			
≤0.07	25.1	5.348*	0.021*
>0.07	14.3		
E-cadherin			
≤3.03	7.7	17.424*	<0.001*
>3.03	23.1		
Vimentin			
≤1.83	25.7	4.447*	0.035*
>1.83	18.5		
Serum TGF-β			
≤595.118	25.7	5.151*	0.023*
>595.118	12.5		

*: Significance was considered at $p \leq 0.05$.

4. Discussion

Breast cancer is the most frequently diagnosed cancer in women, and it is the second leading cause of cancer death in women of all ages. [16] Metastasis occurs via a series of sequential steps, during which the cells acquire an amoeboid-like phenotype, become motile, disseminate, and colonize at distant sites of the body, which in breast cancer are most commonly in liver, lung, bone, and brain. The stages of this transformation are similar to the stages of the developmental process known as EMT. [17]

Previous reports have suggested that DCLK-1 may mark cancer stem cells in gastrointestinal and other cancers. [18] However, it is not clear whether it also plays similar roles in breast cancer. Vimentin, TGF-β and E-cadherin are well recognized for their selective expression. Therefore the aim of the present study was to detect expression level of DCLK1 in breast cancer tissues and study its role in tumorigenesis of breast cancer through its possible association with clinicopathological features and EMT markers (E-cadherin, vimentin and TGF-β).

The present study demonstrated that expression of DCLK-1 in breast cancer tissues was significantly higher than that in normal adjacent non tumor tissues. This result leads us to suggest the role of this gene in tumor growth and development. In line with our results, Wenhua Shi et al [19] showed that the expression of DCLK-1 in cancer tissues was significantly higher than that in normal or adjacent non-tumor tissues. Suggesting that overexpression of DCLK-1 markedly accelerated the pathogenesis and development of cancer.

Doublecortin-like kinase-1 is a microtubule-associated protein that plays key roles in the regulation of neural cell differentiation, migration, and apoptosis during embryonic development. [20] Accumulating evidence suggests that DCLK-1 is a marker of intestinal and pancreatic stem cells and cancer stem cells (CSCs); thus, it is attracting much attention from both gastroenterologists and oncologists. [21,22] In animal models with xenografted tumors of colon cancer and pancreatic cancer cells, gene silencing of DCLK-1 decreased the tumor size. [23] In another study, it was shown that DCLK-1 was involved in not only tumor growth but also invasion and migration. [24] The possible mechanisms underlying the tumor-promoting role of DCLK-1 include downregulation of tumor-suppressing microRNA expression, induction of vascular endothelial growth factor receptor and epithelial-mesenchymal transition (EMT)-related factors, [25] and activation of the oncogenic gene C-MYC in tumor cells. [26]

Furthermore, the current study revealed that tissue DCLK-1 gene expression showed a significant negative correlation with tissue E-cadherin expression and a significant positive correlation with both tissue Vimentin expression and serum TGF-β. These results support a role for DCLK-1 in EMT process. In agreement with our findings, Chandrakesan et al [27] was demonstrated that DCLK-1 supports intestinal tumor growth via enhancing EMT and pluripotency factors. Moreover, previous studies indicated that DCLK-1 over expression could activate EMT in the cells which was likely one of the mechanisms underlying its involvement with metastasis. [28,29]

In this study we analyzed the relationship between DCLK-1 and prognosis by evaluating DFS in patients

group. The analysis of DFS showed that higher levels of tissue DCLK-1 gene expression were significantly associated with poor prognosis in patients with primary breast cancer. This finding agrees with previous study indicating that DCLK-1 is a bad prognostic marker for breast cancer patients. [30] According to this result we can consider tissue DCLK-1 gene expression as a promising prognostic biomarker for breast cancer patients.

E-cadherin is a mechanical adhesion molecule between epithelial cells, it has an important role in establishing the cell polarity, the glandular differentiation, and cellular layering; it is also an important regulator of morphogenesis, thus maintaining the normal epithelial architecture. [31] E-cadherin is considered as a tumor suppressor protein because it was shown that aberrant expression and/or functional losses of this protein are associated with tumorigenesis and tumor progression. [32]

The present study showed that the expression of E-cadherin gene expression in cancer tissues was significantly lower than that in adjacent normal tissues. Down regulation of E-cadherin expression are considered hallmarks of EMT process and have largely been implicated in EMT associated with the acquisition of a migratory/ invasive phenotype by epithelial tumor cells. [33] In line with our finding, Suciu et al [34] found that tumors with low level of E-cadherin expression are more invasive; less differentiated, and have a higher ability to invade lymph nodes.

Different cytoplasmic proteins are a key in the transformation of a normal cell to invasive tumor cell and among these, vimentin is particularly important. It is one of the cytoplasmic intermediate filament proteins which are the major component of the cytoskeleton normally found in embryonic or mesenchymal stem cells. [35] Our study showed that the expression of vimentin in cancer tissues was significantly higher than that in adjacent normal breast tissues. This result is expected since Vimentin is frequently expressed in neoplastic cells with metastatic properties, [36] including breast cancer cells. [37] Hendrix et al [38] demonstrated that expression of Vimentin in human breast cancer cells results in phenotypic interconversion and increased invasive behavior.

TGF-β participation in BC development and metastasis is well-established; however, the clinical meaning of its circulating levels among women with BC is poorly understood. We characterized levels of TGF-β in serum from women with BC and associate them with the main clinical features associated with BC prognosis. [39] The current study displayed that serum TGF-β in BC patients was significantly higher than that in control group. This suggests that TGF-β may have a role in BC progression. In consistent with our finding Ciftci et al [40] and Nacif et al [41] found that serum TGF-β levels in patients with BC were significantly higher than in normal controls which agree with our results. Additionally in Desruisseau et al [42] study he found that 94.3% of BC patients expressed TGF-β protein in breast tumor samples.

Regarding the correlation between EMT markers and clinicopathological characteristic, the current study revealed that E-cadherin gene expression showed significant negative correlation with tumor size while tissue vimentin and serum TGF-β showed significant positive correlation. This may point to the growth advantage for cells possessing low level of E-cadherin gene expression and high expression of vimentin and serum TGF-β levels. This hypothesis is coincided with that reported by Liesheng et al, [43] and Niknami, et al, [44] who demonstrated that low level of E-cadherin gene expression, high level of Vimentin gene expression and high level of serum TGF-β, function to promote cell growth of tumor cells.

In addition the level of tissue E-cadherin was significantly negatively correlated with tumor stage where as the levels of Vimentin gene expression and serum TGF-β were significantly positively correlated with clinical stages of patients. These findings indicated that these parameters may be considered as biological markers for estimating the occurrence and progression of cancer. [45] Whereas axillary lymph node involvement in known as an effective and independent factor in breast cancer prognosis, [46] its negative correlation with E-cadherin gene expression and positive correlation with Vimentin gene expression can help to determine prognosis of breast cancer.

Moreover, it has been found that low level of E-cadherin gene expression and high level of vimentin had significant correlation to the histological grade of the tumor. This led us to hypothesis that these two parameters affect differentiation of cells. These results suggest the possibility that low E-cadherin gene expression and high Vimentin gene expression might be other markers that contribute to increase BC risk. [45] Taken together these results clearly showed the risky role of aberrant E-cadherin expression, increased Vimentin expression as well as serum TGF-β in carcinogenesis and progression of BC.

Furthermore, follow up studies were made to answer the question of whether tissue DCLK-1, E-cadherin, Vimentin gene expression and TGF-β in serum of patients are of prognostic significance and whether these four parameters will be useful in detecting early metastasis in BC. The prognostic importance of tissue E-cadherin, vimentin and serum-TGF-β was assessed by Kaplan-Meier disease free survival curve. The analysis demonstrated that BC patients with decreased tissue E-cadherin and increased tissue vimentin expression and elevated levels of serum TGF-β1 above the cutoff value had shorter mean DFS time than BC patients with increased tissue E-cadherin and decreased tissue vimentin and lower TGF-β. According to the present study we can consider these parameters as bad prognostic markers and useful in detecting early recurrence in breast cancer patients. These results consistent with previous studies indicating that these parameters are indicators of bad prognosis for breast cancer patients [43,47,48].

5. Conclusion

From this study we may conclude the following:-

- Doublecortin like kinase-1 is overexpressed in breast cancer tissues in comparison to normal breast tissues and significantly correlated with EMT markers.
- Invasive breast carcinomas are characterized by decrease of E-cadherin expression. Aberrant E-cadherin expression is correlated negatively with

clinical stage, histological grade, tumor size and axillary lymph nodes involvement.

- Vimentin is overexpressed in breast cancer tissues in comparison to adjacent normal breast tissues suggesting a possible role of vimentin in the development and progression of breast cancer and vimentin overexpression could be useful in the prognosis of breast cancer.
- Our findings offer a preliminary evidence of elevated serum TGF-β levels in breast cancer patients as compared to healthy controls and its high levels correlated positively with clinical stage and tumor size. Moreover, TGF-β1 may be a promising circulating marker for prediction of breast cancers with a poor prognosis. Further prospective studies using greater numbers of patients are required to confirm our findings.

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All authors have contributed significantly to this work.

Conflict of Interest

No conflict of interest is declared.

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