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The role of long noncoding RNAs in patients with Luminal A invasive breast ductal carcinoma

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ABSTRACT

Breast cancer is the most common form of cancer in women around the world. The molecular mechanisms of this heterogeneous disease have been extensively investigated; but yet; It requires a lot of sensitive and specific markers for prognosis and early detection approaches. Non-protein coding RNAs known as lncRNAs have been reported in tumorigenic involvement so they can be used for therapeutic purposes. In the present study, the expression levels of *CCAT1, PDCD4, PDCD4-AS1*, and *MEG3* LncRNA in adjacent tumor and breast tissue in 88 Iranian patients were evaluated by quantitative real-time PCR. CCAT1 was significantly expressed and *PDCD4-AS1* decreased in tumor samples, *PDCD4* and *PDCD4-AS1* showed a positive correlation with each other, higher levels of *PDCD4-AS1* were associated with better survival, tumor samples showed lower levels of *PDCD4* in Showed comparisons with normal tissue. Our findings suggest that lncRNAs play an important role in controlling gene expression after transcription of major tumor suppressors or carcinogenic genes, leading to the development of triple-negative breast cancer (TNBC). In conclusion, this study investigated the prognostic role of lncRNA in breast cancer patients.

1. Introduction

Breast cancer (BC), the leading cause of cancer death worldwide in women, maybe a common and very deadly malignancy [20,24]. Because identifying molecular factors for the treatment of BC may help increase survival, further research has sought to find biological targets that may be used for prognostic and diagnostic approaches [14,29]. However, few genes have been identified as prognostic or therapeutic biomarkers of BC. Among all subtypes of breast cancer, Luminal A has the best prognosis and indicates low grade, low cell proliferation, and high sensitivity to endocrine therapy [7]. Long noncoding RNAs (lncRNAs) typically contain more than 200 nucleotides and cannot be encoded in proteins. However, lncRNAs have been shown to play an important regulatory role in apoptosis, cell proliferation, differentiation, and migration in previous studies [8,11]. Various types of research have shown an association between lncRNA expression and survival, metastasis formation, and clinical recurrence [16,28]. MEG3 is one of the lncRNAs that is continuously regulated in various malignancies of tissue origin such as lung cancer [17], cervical cancer [33], pituitary tumor [4], and uterus cancer [26]. However, the prognostic importance of *MEG3* in BC is still unknown.

Programmed cell death or apoptosis may be an important biological mechanism that occurs during various processes from growth to tissue circulation [13]. One of the protective mechanisms of cells against tumor growth is the removal of defective cells with abnormal gene expression and apoptosis [22]. Any deficiency in apoptosis can lead to diseases such as cancer, and resistance to apoptosis is a key feature of cancer cells [9]. As a result, the expression of genes involved in apoptosis is highly regulated. One of the genes associated with apoptosis is programmed cell death 4 (*PDCD4*), which is regulated after the onset of apoptosis. PDCD4-AS1 could be a double-exon transcription that is related to a genetic material on chromosome 10q24 [12]. PDCD4-AS1 is a long non-coding RNA encoded by the programmed complement cell death strand 4 (*PDCD4*). The *PDCD4* tumor suppressor gene is known to

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Table 1
Patients' demographic information.

Parameters		Values
Age (mean±SD, (range))		51.82±11.25 (32–25)
Site of primary tumor		
Right breast	21 (52.5%)	
Left breast	19 (47.5%)	
Cancer stage (%)		
I		2 (5%)
II		24 (60%)
III		14 (35%)
Overall grade (%)		
I		5 (12.5%)
II		19 (47.5%)
III		14 (35%)
Unknown		2 (5%)
Lymphatic invasion		
Yes		32 (80%)
No		8(20%)
Vascular invasion		
Yes		33 (82.5%)
No		7 (17.5%)
Tumor size (%)		
≤2 cm		5 (12.5%)
>2		35 (87.5%)
Estrogen receptor (%)		
Positive		13 (32.5%)
Negative		4 (10%)
Unknown		23 (57.5%)
Progesterone receptor (%)		
Positive		11 (27.5%)
Negative		4 (10%)
Unknown		25 (62.5%)
Her2/neu expression (%)		
Positive		4 (10%)
Negative		14 (35%)
Unknown		22 (55%)

negatively regulate tumor invasion, neoplastic transformation, and cell proliferation [15] while *PDCD4-AS1* lacks the potential for protein coding, as the protein encoding gene known as *PDCD4* in the opposite direction is coded [30]. Unlike *PDCD4-AS1*, it has not yet been studied. *PDCD4* is a well-known tumor suppressor gene [23].

CCAT1 shows exceed amount of expression in different cancers like hepatocellular carcinoma, gastric cancer, gallbladder cancer, and colon cancer [5,21,31]. However, the therapeutic significance of *CCAT1* in BC has not yet been explored.

A better understanding of the molecular pathway of Luminal A breast cancer and its associated genetic disorders can help us diagnose prognostic and diagnostic biomarkers and provide effective treatment. In this study, the molecular mechanism underlying breast cancer was investigated, focusing on subgroup A luminal with four lncRNA expression in tumor cells and adjacent normal tissues.

2. Materials and methods

2.1. Patients and tissue specimens

In this study, 88 fresh samples of breast cancer, type A and additional non-cancerous tissues were taken from the same patients in Hamadan hospital. The samples were first transferred from the operating room to a 4 °C refrigerator. They were then stored in a freezer at minus 20 °C until delivery. After shipment, the samples were kept at minus 70 °C until RNA extraction. Adjacent tissue samples were identified and identified as normal by referring pathologists. The patients who had received preoperative radiation or chemotherapy were excluded from the study. Demographic information of patients including their clinical and pathological characteristics was considered (Table 1). They also provided an informed written consent form for tissue sample analysis.

We confirm that all experimental protocols have been approved by Shahid Beheshti University of Medical Sciences and that all procedures have been performed in accordance with the relevant instructions and regulations.

2.2. RNA extraction and RT-qPCR analysis

Total cellular RNA was extracted from the tissue samples utilizing Trizol reagent (Invitrogen, Waltham, MA, USA). Frozen tissue samples were transferred to porcelain mortar under the hood for homogenization and pounded well by the mortar. 1 ml of solution was added to the homogenized tissue sample. Then 200 µl of chloroform was added and incubated for 15–20 min at room temperature. Centrifugation was performed at 20 °C at 4 °C for 20 min.

The supernatant was then transferred to another microtube and the same volume of 100% isopropanol was added to the solution. After 10 min of incubation at room temperature and 15 min of centrifugation at 12000*g*, the supernatant was discarded and 1 ml of 60% ethanol was added to the precipitate for washing, and again centrifuged at 7500 rpm. The supernatant was discarded again and the precipitate was dissolved in RNase free water for further experiments and kept at −80 °C until use. The quality and quantities of the RNA extracts were measured using agarose gel electrophoresis and the adsorption rate of 260–280 nm using a Nanodrop device 2000 using manufacturer's protocol for concentration and purity. In order to remove possible DNA contamination, we treated the extracts by DNaseI before RT-PCR performance.

Then, one microgram DNase I treated RNA was incubated with 0.5 µl of random hexamer enzyme for 5 min at 70 °C. In the next step, 1 µl of dNTP mixture and 2 µl of RT buffer were added to the required amount of sterile distilled water, and after 5 min of incubation at 37 °C, 0.5 µl of RT enzyme was added. Incubation of the reaction mixture was performed at 42 °C for 80 min.

In order to synthesize cDNA from the extracted RNA, about 500 ng of RNA was briefly placed with Multiscribe reverse transcriptase (Applied Biosystems), Oligo (dT)primers, and random hexamer primers in a final volume of 10 µl for 30 min at 37 °C. It was then placed at 80 degrees for 5 s to inactivate the enzyme. It should be noted that all the steps were performed on ice and inside the aluminum rack. After finishing the work, the products were stored at a temperature of minus 20 degrees.

LncRNA expression levels were evaluated by Quantitative RT-PCR using the StepOne Plus system and SYBR Green PCR Master Mix according to the following cycling parameters, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 45 s. A negative control was used in each run. The housekeeping gene *HPRT1* was chosen for normalization. The PCR primer sequences for the selected LncRNAs were listed as follows:

CCAT1	
Forward primer	5′-TCACTGACAACATCGACTTTGAAG-3′
Reverse primer	5′-GGAGAAAACGCTTAGCCATACAG-3′
PDCD4:	
Forward primer	5′-TCTGGGAAAGGAAGGGGACTAC-3′
Reverse primer	5′-TTCATAAACACAGTTCTCCTGGTCAT-3′
PDCD4-AS1:	
Forward primer	5′-GGTCAGTGGCCTAGTGAGC-3′
Reverse primer	5′ CAGTCTAATGGGCAGAAGGGC3′
MEG3:	
Forward primer	5′-GCTGGGTCGGCTGAAGAAC-3′
Reverse primer	5′-CGTGGCTGTGGAGGGATTT-3′
HPRT1	
Forward primer	5′-CACTATATTGCCCAGGTTGGT- 3′
Reverse primer	5′-GCGGAAGCGTGTAAAATC-3′

To evaluate the lncRNA relative expression in adjacent normal tissues in comparison with BC tissues, we used melting temperatures, respectively. Each sample was analyzed in duplicate. RNAase free containers, microtubes, tubes, and samplers designed specifically for working with RNA and disposable latex-free gloves were used at all stages.

Table 2
The expression level of lncRNAs is shown in relation to the participants' parameters.

Parameters	PDCD4 up-regulation	PDCD4 down-regulation	P value	PDCD4-AS up-regulation	PDCD4-AS down-regulation	P value	CCAT1 up-regulation	CCAT1 down-regulation	P value
Age			0.35			0.33			0.9
<55	13 (61%)	8 (39%)		9(40%)	12 (60%)		12 (60%)	9 (40%)	
≥55	11 (57%)	8 (43%)		11 (57%)	8(43%)		10 (52%)	9 (48%)	
Site of primary tumor			0.49			0.84			0.9
Right breast	12 (57%)	9 (43%)		8 (39%)	13 (61%)		10 (47%)	11 (53%)	
Left breast	10 (52%)	9 (48%)		7 (37%)	12 (63%)		10 (52%)	9 (48%)	
Stage			0.65			0. 01			0.32
1	0 (0%)	2 (100%)		1 (50%)	1 (50%)		0(0%)	2 (100%)	
2	13 (54%)	11 (46%)		8 (34%)	16 (66%)		14 (58%)	10 (42%)	
3	6 (66.7%)	8 (33.3%)		6 (43%)	8 (57%)		7 (50%)	7 (50%)	
Histological Grade			0. 42			0. 55			0. 6
1	2 (40%)	3 (60%)		2 (40%)	3 (60%)		3 (60%)	2 (40%)	
2	10 (52%)	9 (48%)		6 (32%)	13 (68%)		12 (63%)	7(37%)	
3	7 (50%)	7 (50%)		2 (14%)	12 (86%)		7(50%)	7 (50%)	
Lymphatic invasion			0.64			0.09			0.65
Yes	15 (46%)	17 (54%)		17 (53.125%)	15 (46.875%)		16 (50%)	16 (50%)	
No	4 (66%)	2 (34%)		4 (66.7%)	5 (33.3%)		5 (60%)	3 (40%)	
Vascular invasion			0.78			0.22			0.65
Yes	15 (45%)	18 (55%)		15 (46%)	18 (54%)		16 (49%)	17 (51%)	
No	4 (57%)	3 (43%)		4 (58%)	3 (42%)		4 (66.6%)	3 (33.4%)	
Tumor size			0.02			0.39			1
≤2	2 (40%)	3 (60%)		5 (100%)	0 (0%)		3 (60%)	2 (40%)	
>2	19 (57%)	14 (43%)		10 (29%)	25 (71%)		16 (45%)	17 (55%)	
ER status			0.39			0.89			0.47
Positive	5 (38%)	8 (62%)		6 (46%)	7 (54%)		6 (47%)	7 (53%)	
Negative	2 (50%)	2 (50%)		3 (75%)	1 (25%)		2 (50%)	2 (50%)	
PR status			0.27			0.47			0.2
Positive	5 (45%)	6 (55%)		4 (37%)	7 (63%)		6 (55%)	5(45%)	
Negative	1 (25%)	3 (75%)		2 (50%)	2 (50%)		2 (50%)	2 (50%)	
Her2 status			0.52			0.52			1
Positive	2 (50%)	2 (50%)		3 (75%)	1 (25%)		2 (50%)	2 (50%)	
Negative	8 (58%)	6 (42%)		8 (57%)	6 (43%)		8 (57%)	6 (43%)	

Table 3
The comparison of the LncRNAs relative gene expressions between tumor and healthy samples. Results of Bayesian estimation supersedes the *t*-test.

Gene	Posterior mean diff.	SD	Effect size	P-value	95% HDI
CCAT1	-4.944	3.05	-1.686	<0.0001	[−6.08, −3.81]
PDCD4	-1.92	2.77	-0.719	<0.0001	[−2.95, −0.89]
PDCD4-AS1	-0.97	2.15	-0.461	0.007	[−1.83, −0.14]

*Abbreviations; SD: standard deviation, HDI: highest density interval.

2.3. Statistical analysis

For statistical analysis of data, we used SPSS version 24.0 program (SPSS, Chicago, IL, USA). We used Bayesian estimation to replace the *t*-test (BEST) to test the significance of the mean difference between the two paired groups. A t-student prior family was assumed for parameters with 4000 iterations and 2000 burn-outs. The P values estimated from Frequentist methods including median test.

The Spearman correlation was used to assess the association among relative gene expressions. We used BEST, Rjags, and ggplot2 packages to implement analysis in the software R 4.03. A p-value <0.05 was considered statistically significant.

3. Results

The expression level of LncRNAs considered in this study was measured to evaluate their potential in diagnostic tests. The ROC curves of each LncRNA were plotted. The sensitivity and specificity of each molecule were compared and evaluated. Values above 0.9 were considered excellent.

Simultaneous expression of the studied genes showed that none of them had a significant association with metastasis (Table 2). However, the expression level of the investigated LncRNAs genes indicated a statistically significant difference between the two groups of healthy and tumor samples. Also, based on *t*-test analysis, all the studied genes had completely different expressions in healthy and neoplastic breast tissues; the expression was higher in healthy tissues (Table 3).

Receiver operating characteristic curve (ROC curve) analysis demonstrated that *CCAT1* had higher sensitivity of 87.8% vs. 82.93% in *PDCD4-AS1* and 75.61% in *PDCD4*, and a higher specificity of 90% vs. 47.5% in *PDCD4-AS1* and 72.5% in *PDCD4* (Fig. 1).

The *PDCD4-AS1* expression level was decreased during breast cancer formation and showed to be positively associated with a decreased level of *PDCD4* expression activity in the disease development. The higher levels of *PDCD4-AS1* were associated with higher survival values in a cohort of BC patients. Patient samples showed lower expression levels of *PDCD4* compared to normal tissue (Figs. 2 and 3).

In comparison with adjacent normal tissues, no *MEG3* expression was observed in understudied healthy/tumor samples, so it was removed from the study. The Results of Bayesian estimation supersedes the *t*-test for the comparison of relative gene expression between tumor and healthy samples are shown in Table 3.

4. Discussion

Despite many attempts to find the causes of breast cancer with its heterogeneous nature, successful researchers are still finding new factors involved in its development like the environmental or genetic

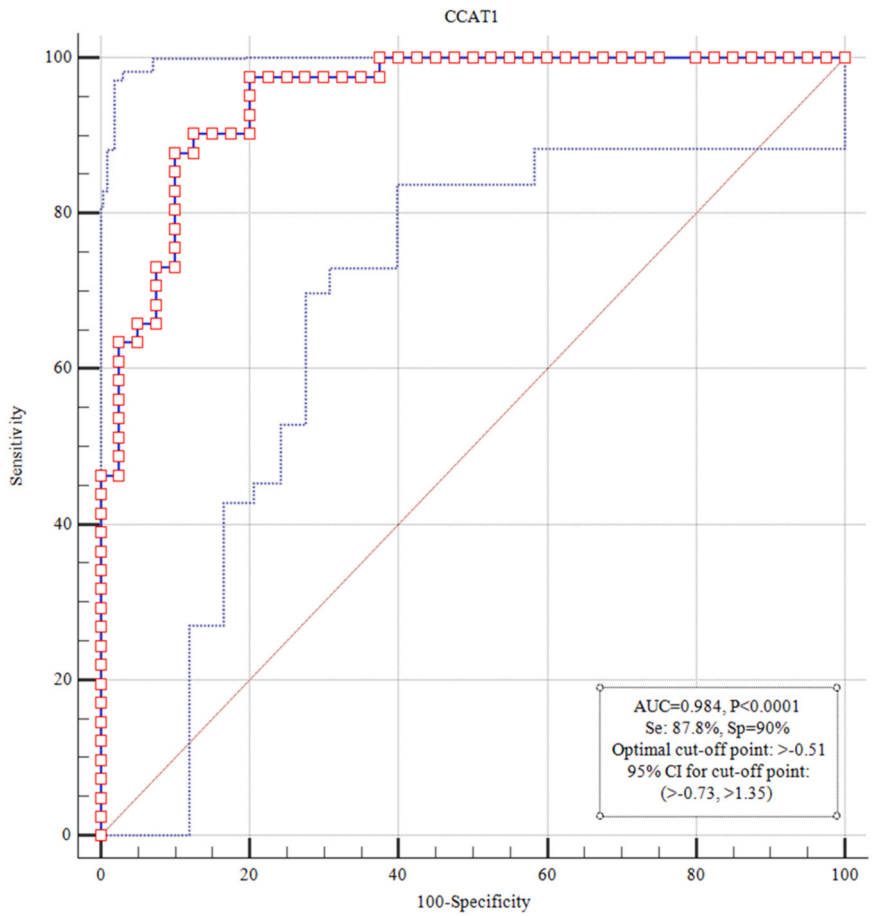

Fig. 1. Receiver operating characteristics curves showing the accuracy of *CCAT1* in distinguishing between breast cancers (Luminal A) patients and healthy ones.

factors that change biochemical pathways leading to unwanted proliferation or apoptosis and cause pathogenesis of the disease. Among the known factors that change the expression of certain genes, are microRNAs that can change the cell signaling pathways by altering the expression of genes involved in the production of growth factors, Wnt, Notch, nuclear factor-kB, Regulate phosphoinositide-3-kinase/Akt, and kinase/mitogen negatively or positively [2]. Today, among the new factors that are seriously discussed in the etiology of the cancer is lncRNA. They affect signaling pathways and contribute to the development of breast cancer by participating in the regulation of gene expression. Therefore, they could be used as significant diagnostic and prognostic biological tools in clinic [1].

An increasing number of new treatments for breast cancer have been improved, such as molecular immunotherapy, gene therapy, and targeted therapy. However, satisfactory therapies have not yet been developed. Breast cancer is still a major public health issue. Recently, various types of research have been performed to investigate the function of irregular lncRNA expression in different types of human cancer, such as BC [25,32].

We have tried to evaluate the molecular role of different lncRNAs to better understand the progression of breast cancer. Recent researches have shown that the lncRNAs play an important role in the development of various disorders, including cancer. In research by Luo et al. [18]. It is reported that MEG3 plays an important role in controlling migration, apoptosis, and cell proliferation by targeting Bcl-2 in prostate cancer. Sun et al. [27] suggested that *MEG3* may be a poor prognostic indicator in BC and that destruction of *MEG3* by siRNA may lead to apoptosis of BC cell proliferation. Braconi et al. [3] showed that ectopic expression level of *MEG3* induced apoptosis in hepatocellular cancer cells. Sun et al. [26] revealed that decreasing *MEG3* inhibited proliferation, invasion,and migration by relying on *p53*'s transcriptional function. All of the data presented above implied that *MEG3* might be a novel molecular marker associated with BC progression and under the regulation of a population depended genomic network of polymorphisms where it plays an independent prognostic role as a biomarker as well as a new potential indicator for BC patients in particular or ethnic populations. Despite all these reports, our results showed that *MEG3* has no expression in either healthy or tumor breast tissue. The fact that this gene was not expressed in any of the samples should be noted that lncRNAs are well influenced by the environment, race and lifestyle, and perhaps *MEG3* is one of them that is not expressed a particular state of health in the Iranian population or the gene has tissue specific expression and has no activity in breast ductal cells. Further research is needed to decide whether *MEG3* expression may be considered as a potential biomarker of BC and whether it affects the survival of BC patients in the Iranian ethnic group.

Recent studies have shown that *CCAT1* is overexpressed in various types of cancer and is involved in a variety of cancer-related cellular mechanisms. However, the prognostic value and clinical significance of *CCAT1* in breast cancer have not been investigated. According to studies by He et al., *CCAT1* is significantly regulated compared to adjacent normal tissues in colorectal cancer, and its overexpression is associated with patients' clinical stage, lymph node metastasis, and postoperative survival rate. In addition, c-Myc may induce *CCAT1* transcription directly by binding to the promoter and increase *CCAT1* expression in invasion and cell proliferation due to colon cancer [10]. In a study by Yang et al., *CCAT1* was overexpressed in gastric cancer. C-Myc induction of *CCAT1* plays an important role in gastric cancer and suggests that *CCAT1* may be used to treat gastric cancer [31]. As in previous research, in the present study, we also found that *CCAT1* expression was

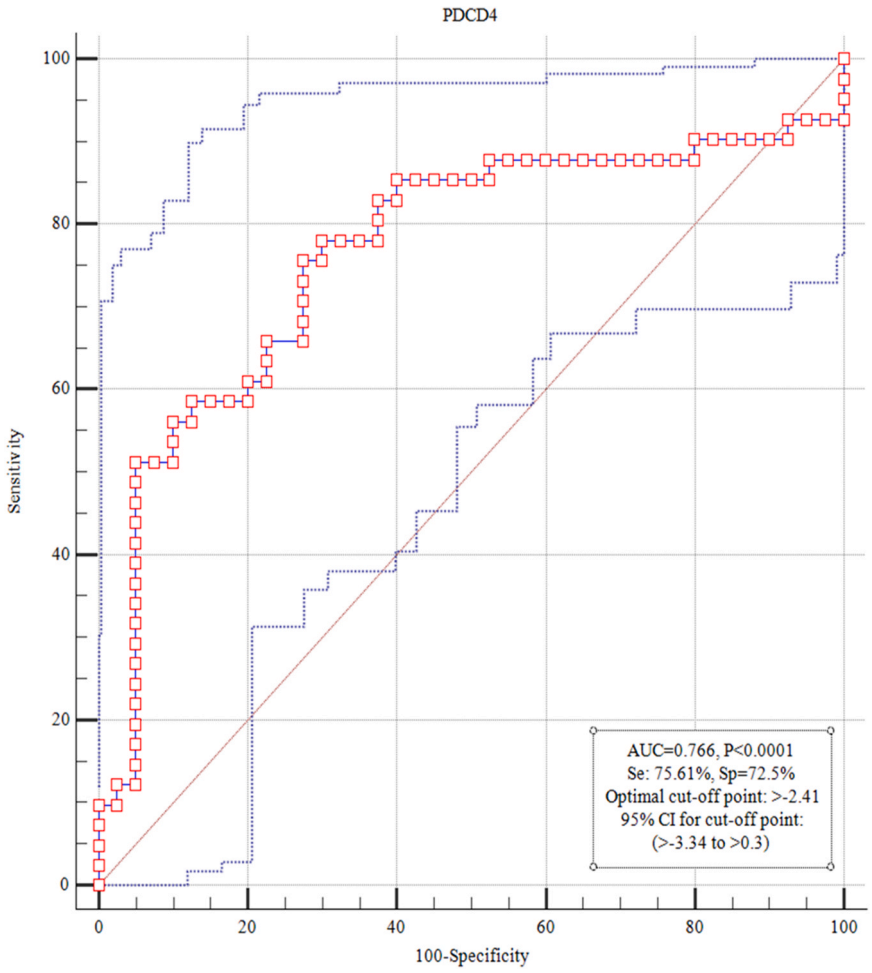

Fig. 2. Receiver operating characteristics curves showing the accuracy of *PDCD4* in distinguishing between breast cancers (Luminal A) patients and healthy ones.

significantly higher in adjacent normal tissues than in BC tumor specimens [p-value −0.73,>1.35]. The correlation of *CCAT1* with various clinical features of BC was examined. We found that overexpression of *CCAT1* was not associated with lymph node metastasis, meaning that *CCAT1* could be involved in invasive disorder and cancer in BC. Further analysis showed that overexpression of *CCAT1* is an independent prognostic marker for overall survival and progression-free survival.

More researches into the molecular pathway by which *CCAT1* contributes to the onset and BC progression are required.

In addition, this study evaluates the clinical relevance of *PDCD4-AS1* and *PDCD4* expressions. These lncRNAs were selected for the following reasons. First, *PDCD4* has previously been identified as a tumor suppressor gene that finds reduced expression in various types of cancer [6, 19]. Second, the *PDCD4* and *PDCD4-AS1* genes are expressed in samples from both Luminal A and BC cell lines. Finally, clinical evidence from BC patients shows that, similar to the *PDCD4* gene, decreased expression of *PDCD4-AS1* is associated with decreased patient survival rate, and this is a good sign of *PDCD4-AS1* tumor-suppressive performance. The results of our research, as in previous researches, showed that the expression levels of PDCD4-AS1 and PDCD4 in tumor tissues were significantly reduced (p-value 0.007) and were co-regulated in BC patients. Moreover, we indicated that a higher rate of *CCAT1* sensitivity and specificity in comparison to the *PDCD4* (sensitivity: 75.61% and specificity: 72.5%) and PDCD4-AS1 (sensitivity: 82.93% and specificity: 47.5%) confirms its strength as a potential biomarker for breast cancer.

Achieving a pattern of expression of co-regulated genes and the effect of their activity as tumor markers on the formation, progression, and changes leading to breast cancer metastasis, such as the discovery of an lncRNA signature, will be useful in novel diagnosis, prognosis, and therapy. Future research is needed to identify the function of other dysregulated lncRNAs in breast cancer biology.

Authors statement

NE and **MT** performed the experiment. **FP** designed and supervised the study. **FR** and **NB** analyzed the data. All the authors read and approved the submitted version.

Conflict of Interest

The authors declare they have no conflict of interest.

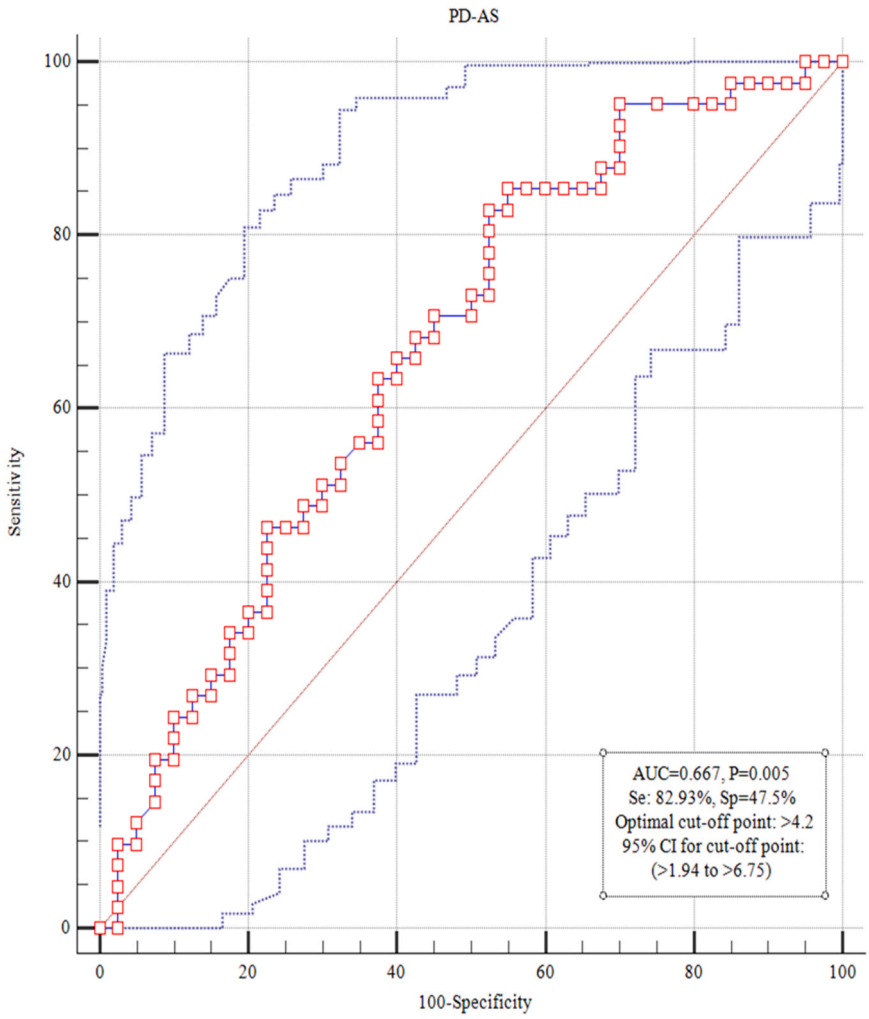

Fig. 3. Receiver operating characteristics curves showing the accuracy of *PDCD4-AS1* in distinguishing between breast cancers (Luminal A) patients and healthy ones.

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