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Original Article

Low expression of long noncoding RNA GAS6-AS1 as a novel biomarker of poor prognosis for breast cancer

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Abstract: Background: Breast cancer is the most frequent cancer for women in the worldwide. Recent studies showed that long noncoding RNAs might play important roles in a broad range of biological processes. The aim of the present study was to investigate the potential correlation between lncRNA GAS6-AS1 expression level and clinicopathological characteristics as well as prognosis in breast cancer. Methods: The expression of GAS6-AS1 was analyzed in 90 pairs of breast cancer tissues and their matched nontumor adjacent tissues (NATs) as well as 4 breast cancer cell lines by real-time PCR. The correlation between expression of GAS6-AS1 and clinicopathological characteristics as well as prognosis was analyzed by non-parametric test and log-rank test. Result: The results showed that GAS6-AS1 expression was significantly downregulated in breast cancer cell lines and breast cancer tissues compared with their matched NATs. The expression of GAS6-AS1 was correlated with lymph node metastasis (P=0.03) and histologic grade (P=0.01). The Kaplan-Meier curves indicated that the overall survival (OS) and disease-free survival (DFS) were all significantly poor in low GAS6-AS1 expression breast cancer patients. Moreover, the expression of lncRNA GAS6-AS1 was found to be an independent prognostic factor of breast cancer by Cox's regression. The ROC curve indicated that GAS6-AS1 may be a potential biomarker for diagnosis of breast cancer. Conclusion: Our research indicated that lncRNA GAS6-AS1 is significantly downregulated in breast cancer cell lines and tissues. GAS6-AS1 may represent a new marker for prognosis of breast cancer.

Keywords: LncRNA GAS6-AS1, breast cancer, biomarker, prognosis

Introduction

Breast cancer is one of the most frequently cancers of women in the worldwide [1]. The morbidity of breast cancer is on rising year by year in China [2]. The treatment of breast cancer usually includes resection, cytotoxic chemotherapy, hormonal therapy, immunotherapy and targeted therapy [3]. In spite of that the survival has been improved by multi-purpose therapies, a large number of patients are still not cured due to recurrence and metastasis which may lead to late stages and death. Hence, to explore new molecular biomarkers for guiding diagnostic and therapeutic strategies remain an important area in the management of breast cancer.

The protein coding genes in human genome are less than 2% of all genome sequence, almost over 90% gene are transcribed into non-coding RNAs [4]. Recent evidence is accumulating that non-coding RNAs (lncRNAs) are involved in many human diseases [5]. Although the short non-coding RNAs have attracted attention from researchers, long non-coding RNAs are proved to play important roles in regulation of cancer [6]. LncRNAs are a kind of transcripts which are longer than 200 bases and have no protein coding ability. The expression of lncRNAs are dysregulated in different cancers, and they are also associated with biological behavior and prognosis of cancers [7]. More and more lncRNAs are researched to be associated with breast cancer. For instance, the HOX transcript antisense RNA (HOTAIR) was overexpression in patients with breast cancer who might have worse outcomes such as metastasis and poor prognosis [8]. Another sample is lincRNA-ROR, which was reported upregulated breast cancer. The research found

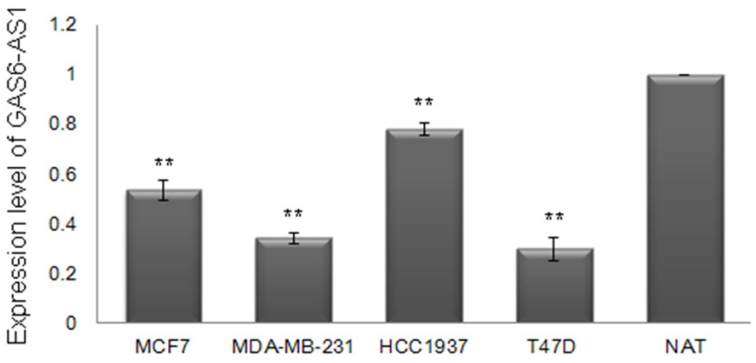

Figure 1. The expression levels of GAS6-AS1 in breast cancer cell lines. The GAS6-AS1 expression in breast cancer cell lines was compared with one case of NATs by real-time PCR analysis. GAPDH was used as the internal control. NAT, nontumor adjacent tissue.

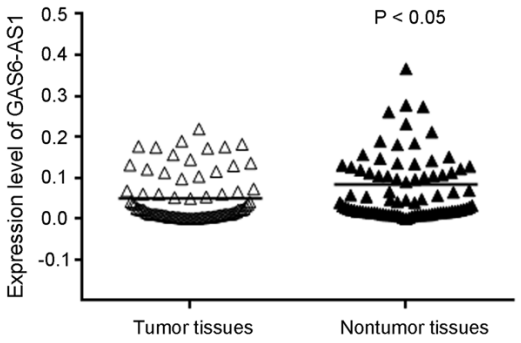

Figure 2. The expression levels of GAS6-AS1 in breast cancer tissues and their NATs. The GAS6-AS1 expression in breast cancer was compared with NATs using the data from real-time PCR (N=90, P<0.001). GAPDH was used as the internal control. Smaller $2^{-\Delta CT}$ value indicates lower expression.

that lincRNA-ROR played an important role in regulation of EMT and could promote cancer progression and metastasis in breast cancer [9]. So novel biomarkers of breast cancer such as lncRNAs are still important and useful for its treatment.

Previous studies found the growth arrest-specific gene 6 (GAS6) was upregulated in breast cancer [10]. GAS6 is a ligand for the Axl/Tyro3 family of receptor tyrosine kinases, and it can bind to these receptors and induce receptors phosphorylation [11]. In many kinds of human tumors, the receptors were proved to be overexpression, such as colon cancer and breast cancer [12, 13]. Especially in breast cancer, GAS6 has also been proved to be upregulated and associated with β-catenin which is a key factor of the epithelial-mesenchymal transition (EMT) [14].

From the bioinformatics analysis and previous studies, we noticed a novel lncRNA GAS6-AS1 (GAS6 antisense RNA 1). This lncRNA is located at 13q34 and transcribed from the antisense of GAS6. The objective of the present study was to investigate the expression of GAS6-AS1 in breast cancer and their matched nontumor adjacent tissues (NATs). Moreover, we aimed to explore the correlation between the expression of GAS6-AS1 and the clinicopathological characteristics, and try to provide new target set for the diagnosis and treatment of breast cancer.

Methods

Human tissue samples

A total of 90 pairs of human breast tumor samples and their pair-matched NATs were obtained from patients who underwent surgical resection at the Liaoning Cancer Hospital and Institute between 2009 and 2010 and were diagnosed to be breast cancer based by histopathological examination. The tissue samples were taken mastectomy under the supervision of the pathologist. The fresh tissue samples were immediately snap frozen after resection at -80°C. One part of each sample was examined by H.E. staining method for histopathological examination. The clinical information was collected on all donators in our study. The tumors were assessed according to the seventh TNM staging of the International Union against Cancer (UICC)/American Joint Committee on Cancer (AJCC) system. The estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor (HER2) status were also assessed in our study according to the ASCO breast cancer guideline. The study was approved by the Research Ethics Committee of Liaoning Cancer Hospital and Institute, and informed consent was obtained from all patients.

Cell lines and culture conditions

Human breast cancer cell lines MCF7, MDA-MB-231, HCC1937 and T47D were purchased

Table 1. Relationship between GAS6-AS1 and clinicopathological characteristics in breast cancer patients

Characteristics	No. of patients	Expression level	*P* value
Age (years)			0.209
≤50	51	0.72 (0.18-1.81)	
>50	39	0.37 (0.13-0.77)	
Tumor size (cm)			0.180
<2	30	092 (0.15-1.71)	
≥2	60	0.44 (0.18-0.85)	
Menopausal status			0.288
Pre	39	0.78 (0.24-1.80)	
Post	51	0.42 (0.11-0.87)	
Node status			0.030*
Negative	38	0.93 (0.35-2.06)	
Positive	52	0.34 (0.13-0.74)	
Histologic grade			0.010*
Good	39	0.24 (0.08-0.50)	
Poor	51	0.79 (0.40-1.81)	
ER status			0.527
Negative	45	0.51 (0.15-1.23)	
Positive	45	0.43 (0.16-1.17)	
PR status			0.509
Negative	40	0.50 (0.13-1.15	
Positive	50	0.49 (0.16-1.27)	
HER-2 status			0.576
N0	41	0.48 (0.14-1.18)	
N1	49	0.50 (0.18-1.28)	
TNM stage			0.109
I	15	0.48 (0.29-1.86)	
II	23	0.90 (0.13-2.67)	
III	33	0.37 (0.14-0.81)	
IV	19	0.47 (0.18-0.87)	

A Median of relative expression, with 25th-75th percentile in parenthesis; P<0.05 considered to be statistical significance. *Indicated statistical significance (P<0.05).

from the Institute of Biochemistry and Cell Biology at the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA). They were all cultured with 10% fetal bovine serum (FBS) at 37°C and 5% CO_2.

RNA isolation and real-time PCR

Total RNA from the specimens and cultured cells was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. The purity and concentration of total RNA were detected by UV spectrophotometry (A260/A280 >1.9). The total RNA was used to transcribe reversely to cDNA by Reverse Transcription Kit (Takara, Dalian, China). Roche LightCycler 480 II Real-Time PCR system (Roche, Switzerland) was used to quantify GAS6-AS1. 12.5 microliters of SYBR Premix ExTaq II (Takara) was added into mix according to the manufacturer's instructions. The expression of lncRNA was calculated relative to GAPDH. The results were calculated with the method of $2^{-\Delta\Delta CT}$ that the ΔCT was the difference in threshold cycle values [15]. The sequences of primers were as follows: 5'-AGCTACCCGGCTTGTTGTGG-3' (sense) and 5'-CTGGTCCTGGTCCTCGTTTCC-3' (antisense) for GAS6-AS1; 5'-CGGATTTGGTCGTATTGGG-3' (sense) and 5'-CTGGAAGATGGTGATGGGATT-3' (antisense) for GAPDH.

Statistical analysis

Data is presented as mean ± SD from at least three separate experiments. Statistical analysis was performed using Student's t-test, non-parametric test. Mann-Whitney U test between two groups and Kruskall-Wallis test for three or more groups. Receiver operating characteristic (c) curve was established to evaluate the diagnostic value for differentiating between breast cancer and benign diseases. To separate cancer tissues from nontumor tissues, the criterion for selection of cut-off point was the maximum of Youden index, which was defined as maxc[Sen(c)+Spe(c)-1], where c is the cut point. At the cut-off point, the sensitivity, specificity, as well as positive and negative predictive values of the breast cancer tissues and benign tissues were calculated separately. The Kaplan-Meier method was used to estimate survival curves, and the statistical different between survival curves was estimated by the log-rank test. Differences were considered statistically significant at P<0.05. Statistical analysis was performed using SPSS 18.0 computer software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, LaJolla, CA, USA).

Results

The characteristics of the subjects

The median age of patients with breast cancer was 54.5 years (range, 24-78 years), and 52

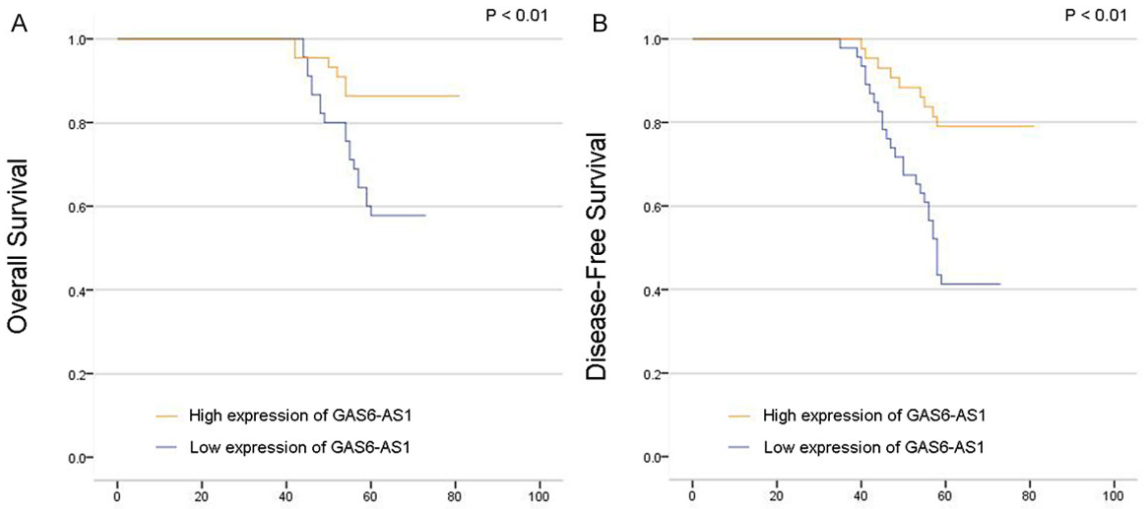

Figure 3. Kaplan-Meier survival curves of patients with breast cancer based on GAS6-AS1 expression levels. A. Overall survival of patients in lower expression group were poorer than in higher expression group; B. Disease-free survival of patients in low and high expression groups.

Table 2. Univariate regression model of prognostic covariates in breast cancer patients for OS

Variable	HR	β	95% CI		*P* value
			Lower	Upper	
Age (≤50/>50)	1.656	0.504	0.755	3.632	0.208
Menopause (positive/negative)	1.866	0.624	0.804	4.326	0.146
Tumor size (<2/≥2)	2.756	1.014	0.945	8.035	0.063
Lymph node (positive/negative)	1.417	0.348	0.626	3.207	0.403
Histologic grade (poor/good)	0.449	-0.801	0.202	0.998	0.047*
HER-2 status (positive/negative)	0.985	-0.015	0.449	2.159	0.970
TNM (I+II/III+IV)	1.151	0.184	0.517	2.561	0.731
GAS6-AS1 (down/up)	3.507	1.255	1.399	8.790	0.027*

P<0.05 considered to be statistical significance. *Indicated statistical significance (P<0.05).

patients (57.78%) had lymph node metastasis. All included patients had complete follow-up, the overall survival (OS) was used to evaluate the length of time between the surgery and death, and the disease-free survival (DFS) was used to evaluate the time between the surgery and death caused by breast cancer (Supplementary Table 1).

The expression of GAS6-AS1 in breast cancer cell lines and the non-tumor adjacent tissue

In order to detect the expression of GAS6-AS1 in breast cancer, we first detected the expression of GAS6-AS1 in four kinds of breast cancer cell lines compared with one case of nontumor adjacent tissues. As shown in **Figure 1**, we found that GAS6-AS1 was expressed much lower in all four breast cell lines than the normal breast tissue. These results indicated that GAS6-AS1 might have the function of antitumor in breast cancer.

GAS6-AS1 show down regulation in breast cancer tissues

To further examine the expression of GAS6-AS1 in breast cancer, we detected the expression of GAS6-AS1 in 90 pairs of breast cancer tissues and their matched NATs. As shown in **Figure 2**, GAS6-AS1 expression level was determined by real-time PCR, and there was significantly different expression of GAS6-AS1 between the tumor and NATs (P<0.001, Student's *t*-test). The expression levels in cancer tissues were much lower than the corresponding normal tissues. Furthermore, there were 62 of 90 (68.9%) cancer tissues which had lower expre-ssion of GAS6-AS1 than their corresponding NATs. These results further suggested that the lncRNA GAS6-AS1 was down regulated and might be a tumor suppressor in breast cancer.

Table 3. Univariate regression model of prognostic covariates in breast cancer patients for DFS

Variable	HR	β	95% CI		*P* value
			Lower	Upper	
Age (≤50/>50)	1.613	0.478	0.838	3.105	0.152
Menopause (positive/negative)	1.498	0.404	0.759	2.959	0.244
Tumor size (<2/≥2)	2.133	0.758	0.972	4.685	0.059
Lymph node (positive/negative)	1.404	0.339	0.711	2.772	0.328
Histologic grade (poor/good)	0.546	-0.606	0.282	1.054	0.071
HER-2 status (positive/negative)	0.892	-0.114	0.464	1.715	0.733
TNM (I+II/III+IV)	1.208	0.189	0.866	1.686	0.265
GAS6-AS1 (down/up)	3.567	1.272	1.675	7.597	0.031*

P<0.05 considered to be statistical significance. *Indicated statistical significance (P<0.05).

Table 4. Multivariate regression model of prognostic covariates in breast cancer patients for OS

Variable	HR	β	95% CI		*P* value
			Lower	Upper	
Lymph node (positive/negative)	0.405	-0.904	0.084	1.952	0.260
Histologic grade (poor/good)	0.613	-0.490	0.227	1.652	0.333
HER-2 status (positive/negative)	1.097	0.093	0.475	2.537	0.828
TNM (I+II/III+IV)	1.450	0.372	0.742	2.835	0.278
GAS6-AS1 (down/up)	3.519	1.258	1.236	10.020	0.018*

*Indicated statistical significance (P<0.05).

Relationship between the expression of GAS6-AS1 and clinicopathological characteristics in breast cancer

Next, we analyzed the potential correlation between the expression of GAS6-AS1 and the clinicopathological characteristics in breast cancer, such as age, menopausal status, tumor size, lymph node status, HER-2 status and so on. To find the relationship between GAS6-AS1 expression and clinicopathologic characteristics, we categorized the expression level of GAS6-AS1 as low or high with their median values. Of all included 90 patients, 46 cases were in the GAS6-AS1 down regulated group, and 44 cases were in the GAS6-AS1 up regulated group. The results shown that the expression of GAS6-AS1 in breast cancer was significantly correlated with lymph node metastasis (P=0.030) and histologic grade (P=0.010). However, the GAS6-AS1 expression in breast cancer had no association with other characteristics, such as age (P=0.209), tumor size (P=0.180), menopausal status (P=0.288), ER status (P=0.527), PR status (P=0.509), HER-2 status (P=0.576) and TNM stage (P=0.109) (**Table 1**).

Association between GAS6-AS1 expression and patients' survival

All patients included in our study were followed up for at least 60 months. The OS and DFS curves in down regulated and up regulated groups were shown in **Figure 3**. The OS was significantly lower in patients with down regulated GAS6-AS1 than patients with up regulation (P<0.010) (**Table 2**). The DFS was also significantly worse in patients with lower expression of GAS6-AS1 than patients with higher expression (P<0.010) (**Table 3**). The univariate analysis of prognostic parameters for OS was performed by Log-rank test and multivariate analysis was using Cox's regression model. As shown in **Table 4**, the expression level of GAS6-AS1 was an independent prognosis factor in breast cancer for overall survival.

The diagnostic value of lncRNA GAS6-AS1 for breast cancer

In order to explore the diagnostic value of GAS6-AS1 in breast cancer, we used the matched NATs as control to draw a ROC curve. As shown in **Figure 4**, the cut off value was 5.21 and the area under the ROC curve was 0.646 (95% CI=0.565-0.726, P=0.001). The specificity and sensitivity were 0.811 and 0.478. The Youden index was 0.289.

Discussion

Recent years, breast cancer is the leading cause of cancer death for women in the worldwide. Especially in the People's Republic of China, the morbidity of breast cancer has increased year by year with in the last ten years [16]. In spite of that the current comprehensive treatment for breast cancer was proved

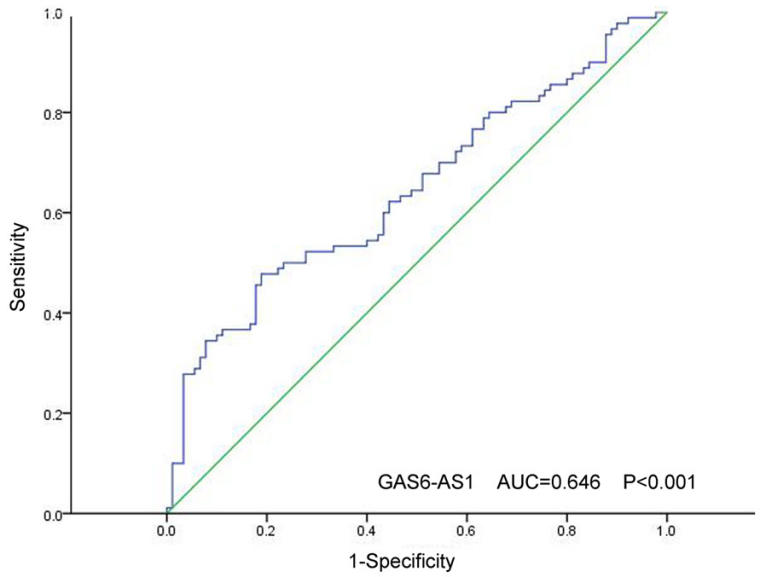

Figure 4. ROC curve of patients with breast cancer based on GAS6-AS1 expression in tumor tissues and NATs.

effective to prolong survival, recurrence and metastasis are still key factors for prognosis. Hence, effective tumor biomarkers are needed to render a service for the early diagnosis of breast cancer [17]. Several biomarker for breast cancer are already used in the diagnosis such as BRCA1/2 and CA27.29 [18]. But the specificity and sensitivity of these biomarkers are not enough for breast cancer [19]. Recently, long noncoding RNAs are studied well for their aberrant expression in cancers. With the contribution of GENCODE team on the ENCODE project, many lncRNAs were indicated to play important roles in cell signaling pathways. Moreover, a large number of studies support that many lncRNAs have tissue specific expression especially in cancer and could be used to predict prognosis of tumors as biomarkers [20, 21].

GAS6-AS1 is a novel lncRNA which is located at 13q34 and transcribed from the side chain of GAS6. Liang et al [22] first reported that GAS6-AS1 was dysregulated expression in non-small cell lung cancer and it was an independent prognosis factor. In our present study, we explored the correlation between lncRNA GAS6-AS1 expression level and clinicopathological characteristics as well as their prognosis of patients with breast cancer. To data, our study is the first time to report the expression level of GAS6-AS1 in breast cancer tissues and their matched NATs. Our results indicated that this lncRNA was down regulated in breast cancer tissues and breast cancer cell lines. This found shown that GAS6-AS1 may be a potential tumor suppressor in breast cancer. Meanwhile, we also found that decreased expression of GAS6-AS1 was associated with lymph node metastasis and histologic grade. These results indicated that lncRNA GAS6-AS1 might play an important role in the process of tumorigenesis and progression of breast cancer. However, the expression of GAS6-AS1 had no significant association with ER and PR status in our present study. Although the ROC curve indicated the potential function of diagnosis, more work is still needed in future research with a larger sample size to conform the function of lncRNA GAS6-AS1 as tumor suppressor and biomarker in breast cancer.

We also analyzed the influence of GAS6-AS1 expression level for overall survival and disease-free survival in breast cancer patients. As shown in the results section, patients in lower GAS6-AS1 group had worse survival than patients in higher GAS6-AS1 group for both OS and DFS. Furthermore, we analyzed the prognostic parameters for OS and DFS. The results showed that low expression of GAS6-AS1 was significantly associated with a worse OS and DFS, and we also found that GAS6-AS1 was an independent prognosis factor for OS in addition with histologic grade.

As we all know, a lot of factors can influence the expression of lncRNAs. For instance, Liu et al [23] reported that the lncRNA loc285194 was dysregulated in colorectal cancer. They found that p53 could regulate the expression of lncRNA loc285194 at the upstream region directly. The expression of lncRNAs could be also regulated by epigenetic modification, such as methylation, acetylation and microRNAs (miRNAs). Recently, the competing endogenous RNA (ceRNA) attracted focus of researchers on the research about cancer. As an important

part of ceRNA, miRNAs could influence the expression of lncRNAs through binding with their special binding sits [24]. Meanwhile, the dysregulation of lncRNAs might induce the expression of mRNA in several kinds of cancers. For example, lncRNA MALAT1 could combine with SR-clan splicing proteins and lead to tumors through controlling the splicing channel of pre-mRNA [25] Liang et al [26] found that the expression of GAS6-AS1 was associated with GAS6 in non-small cell lung cancer. At the same time, GAS6 was reported upregulated in breast cancer. All these evidence might provide direction for us in the further study on GAS6-AS1, that lncRNA GAS6-AS1 might influence the tumorigenesis and progression of breast cancer visa binding with GAS6 gene.

Conclusions

Our present study firstly reported that lncRNA GAS6-AS1 was dysregulated expression in breast tumor and was an independent prognosis factors for the patients. The results from our study might also provide potential biomarker associated with diagnosis of breast cancer. However, more studies on the molecular mechanism are still needed in the future.

Disclosure of conflict of interest

None.

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Supplementary Table 1. The original data for analysis

Sample number	ΔCtT	ΔCtN	$2^{-\Delta\Delta Ct}$	Year	Tumor size	Menopausal status[a]	Histologic grade[b]	Node status[c]	pTNM stage	ER[c]	PR[c]	HER2	Survival	Survival status	DFS	DFS status
1	2.19	4.12	3.8106	43	1.00	N	P	N	2	N	P	2	69	0	69	0
2	4.58	3.37	0.4338	45	5.00	N	G	P	3	P	N	1	69	0	69	0
3	2.88	6.80	15.0671	55	3.50	Y	P	N	2	P	P	2	69	0	69	0
4	4.06	2.47	0.3314	33	3.00	N	G	P	3	P	P	2	69	0	69	0
8	3.93	4.23	1.2340	40	5.00	N	P	N	2	N	P	1	69	0	69	0
9	5.61	4.67	0.5224	50	6.00	Y	P	P	4	N	P	2	52	1	40	1
10	6.69	5.96	0.6029	65	2.00	Y	P	P	4	N	N	1	50	1	41	1
11	6.81	8.88	4.2086	56	7.00	Y	P	N	2	N	N	2	69	0	69	0
12	21.65	20.73	0.7457	60	7.50	Y	P	P	3	P	N	2	69	0	69	0
13	9.83	7.12	0.1525	43	1.00	N	G	P	3	N	P	1	73	0	73	0
14	7.89	2.24	0.0199	58	1.00	Y	G	P	4	P	P	2	45	1	33	1
15	8.39	6.33	0.2407	44	7.50	N	G	P	3	N	N	1	73	0	58	1
16	7.92	6.37	0.3404	59	1.00	Y	G	P	3	N	N	2	69	0	69	0
17	10.52	6.83	0.0773	47	0.50	N	G	N	1	P	P	1	60	1	57	1
18	7.85	4.66	0.1093	52	1.30	Y	P	P	3	N	N	1	73	0	73	0
19	6.94	5.37	0.3367	60	2.00	Y	P	N	1	N	P	1	71	0	71	0
20	8.23	6.89	0.3975	65	2.50	Y	P	P	4	P	N	1	55	1	45	1
21	9.20	6.73	0.1801	32	7.00	N	G	P	4	P	N	2	54	1	50	1
22	7.76	6.17	0.3320	41	3.00	N	G	P	3	P	P	2	44	1	35	1
24	4.27	6.76	5.6158	42	1.00	N	P	N	1	N	P	2	73	0	73	0
25	5.06	8.80	13.3494	64	1.50	Y	P	N	1	N	N	1	73	0	73	0
26	4.34	3.07	0.4160	51	7.00	Y	G	P	3	P	P	2	69	0	69	0
27	5.40	3.95	0.3665	56	7.50	Y	G	P	3	P	P	1	73	0	73	0
28	4.83	2.99	0.2793	50	2.00	N	G	P	4	N	N	2	73	0	73	0
29	7.55	2.73	0.0354	41	3.50	N	G	N	2	N	N	1	59	1	48	1
30	2.40	3.26	1.8085	30	1.00	N	P	P	3	P	P	2	73	0	73	0
31	7.88	3.15	0.0377	70	0.50	Y	G	P	1	P	P	2	44	1	39	1
32	6.63	6.48	0.9038	63	2.50	Y	P	P	2	P	P	1	69	0	69	0
33	8.40	3.04	0.0244	58	5.00	Y	P	N	2	P	N	2	48	1	44	1
34	6.01	5.76	0.8424	24	1.00	N	P	N	3	N	N	1	72	0	72	0
35	6.55	6.07	0.7180	50	0.50	N	P	P	4	N	N	2	73	0	73	0
36	3.15	3.30	1.1052	50	1.00	N	P	N	4	N	N	1	72	0	72	0
37	5.50	5.17	0.7945	36	2.50	N	P	N	2	P	P	2	69	0	69	0

38	7.79	6.00	0.2902	58	5.50	Y	P	P	1	P	P	2	45	1	34	1
39	5.50	7.54	4.1122	53	3.00	N	P	P	3	N	N	2	73	0	73	0
40	6.23	5.78	0.7276	60	2.20	Y	P	N	3	N	P	1	42	1	39	1
41	6.62	5.99	0.5158	41	6.00	N	P	N	1	N	N	2	71	0	71	0
42	10.04	5.52	0.0428	58	2.50	Y	G	P	3	N	N	1	46	1	46	1
43	6.50	5.44	0.4800	43	4.00	N	G	N	1	N	N	1	71	0	71	0
44	8.50	7.50	0.4991	47	5.00	N	G	P	3	P	P	2	59	1	43	1
45	9.78	6.88	0.1342	58	3.50	Y	G	P	2	N	P	1	69	0	69	0
46	4.06	4.47	1.3308	27	1.00	N	P	N	3	P	N	2	75	0	5	0
47	5.51	5.96	1.3632	41	4.50	N	P	N	2	N	P	1	71	0	71	0
48	6.62	4.42	0.2174	58	1.50	Y	G	P	4	P	P	2	73	0	73	0
49	2.51	3.93	2.6736	39	1.00	N	P	N	2	P	P	1	70	0	70	0
50	7.44	3.85	0.0830	52	4.00	N	G	P	3	N	N	2	55	1	47	1
51	6.50	6.09	0.7535	78	3.00	Y	P	N	2	N	N	1	69	0	69	0
52	10.67	6.98	0.0777	46	0.50	N	G	P	4	N	P	2	37	0	73	0
53	5.91	5.58	0.7979	66	4.50	Y	P	N	2	N	P	1	69	0	69	0
54	6.56	6.21	0.7848	35	4.00	N	P	P	3	P	N	2	73	0	73	0
55	10.33	5.21	0.0287	65	4.50	Y	P	N	2	P	N	1	69	0	69	0
56	4.61	6.00	2.6313	42	2.00	N	P	P	3	P	P	2	65	0	65	0
58	5.56	5.59	1.0218	44	1.00	N	P	N	2	P	P	2	63	0	63	0
60	7.63	2.40	0.0266	52	3.00	Y	G	P	3	P	N	2	73	0	73	0
61	7.11	6.74	0.7716	69	1.60	Y	G	N	2	P	P	2	42	1	38	1
62	6.46	6.75	1.2250	61	1.50	Y	G	P	4	N	N	1	69	0	69	0
63	5.51	6.66	2.2327	38	4.00	N	P	P	4	N	N	1	54	1	47	1
64	4.22	5.76	2.9033	47	2.00	Y	P	P	3	P	P	1	81	0	81	0
65	9.79	5.83	0.0644	63	0.50	Y	G	P	4	P	P	1	69	0	58	1
66	8.79	3.48	0.0253	77	4.00	Y	G	P	3	N	N	2	46	1	40	1
67	4.05	3.17	0.5442	78	4.00	Y	P	P	3	N	N	1	69	0	69	0
68	2.93	1.85	0.4736	56	1.70	Y	P	P	4	P	P	2	67	0	67	0
69	6.83	4.95	0.2711	60	2.00	Y	P	N	2	P	P	1	56	1	53	1
70	6.20	6.43	1.1683	65	2.00	Y	P	N	1	N	N	2	73	0	73	0
71	6.48	2.77	0.0767	45	2.00	N	P	N	1	P	P	2	67	0	67	0
73	7.08	4.40	0.1559	66	1.00	Y	G	P	3	N	P	2	67	0	67	0
74	2.54	5.66	8.6710	63	3.00	Y	G	N	2	N	P	1	54	1	54	1
75	3.12	5.35	4.7145	63	6.00	Y	P	N	2	P	N	2	67	0	67	0
76	2.52	3.27	1.6819	65	0.50	Y	P	N	2	P	P	1	73	0	73	0

78	8.20	3.23	0.0317	61	4.00	Y	G	P	4	N	P	2	54	1	41	1
92	2.50	0.46	0.2426	58	2.00	Y	G	P	3	P	P	1	73	0	73	0
93	3.28	4.14	1.8140	32	1.50	N	G	P	3	N	N	2	69	0	69	0
94	2.79	2.97	1.1347	45	1.00	N	P	N	1	P	P	1	67	0	67	0
96	3.05	1.94	0.4657	56	2.00	Y	P	N	1	N	N	2	69	0	69	0
97	5.32	2.44	0.1359	55	1.50	Y	P	P	3	P	P	1	73	73	0	1
98	0.67	2.11	2.7132	34	1.40	N	G	P	4	P	P	2	73	0	73	0
99	5.38	2.90	0.1790	45	2.10	N	P	P	4	P	P	2	69	0	69	0
100	2.95	3.26	1.2374	39	1.50	N	P	N	2	P	P	2	73	0	73	0
101	6.92	5.75	0.4440	66	5.00	Y	P	P	3	N	N	2	60	0	60	0
102	6.95	3.38	0.3367	56	5.90	Y	G	P	3	N	N	2	69	0	69	0
103	3.78	2.82	0.5129	54	2.20	Y	G	P	4	N	P	1	73	0	73	0
104	2.46	2.94	1.8603	47	6.00	N	G	N	1	P	P	1	69	0	69	0
105	4.76	0.25	0.0438	68	5.50	Y	P	N	2	P	N	1	48	1	42	1
106	6.02	1.45	0.0422	77	5.50	Y	P	N	2	N	P	1	57	1	50	1
107	2.68	2.68	1.0031	76	1.00	Y	G	P	3	P	P	2	60	0	58	1
108	8.74	2.89	0.0174	65	3.00	Y	G	P	3	N	N	2	49	1	41	1
109	8.17	5.01	0.1114	46	7.00	N	G	P	3	P	P	1	57	1	45	1
110	4.82	4.63	0.8727	69	2.00	Y	G	P	4	P	N	2	69	0	69	0
111	3.36	1.87	0.3549	53	3.00	N	P	N	1	N	P	1	69	0	58	1
112	3.88	6.07	4.5751	55	3.00	Y	P	N	1	N	N	2	69	0	69	0

a, Y indicated yes and N indicated no; b, G indicated good and P indicated poor; c, P indicated positive and N indicated negative.