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Original Article

High expression of Sirt7 served as a predictor of adverse outcome in breast cancer

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Abstract: Objective: Sirt7, as one of the seven Sirtuin family members, which plays distinct roles in cancer progression, is bringing emerging attention due to its oncogenic characteristic. The expression of Sirt7 in breast cancer remained unclear, and the aim of this study was to elucidate its role in breast cancer. Methods: A total of 188 cases included in this study were immunohistochemically evaluated for Sirt7, and western blot assay was used to assess its expression in breast cell lines as well as 36 breast cancer tissues and 36 paired non-cancerous tissues. Results: Upregulation of Sirt7 was found in breast cancer cell lines and breast cancer tissues ($P < 0.001$) by western blot analysis. Sirt7 was highly expressed in breast cancer tissue samples (67.8%) compared to adjacent normal breast tissues (31.8%) by immunohistochemical assay. It was also observed that the high expression level of Sirt7 was significantly correlated with high histological grade ($P = 0.039$) and negatively related to overall survival ($P = 0.006$). Sirt7 proved to be an independent prognostic factor ($P = 0.007$) in breast cancer. Conclusions: Sirt7 expression was implicated with high histological grade and independently predicted poor clinical outcome in patients with breast cancer, suggesting that Sirt7 might play a role in the malignant progression of breast cancer.

Keywords: Breast cancer, immunohistochemistry, oncogenes, tumour markers

Introduction

Breast cancer is the most frequent malignancies for females in China, whereas it enjoys a relatively favourable prognosis, being the fourth common cause of cancer-related mortality [1]. According to its various immunophenotypes, breast cancer is known to be a very heterogeneous disease [2]. Different subtypes of breast cancer that are classified based on ER/PR and Her2 status, lead to distinct outcomes [3]. More and more pathological indicators have been proved to be capable of predicting overall survival of patients with breast cancer in recent years.

Sirtuins are NAD-dependent deacetylases, most subtypes of which have been clearly studied in catalytic activities, while Sirt7 has been the least well understood for decades [4]. Sirtuins have been indicated to be associated with cellular aging, cell cycle and metabolism, which aroused researchers' interest on their roles in tumorigenesis [5]. Sirt7 was reported to locate and function in nucleus, but according to an article published lately, there was a role for Sirt7 in cytoplasm as well [6]. It was found that this protein possessed a steady-state cytoplasmic pool, possibly suggestive of a result from postranslational modification. Besides, the presence of nucleolar Sirt7 in young fibroblast was prominent, but it decreased as cell aged. Furthermore, nucleolar Sirt7 was indicated to be associated with replicative senescence in fibroblasts.

Sirt7 has been reported to be involved in several cancers [7-11], but the mechanism how Sirt7 promotes the cancer process remains veiled. However, some views have been proposed regarding how Sirt7 is linked to tumorigenesis [12]. It was suggested that Sirt7, localized to chromatin, might deacetylate the tumor suppressor p53. But the protein was eventually

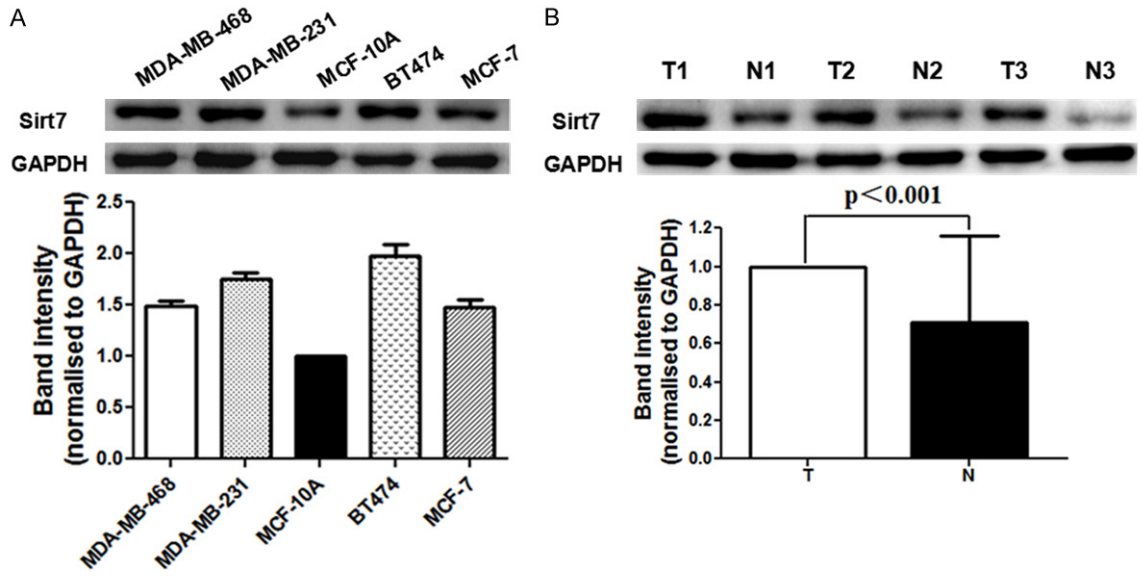

Figure 1. A. Expression of Sirt7 in 5 breast cancer cell lines and 1 breast epithelial cell line in Western Blot assay. The mean expression level of Sirt7 in MDA-MB-468, MDA-MB-231, BT-474 was significantly high, while MCF-7 and MCF-10A had lower mean expression level. GAPDH was used as a control. B. 3 representative pairs of breast cancer and adjacent tissues in Western blot analysis. T stood for cancer, N stood for non-cancer. Cancer tissues had more abundant expression of Sirt7 in comparison to their non-tumoral counterparts. GAPDH was used as a control (P = 0.014, pair t test).

found to scarcely have deacetylase activity on p53 [13]. Recently a published article pointed out that, Sirt7 deacetylated H3K18Ac selectively, depletion of which leaded to aggressive cancer phenotypes and poor prognosis of patients with prostate cancer. And the deacetylation of H3K18Ac was the key to keep the essential features of cancer cells, while the overexpression of Sirt7 did not bring malignant transformations [14]. Sirt7 also regulates the RNA Pol I machinery, but how it mediates tumorigenesis remains to be elucidated [15]. A research aiming to investigate the influence of Sirt7 on hepatocellular carcinoma showed that, knockdown of Sirt7 inhibited in vitro liver cancer cell growth, and reduced the tumor growth rate in a mouse xenograft model. Further study on the relevant mechanism suggested that depletion of Sirt7 restored transcriptional activation of $p21^{WAF1/Cip1}$ through miR-125a-5p and miR-125b, and this brought cell cycle arrest to liver cancer cell lines [7]. This illuminated in another way how Sirt7 promoted cancer progression.

It has been found that the expression of Sirt7 was increased in the breast cancer cell line, MCF-7, the primary mammary epithelial cell line HMEC, as well as the immortalized non-tumorigenic MCF-12A breast cell line by polymerase chain reaction (PCR) analysis. In addition, Sirt7 was implicated with node-positive breast cancer, indicating a possible role in breast cancer metastasis [11]. Furthermore, Sirt7 was found overexpressed in colorectal cancer, and correlated with tumor stage and poor patients' survival [8]. Nevertheless, the role of Sirt7 in breast cancer is yet to be elucidated, and whether it can be a prognostic factor in patients with breast cancer remains unclear.

Materials and methods

Cell lines and sample collection

Breast epithelial cell line MCF-10A and metastatic breast cancer cell lines including MCF-7, MDA-MB-231, BT474 and MDA-MB-468 were purchased from American Type Culture Collection (ATCC) and preserved in Central Laboratory in Southern Medical University (Nan Fang Hospital). MDA-MB-231 and BT474 were maintained in RPMI 1640 medium (Hyclone) containing 10% fetal bovine serum (Thermo). MCF-10A, MCF-7 and MDA-MB-468 were maintained in DMEM medium (Hyclone) containing 10% fetal bovine serum. All the cell lines were incubated in a humidified chamber with 5% CO_2

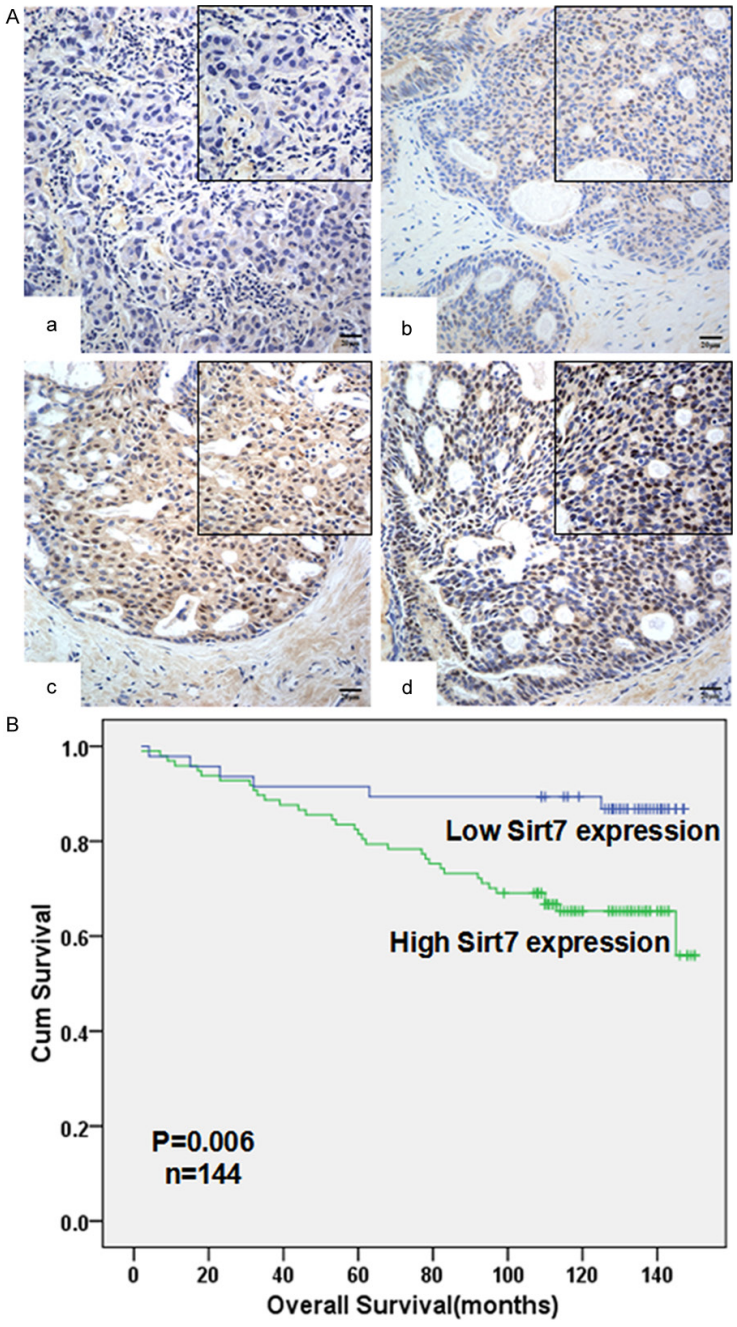

Figure 2. A. Expression of Sirt7 in breast cancer tissues. **Figure 2A**, **2B** showed low nucleolar expression of Sirt7 in breast cancer tissues while **Figure 2C**, **2D** represented high ones. Scale bar, 20 μm. B. Kaplan-Meier survival curve, with data from 144 patients with breast cancer. Patients with breast cancer expressing low level of Sirt7, achieved better survival compared to those with high expression of Sirt7 (P = 0.006, log-rank test).

at 37°C. A total of 180 paraffin-embedded breast cancer and 44 adjacent normal tissue samples were obtained from the Nanfang Hospital Affiliated to Southern Medical University, Guangzhou, China. These cases were from 188 females with ages between 28 and 83 years. Prior consent from patients and approval from the Ethics Committees of Nanfang Hospital was obtained for using these clinical materials for research purposes. All specimens had confirmed pathological diagnosis and were classified according to the American Joint Committee on Cancer (AJCC) criteria.

Western blot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0; 1 mM ethylenediaminetetraacetic acid, pH 8.0; 5 mM DTT; 2% sodium dodecyl sulfate (SDS)), and protein concentration was determined using BCA assay (Beyotime, Beijing, China). Total protein (30 μg) was resolved with the use of a 10% SDS-polyacrylamide gel electrophoresis and electro-transferred to polyvinylidene fluoride membranes (Invitrogen). Membranes were blocked with 3% BSA in Tris-buffered saline (pH 7.5), followed by immunoblotting overnight at 4°C with Sirt7 (1:1000, Proteintech Group, Inc, Chicago, USA) or GAPDH (Santa Cruz Biotechnology, CA, USA) antibody. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody (Zhongshan, Beijing, China). Signals were detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Immunohistochemistry and evaluation of staining

Paraffin sections (4 μm) from samples were deparaffinized in 100% xylene and rehydrated in descending ethanol series and water according to standard protocols. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100°C. Endogenous peroxidase activity

Table 1. Expression of Sirt7 protein in the breast cancer tissues and normal breast tissues

Pathological Type	n	Sirt7 Expression		X^2	*P* value
		Low (n%)	High (n%)		
Breast Cancer Tissue	180	58 (32.2)	122 (67.8)	19.168	1.197E-5
Normal Breast Tissue	44	30 (68.2)	14 (31.8)		

and nonspecific antigen were blocked with peroxidase-blocking reagent containing 3% hydrogen peroxide and serum, followed by incubation with rabbit anti-human Sirt7 antibody (1:100) for overnight at 4°C. After washing, the sections were incubated with biotin-labeled goat anti-rabbit antibody for 20 min at room temperature, and subsequently were incubated with streptavidin-conjugated horseradish peroxidase (Maixin, Fuzhou, China). The peroxidase reaction was developed using 3, 3-diaminobenzidine chromogen solution in DAB buffer substrate. Sections were visualized with DAB and counterstained with hematoxylin, mounted in neutral gum and analyzed using a bright field microscope. IHC results were assessed by two independent pathologists who were blinded to the origination of the samples and subject outcome. The nuclear staining results for Sirt7 protein was semi-quantitatively expressed by an immunohistochemical score combined with the percentage of tumor cells showing specific immunoreactivity [16, 17]. Each specimen was assigned a score according to the percentage of positive cells (none, 0; 1%-9%, 1; 10%-50%, 2; 51-100%, 3), and staining intensity (none, 0; weak, 1; moderate, 2; strong, 3). The staining intensity and average percentage of positive tumor cells were assayed for 10 independent high magnification (× 400) fields. Points for percentage of positive cells and staining intensity were multiplied, and these samples were then attributed to two groups according to their overall score: low expression, 0-4 points; and high expression, 6-9 points.

Statistical analysis

All quantified data represented an average of at least three samples. SPSS 16.0 and Graph Pad Prism 5.0 software were used for statistical analysis. All data are expressed as mean ± SD. Significance was established by paired t-test or Chi square test as appropriate. Kaplan-Meier and log-rank tests were used to compare patient survival and to create survival curves based on high and low Sirt7 IHC scores. Multivariate survival analysis was performed for all parameters that were significant in univariate analyses using the Cox regression model. $P < 0.05$ was considered statistically significant.

Results

Sirt7 expression was upregulated in human breast cancer cell lines and breast cancer tissues

Sirt7 expression was first examined by western blotting in one breast epithelial cell line and four breast cancer cell lines. Among the four breast cancer cell lines, MDA-MB-231, BT474 and MDA-MB-468 showed highest expression of Sirt7, while MCF-7 demonstrated relatively lower Sirt7 expressions (**Figure 1A**). Furthermore, Sirt7 protein levels of surgical samples were investigated by western blotting and found to be upregulated in 36 cases of breast cancer tissues, as compared to 36 matched adjacent normal breast tissues ($P < 0.001$) (**Figure 1B**).

In order to determine Sirt7's expression level and its correlation with other indexes in surgical samples, we performed immunohistochemical studies on Sirt7 protein in 180 archived paraffin-embedded breast cancer samples and 44 adjacent non-cancerous specimens. The representative results from the IHC analysis were shown in **Figure 2A**. Sirt7 protein was highly expressed in 67.8% (122/180) of breast cancer tissues, while only in 31.8% (14/44) of adjacent normal breast tissues ($P < 0.001$) (**Table 1**).

Sirt7 was correlated with patient's age and histological grade

Then we determined the relationship between Sirt7 expression and the clinicopathological characteristics of 180 breast cancer tissue samples with associated clinical details (**Table 2**). We observed that expression level of Sirt7 was significantly correlated with patient's age ($P = 0.008$) and histological grade ($P = 0.039$). Breast cancer tissues from elder females were expected to have higher Sirt7 expression level, and tumors with lower pathological grade tend-

Table 2. Relationship between clinicopathological characteristics and Sirt7 expression levels in individuals with breast cancer (n = 180)

Pathological Parameters		n	Sirt7 Expression		X^2	*P* value
			Low (n%)	High (n%)		
Age (years)	≤ 50	92	38 (41.3)	54 (58.7)	7.107	0.008
	> 50	88	20 (22.7)	68 (77.3)		
Tumor Size (cm)	T1-2 (≤ 5)	162	51 (31.5)	111 (68.5)	0.407	0.523
	T3 (> 5)	18	7 (38.9)	11 (61.1)		
TNM Stage	I	104	35 (33.7)	69 (66.3)	0.284	0.868
	II	65	20 (30.8)	45 (69.2)		
	III	11	3 (27.3)	8 (72.7)		
Histological Grade	G1	42	19 (45.2)	23 (54.8)	4.250	0.039
	G2-3	139	39 (28.3)	99 (71.7)		
Lymph Node Metastasis#	N0-1	127	42 (33.1)	85 (66.9)	0.142	0.706
	N2-3	53	16 (30.2)	37 (69.8)		
ER	(-)	60	21 (35.0)	39 (65.0)	0.318	0.573
	(+)	120	37 (30.8)	83 (69.2)		
PR	(-)	88	29 (33.0)	59 (67.0)	0.042	0.837
	(+)	92	29 (31.5)	63 (68.5)		
HER2	(-)	136	46 (33.8)	90 (66.2)	0.653	0.419
	(+)	44	12 (27.3)	32 (72.7)		
Ki67	(-)	113	37 (32.7)	76 (67.3)	0.038	0.846
	(+)	67	21 (31.3)	46 (68.7)		

ed to express less Sirt7. We also noticed a trend but not statistically significant that high level of Sirt7 often occurred with advanced TNM stage and more lymph node metastasis. However, we did not find any significant associations between Sirt7 expression and tumor size, ER status, PR status, HER2 status or Ki67.

Upregulated expression of Sirt7 was associated with decreased survival in breast cancer

We also investigated the prognostic value of Sirt7 expression for breast cancer patients using Kaplan-Meier analysis with the log-rank test. In 144 breast cancer cases, we observed that the level of Sirt7 protein expression was significantly correlated with overall survival, as patients with higher Sirt7 expression had worse survival (P = 0.006) (**Figure 2B**). Univariate analysis displayed that advanced TNM stages, more lymph node metastasis and ER-negative status were also significantly correlated with decreased survival (P = 0.030, P = 0.023 and P = 0.004, respectively) (**Table 3**). To determine whether Sirt7 is an independent prognostic factor for breast cancer, we performed multivariate analysis of Sirt7 expression level adjusted for TNM stage, lymph node metastasis, ER status of breast cancer patients using Cox proportional-hazards model. The results indicated that increased Sirt7 expression was a prognostic factor for predicting poor outcomes of patients with breast cancer (P = 0.007) (**Table 4**).

Discussion

Recent studies have related Sirtuin Family to malignant progression of tumors, and Sirt7 was shown to be involved in the lymph node metastasis of breast cancer [11]. But the relation between this protein and the prognosis of breast cancer has not been investigated yet. In our study, we observed an overwhelming expression of Sirt7 in breast cancer tissues compared to adjacent normal breast tissues in both immunohistochemical assay and western blot analysis. And in breast cell lines, Sirt7 tended to have higher expression levels in more invasive cell lines, while in MCF-10A, a non-tumorigenic breast epithelial cell line, Sirt7 was poorly expressed. All these results suggested that Sirt7 might function as an oncogene in breast cancer, and might participate in tumor inva-

Table 3. Univariate prognostic factors in Kaplan-Meier survival analysis (n = 144)

Characteristics	N	Deaths	Mean	SE	Log-rank	*P* value
Age (years)						
≤ 50	68	14	128.630	5.472	2.953	0.086
> 50	76	26	119.237	5.290		
Tumor Size (cm)						
T1 (≤ 2)	32	7	129.448	6.341	3.421	0.181
T2 (2-5)	98	27	125.648	4.306		
T3 (> 5)	14	6	90.786	17.755		
TNM Stage						
I-II	97	22	130.600	3.851	4.719	0.030
III	47	18	108.364	8.099		
Histological Grade						
G1	39	9	126.446	6.880	0.709	0.400
G2-G3	105	31	122.164	4.548		
Lymph Node Invasion						
N0-N1	101	23	130.080	3.879	5.171	0.023
N2-N3	43	17	107.073	8.375		
ER						
Negative	51	21	107.588	7.359	8.255	0.004
Positive	93	19	131.676	4.036		
PR						
Negative	73	24	117.536	5.891	2.215	0.137
Positive	71	16	130.013	4.727		
HER2						
Negative	116	34	123.128	4.209	0.443	0.506
Positive	28	6	126.357	9.064		
Ki67						
Negative	104	29	124.033	4.461	0.003	0.957
Positive	40	11	123.125	7.401		
Sirt7						
Negative	47	6	133.715	5.463	7.516	0.006
Positive	97	34	117.559	4.868		

Table 4. Prognostic factors in the Cox proportional hazards model (n = 144)

Parameters	RR	95% CI	Wald	*P* value
Age (years)				
Tumor Size (cm)				
Lymph Node Metastasis#	1.876	0.240-14.668	0.360	0.549
Histological Grade				
TNM Stage	0.998	0.129-7.738	3.110E-6	0.999
ER	0.418	0.222-0.786	7.332	0.007
PR				
HER2				
Ki67				
Sirt7	3.342	1.392-8.020	7.296	0.007

P < 0.05: indicating statistical significance; RR: relative risk; Wald: Wald value.
#: Including two groups, N0-N1 and N2-N3.

sion, which has been shown in the study of colorectal cancer [8].

In our Kaplan-Meier survival analysis, patients with higher Sirt7 expression levels had poorer prognosis in comparison to patients with lower ones. Further Sirt7 was found to predict poor outcome in breast cancer independently in multivariate analysis. Thus we tried to connect this result with established studies on Sirt7. It has been noted that overexpression of Sirt7 was responsible for inactivation of H3-K18Ac, which contributed to maintaining aggressive features of prostate cancer cells and predicted risk of prostate cancer recurrence [18]. And it has been reported that lower levels of H3K18Ac correlated significantly with poorer survival probabilities in both lung and kidney cancer patients. We found in our study that Sirt7 located dominantly in cell nucleus of breast cancer, probably by which Sirt7 functioned as a NAD-dependent H3K18Ac deacetylase. In addition, Sirt7 was associated with high histologic grade. Therefore, it was reasonable to assume that, Sirt7 reduced survival of patients with breast cancer by deacetylating H3K18Ac. However, we still need further experiments to investigate the possible downstream factors, and how these factors interact with each other.

We also showed that, breast cancer tissues with higher grade and from patients of older age displayed increased Sirt7 expression. In vitro study on colorectal cancer indicated that Sirt7 promoted epithelial mesenchymal transition (EMT) [8], and loss of epithelial features and transition into mesenchymal-like cells were concerned with invasion of breast cancer and higher histologic grade. This might partly explain how Sirt7 became a negative prognostic factor for patients with breast cancer. However, how Sirt7 had effects on cancer

grade was still unknown to us, and further study at cellular level might possibly elucidate this process. We also saw a trend that, upregulated Sirt7 was related to advanced TNM stage and lymph node metastasis status. But we need more clinical samples to confirm whether there is a significant trend between Sirt7 and TNM stage or lymph node metastasis. Sirt7 has been linked to the promotion of the liver cancer cell growth [7], but we were not able to find an obvious relation between Sirt7 and Ki67, a pathological index indicating the capability of cancer proliferation. Neither did we see any significant correlation between Sirt7 and Her2 or ER status, which are associated with the outcome of breast cancer [19, 20]. Possibly Sirt7 had impact on survival of patients with breast cancer independent of Her2 or ER status.

In summary, we found in our study that Sirt7 was abundantly expressed in both breast cancer cell lines and tissues, while their non-cancerous counterparts showed relatively low expression level. Sirt7 was supposed to independently predict an unfavorable prognosis in breast cancer, as well as associated with advanced cancer histologic grade. Furthermore, Sirtuins as drug targets in malignant tumors have been preliminarily studied on [21], and an inhibitor targeting Sirt7 may be a promising medicine for breast cancer in the future.

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Disclosure of conflict of interest

None.

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