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Original Article

High Survivin and Low Zinc Finger of the Cerebellum 1 Expression Indicates Poor Prognosis in Triple-negative Breast Carcinoma

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ABSTRACT

Purpose: Triple-negative breast carcinoma (TNBC) is accompanied with high risk of metastasis and recurrence. The present study aimed to explore the clinicopathological and prognostic roles of putative tumor-related genes in patients with TNBC.
Methods: Thirty pairs of frozen-thawed tumors were used to select reliable indicators via real-time quantitative polymerase chain reaction (RT-qPCR). Then, 150 pathology specimens were used to evaluate the expression of proteins in TNBC through immunohistochemistry. In addition, Kaplan-Meier curves and Cox regression analysis were also performed to analyze the overall survival and disease-free survival.
Results: RT-qPCR results indicated that among all the proteins analyzed using fresh-frozen TNBC samples, the expression levels of only Survivin and zinc finger of the cerebellum 1 (ZIC1) were obviously different from those in the corresponding normal tissues. Survivin and ZIC1 expression had opposite effects on the clinicopathological diagnosis and prognostic assessment in TNBC patients. Further, there was a negative correlation between Survivin and ZIC1 expression. In addition, the "Survivin-positive ZIC1-negative group" was associated with histologic grade, lymph node metastasis, and TNM staging ($p < 0.001$) and this was also an independent factor for evaluating the prognosis of TNBC in patients.
Conclusion: In summary, the expression levels of Survivin and ZIC1 in TNBC are different from those in normal tissues and are negatively correlated mutually. The combined detection of Survivin and ZIC1 expression levels could allow better comprehensive diagnosis and prognostic evaluation for TNBC patients.

Keywords: Prognosis; Survivin; Triple negative breast neoplasms; ZIC1 protein, human

INTRODUCTION

Breast carcinoma is one of the leading causes of cancer-related deaths among women and infiltrative ductal carcinoma is one of the most common types of breast cancer [1]. Breast cancer is defined as triple-negative breast carcinoma (TNBC) when there is simultaneous loss of expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal

Conflict of Interest
The authors declare that they have no competing interests.

Author Contributions
Data curation: Shi CT; Funding acquisition: Shi CT; Investigation: Shi CT, Ma J, Shi QF, Zhang Y, Wang HN; Methodology: Shi CT, Shi QF, Zhang Y, Wang HN; Project administration: Shi CT; Software: Ma J, Shi QF, Zhang Y, Wang HN; Supervision: Ma J, Zhang Y; Writing - original draft: Shi CT, Ma J; Writing - review & editing: Wang HN.

growth factor receptor (HER2) and this is an aggressive breast cancer subtype characterized by poor prognosis [2]. Although patients with TNBC are subjected to major treatments such as radical mastectomy and postoperative chemotherapy, the prognosis remains dismal mainly due to metastasis and recurrence [3]. Thus, it is critical to develop novel markers for the prognostic evaluation of patients with TNBC.

Recent studies have reported that inhibitors of apoptosis (IAP) proteins and eukaryotic initiation factor 3 (eIF3) subunits promote growth and invasion of breast cancer cells by regulating several signaling pathways, such as Akt/PI3K/mTOR, JAK/STAT and SAPK/JNK [4-6]. On the other hand, non-metastatic genes (such as *NM23* genes) conspicuously suppress the metastasis of breast cancer cells but are also related to cell growth, differentiation and tumor pathogenesis [7,8]. A few studies have revealed that the zinc finger of the cerebellum (ZIC) family proteins are associated with the development and progression of breast cancer [9,10]. Dysregulation of these proteins has been associated with metastasis and poor prognosis in patients with breast carcinoma [11-14]. These reports imply that the expression of IAPs, eIF3 subunits, *NM23* genes and ZIC family proteins are essential for carcinogenesis and can be targeted by novel therapies against breast cancer.

As breast cancer has remarkable inductive or suppressive effects on the expression of several proteins, our research aimed at selecting reliable indicators and analyzing their correlativity using real-time quantitative polymerase chain reaction (RT-qPCR). Moreover, these potential bio-markers were detected in 150 cases of triple-negative breast infiltrative ductal carcinoma evaluated through immunohistochemistry (IHC) which was conducted to explore the clinicopathological and prognostic roles of these putative indicators in triple-negative breast infiltrative ductal carcinoma patients.

METHODS

Tissue samples

After obtaining ethics approval from the Wuxi Xishan People's Hospital Ethics Committee (No. XSL2010008), 150 patients undergoing radical mastectomy at Wuxi Xishan People's Hospital from 2010 to 2012 were enrolled for this study. Every patient signed an informed consent form. The selection criteria for this study with consecutive patient participation is listed as follows: 1) patients were diagnosed with triple-negative breast infiltrative ductal carcinoma; 2) complete clinical and pathological data were available; and 3) no chemotherapy or radiotherapy was done before surgery. While all the patients in this study received radical mastectomy, they did not undergo breast-conserving surgery. All of the patients received doxorubicin (60 mg/m^2) and cyclophosphamide (600 mg/m^2) for the first 4 cycles and paclitaxel (80 mg/m^2) for the next 4 cycles after surgery. A single cycle of chemotherapy lasted for 21 days. The mean age of the patients was 54.82 ± 13.07 years and the median follow-up duration was 60 months (3–60 months). Then, 30 pairs of fresh-frozen TNBC samples and matched normal tissues were obtained from Wuxi Xishan People's Hospital and these were also used for mRNA extraction.

Real-time quantitative polymerase chain reaction

Total RNA was extracted using Trizol regent (Thermo Fisher Scientific, Waltham, USA), and 2 μg of RNA was reverse transcribed using the miScript II RT Kit (Invitrogen, Berkeley, USA). Then,

qPCR was performed using an iQ5 real-time PCR detection system (Bio-Rad, Berkeley, USA) and SYBR Premix Ex Taq™ kit (Takara, Tokyo, Japan). The PCR primers were designed as shown in **Table 1**. The PCR cycling conditions were as follows: 1) 94°C for 4 minutes, 2) 40 cycles of 95°C for 1 minute, 3) 60°C for 1 minute, and 4) 72°C for 1 minute. The relative gene expression was calculated using $2^{-\Delta\Delta Ct}$ and $\Delta\Delta Ct = (Ct_{Tumor\text{-}x} - Ct_{Tumor\text{-}GAPDH}) - (Ct_{Normal\text{-}x} - Ct_{Normal\text{-}GAPDH})$.

Immunohistochemistry and evaluation of immunohistochemical staining

Paraffin-embedded sections were used for Survivin and ZIC1 immunohistochemical staining with a SP Rabbit & Mouse HRP Kit (CWBIO, Beijing, China). Survivin (bs-0615R) and ZIC1 (bs-11609R) rabbit polyclonal antibodies (BIOSS, Beijing, China) were both diluted at a concentration of 1:200 in phosphate-buffered saline (PBS). PBS without primary antibodies was used as negative control. Two pathologists (Qi-Feng Shi and Ye Zhang) independently evaluated the scores through a semi-quantitative assessment system and assigned the immunoreactivity score (IRS), which was obtained by combining the score of staining intensity (0, no staining; 1, mild staining; 2, moderate staining; and 3, strong staining) and the percentage of cells ('0–100%' = '0–10'). Any disagreement was resolved by discussion. The IRS was calculated by multiplying the staining intensity and percentage of cells, and the protein expression was considered positive only when IRS > 10.

Statistical analysis

Pearson's χ^2 was used to analyze the association between protein expression and clinicopathological characteristics. Continuous variables expressed as x ± SD were analyzed by a paired *t*-test. Linear regression analysis and Pearson correlation analysis were used to assess the association between the protein expression levels. Cox regression analysis and Kaplan-Meier curves with log Rank test were used to analyze the overall survival (OS) and disease-free survival (DFS). A $p < 0.05$ was considered statistically significant. SPSS 20.0 (IBM, Armonk, USA) and GraphPad 6.0 (GraphPad Software, San Diego, USA) were used for statistical analyses.

Table 1. The PCR primers for the IAPs (Survivin and livin), eIF3 subunits, *NM23* genes, ZIC family genes and GAPDH

Genes	Forward primer (5′-3′)	Reverse primer (5′-3′)
Survivin	ATGTCCATTTTTCAGGTTCTCTAAG	GCACTGCTGTCTCTACTTTCC
Livin	GCACTTGGCACTGTCTTTAGG	CTGGGCATATTCTGAGATTGG
NME1	GAGGACCAGGCTGTAGGAAATC	GCAATGCAACAATATGAAGTAACCA
NME2	CCACCTCTTATTCATAGACCCA	AGATTCAAAGCCAGGCACCAT
NME3	ACCAACTTGAAGCCCTTCCTC	TGCTGACCATCTTCGCTAACC
eIF3α	GAACCAAAGAAGTCAAACGAG	GCGAGTCACAAAGGTTCTAAA
eIF3c	CTTCATCCATTCGTTCCACCA	GATCAAGTTCAATATCATCGCCTCT
eIF3d	TCGTGCTCACAACGGACAATA	GGAGGCAACCTACATCAACCA
eIF3e	TTGCTGATAGGGTGAGACTGC	GGTGGCTTGTCTTGAGGATTT
eIF3g	CTTTATGTTTCCGTTGATGAC	ATGCCTACTGGAGACTTTGAT
eIF3h	ATGGGATCATAAATGAGAACGAC	GGCTTGAAATTACCAACTGCT
ZIC1	GCGTCCTTTTGTGGATCTTTAA	AGTAATCACATCTGCTTCTGGG
ZIC2	ACACTCCTCCCAGAAGCAGAC	GCAACTGAGCAATCCCAAGAA
ZIC3	AGACTGTCCCGGATACCAAGC	CAACAGCAGCGACCGTAAGAA
ZIC4	GCCTTTTCCCAGAGGGTATTA	CCTTTCTTTCCTGATTTGTGC
ZIC5	TCCCCACTGATGAGTAACCAA	AAGAAACATTCCCATGTCCAC
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

PCR = polymerase chain reaction; IAP = inhibitors of apoptosis; eIF3= eukaryotic initiation factor 3; NM23 = non-metastatic 23; ZIC = zinc finger of the cerebellum; GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

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RESULTS

Expression of bio-markers in TNBC

We calculated the relative expression levels of IAPs, eIF3 subunits, *NM23* genes and ZIC family proteins using RT-qPCR to identify the differentially expressed genes which might contribute to the development or progression of TNBC (**Figure 1**). We observed that only the expression of Survivin was obviously higher in the tumors compared to that in the corresponding normal tissues ($p < 0.001$, **Figure 1**), whereas the expression of ZIC1 was significantly lower in the tumors than that in the normal tissues ($p < 0.001$, **Figure 1**). Using this method, we evaluated the Survivin and ZIC1 expression levels in 150 cases of TNBC.

Expression of Survivin and ZIC1 in TNBC

Survivin was expressed in both the nucleus and cytoplasm in all cases and therefore, the average score for the cytoplasm and nucleus was used to assess the IRS for Survivin ([IRScytoplasm+IRSnucleus]/2, **Figure 2A and B**). However, ZIC1 was expressed only in the cytoplasm (**Figure 2C and D**). The positivity rate of Survivin in TNBC was higher than that in normal tissues (80.7% vs. 19.3%, $p < 0.001$), whereas the positivity rate of ZIC1 in TNBC was lower than that in normal tissues (37.3% vs. 62.7%, $p < 0.001$). The average score of Survivin in TNBC (IRS = 17.88 ± 7.42) was significantly higher than that in normal tissues (IRS = 6.37 ± 4.22, $p < 0.001$, **Figure 2E**), while the average score of ZIC1 in TNBC (IRS = 7.89 ± 7.20) was obviously lower than that in normal tissues (IRS = 12.83 ± 3.97, $p < 0.001$, **Figure 2E**). In addition, there was a negative correlation between Survivin and ZIC1 expression ($r = -0.3768$, $p < 0.001$, **Figure 2F**).

Relationship between Survivin/ZIC1 and clinicopathologic parameters in TNBC

According to the IRS, 121 patients were included in the "Survivin-positive group" and 56 cases were enrolled in the "ZIC1 positive group." In **Table 2**, it was shown that the positive expression of Survivin was positively related to histologic grade ($p = 0.002$), lymph node metastasis ($p < 0.001$) and TNM staging ($p < 0.001$), while the positive expression of ZIC1 was negatively related to histologic grade ($p < 0.001$), lymph node metastasis ($p = 0.002$)

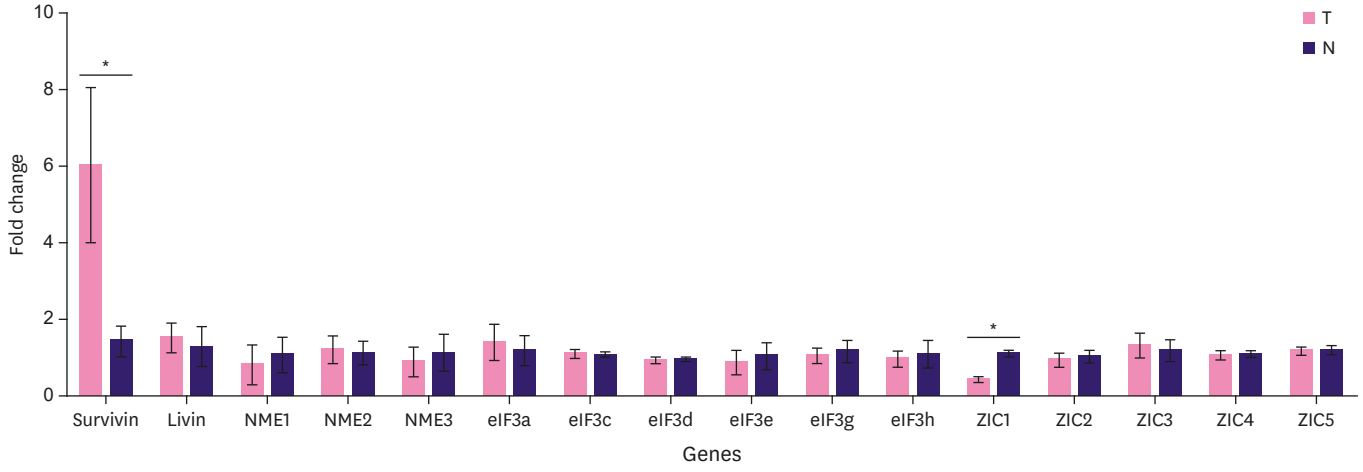

Figure 1. The result of RT-qPCR of IAPs (Survivin and livin), eIF3 subunits, *NM23* genes and *ZIC* family genes.
RT-qPCR = real-time quantitative polymerase chain reaction; IAP = inhibitors of apoptosis; eIF3 = eukaryotic initiation factor 3; NM23 = non-metastatic 23; ZIC = zinc finger of the cerebellum.
*$p<0.001$.

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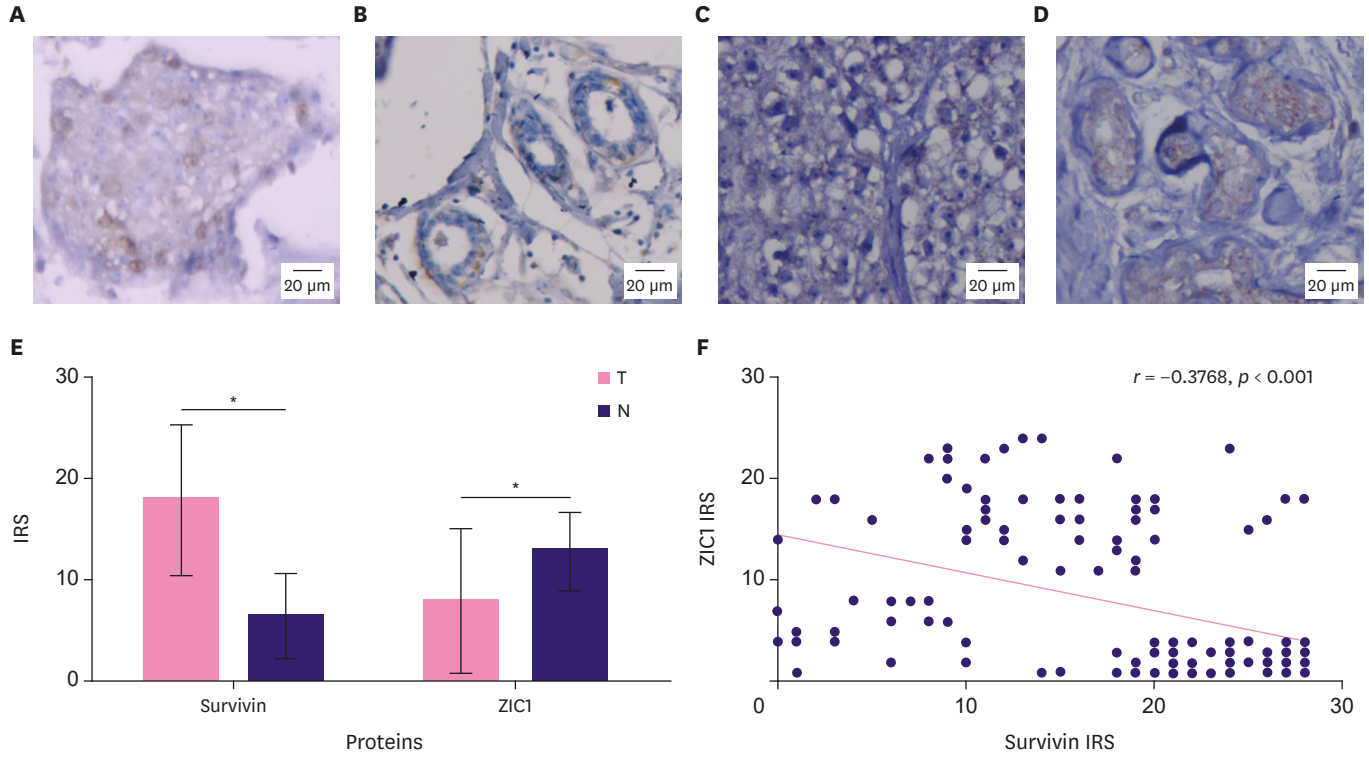

Figure 2. The expression of Survivin and ZIC1 in TNBC patients. (A) Survivin protein was observed in the nucleus and cytoplasm of TNBC (IHC for Survivin, ×400); (B) Survivin protein was observed in the nucleus and cytoplasm of normal tissues (IHC for Survivin, ×400); (C) ZIC1 protein was observed in the cytoplasm of TNBC (IHC for ZIC1, ×400); (D) ZIC1 protein was observed in the cytoplasm of normal tissues (IHC for ZIC1, ×400); (E) Comparison of Survivin IRS and ZIC1 IRS in TNBC with normal tissues; (F) a negative correlation between Survivin and ZIC1 expression, $r = -0.3768$, $p < 0.001$.
IRS = immunoreactivity score; ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma; IHC = immunohistochemistry.
*$p < 0.01$.

Table 2. Associations of Survivin or ZIC1 expression with clinicopathological features of 150 patients with TNBC

Parameters	No.	Survivin expression				ZIC1 expression			
		Positive (%)	Negative (%)	χ^2	*p*-value	Positive (%)	Negative (%)	χ^2	*p*-value
Total	150	121 (100.0)	29 (100.0)			56 (100.0)	94 (100.0)		
Age (yr)				1.404	0.236			3.623	0.057
≤ 50	68	52 (43.0)	16 (55.2)			31 (55.4)	37 (39.4)		
> 50	82	69 (57.0)	13 (44.8)			25 (44.6)	57 (60.6)		
Tumor size				1.840	0.175			0.100	0.752
≤ 5 cm	94	79 (65.3)	15 (51.7)			36 (64.3)	58 (61.7)		
> 5 cm	56	42 (34.7)	14 (48.3)			20 (35.7)	36 (38.3)		
Histologic grade				12.411	0.002			20.651	< 0.001
1	52	34 (28.1)	18 (62.1)			32 (57.1)	20 (21.3)		
2	65	59 (48.8)	6 (20.7)			14 (25.0)	51 (54.3)		
3	33	28 (23.1)	5 (17.2)			10 (17.9)	23 (24.4)		
Lymph node metastasis				15.017	< 0.001			10.054	0.002
Positive	89	81 (66.9)	8 (27.6)			24 (42.9)	65 (69.2)		
Negative	61	40 (33.1)	21 (72.4)			32 (57.1)	29 (30.8)		
TNM staging*				15.841	< 0.001			21.167	< 0.001
I	34	20 (16.5)	14 (48.3)			24 (42.9)	10 (10.6)		
II	78	71 (58.7)	7 (24.1)			20 (35.7)	58 (61.7)		
III	38	30 (24.8)	8 (27.6)			12 (21.4)	26 (27.7)		
Menopausal status				1.395	0.237			3.513	0.061
Premenopausal	63	48 (39.7)	15 (51.7)			29 (51.8)	34 (36.2)		
Postmenopausal	87	73 (60.3)	14 (48.3)			27 (48.2)	60 (63.8)		

ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma.
*The 8th edition of American Joint Committee on Cancer.

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and TNM staging ($p < 0.001$) of the tumor. In addition, Survivin positive and ZIC1 negative expression was associated with histologic grade, lymph node metastasis and TNM staging ($p < 0.001$, **Table 3**).

Overall survival

In the Kaplan-Meier analysis (**Figure 3A and B**), the overall survival rate of "Survivin positive group" was significantly lower than "Survivin negative group" ($p = 0.001$), while the overall survival rate of "ZIC1 positive group" was significantly higher than "ZIC1 negative group" ($p = 0.003$). In univariate analysis of the Cox regression analysis (**Table 4**), Survivin, ZIC1, tumor histologic grade, lymph node metastasis and TNM staging were associated with OS ($p < 0.01$). In multivariate analysis, Survivin expression (hazard ratio [HR], 5.33; 95% CI, 3.13–9.09; $p < 0.001$), ZIC1 expression (HR, 0.71; 95% CI, 0.63–0.79; $p < 0.001$), tumor histologic grade (HR, 2.81; 95% CI, 2.51–3.15; $p < 0.001$), lymph node metastasis (HR, 3.43; 95% CI, 2.01–5.85; $p < 0.001$) and TNM staging (HR, 4.41; 95% CI, 2.58–7.52; $p < 0.001$) were the five independent prognostic factors.

Disease-free survival

In **Figure 3C and D**, the DFS rate of patients with positive expression of Survivin was significantly lower than that of patients with negative expression ($p = 0.001$), while the DFS of ZIC1 positive expression was obviously higher than the negative expression ($p = 0.002$). In Cox regression analysis (**Table 5**), Survivin expression, ZIC1 expression, tumor histologic grade, lymph node metastasis and TNM staging ($p < 0.001$) were also 5 independent factors of DFS.

Role of Survivin combined with ZIC1 on OS and DFS

As shown in **Figure 4A and B**, the OS and DFS of the "Survivin-positive ZIC1-negative group" were both significantly lower than those in the other groups ($p < 0.001$). In multivariate Cox regression analysis (**Table 6**), Survivin positivity and ZIC1 negativity were independent factors of OS and DFS ($p < 0.001$).

Table 3. Associations of Survivin positive and ZIC1 negative expression with clinicopathological features of 150 patients with TNBC

Parameters	No.	Survivin positive and ZIC1 negative (%)	Other expression (%)	χ^2	*p*-value
Total	150	76 (100.0)	74 (100.0)		
Age (yr)				0.032	0.858
≤ 50	68	35 (43.0)	33 (55.2)		
> 50	82	41 (57.0)	41 (44.8)		
Tumor size				2.180	0.140
≤ 5 cm	94	52 (65.3)	42 (51.7)		
> 5 cm	56	24 (34.7)	32 (48.3)		
Histologic grade				15.815	< 0.001
1	52	19 (28.1)	33 (62.1)		
2	65	45 (48.8)	20 (20.7)		
3	33	12 (23.1)	21 (17.2)		
Lymph node metastasis				24.562	< 0.001
Positive	89	60 (66.9)	29 (27.6)		
Negative	61	16 (33.1)	45 (72.4)		
TNM staging*				21.242	< 0.001
I	34	8 (16.5)	26 (48.3)		
II	78	53 (58.7)	25 (24.1)		
III	38	15 (24.8)	23 (27.6)		
Menopausal status				0.404	0.525
Premenopausal	63	30 (39.7)	33 (51.7)		
Postmenopausal	87	46 (60.3)	41 (48.3)		

ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma.
*The 8th edition of American Joint Committee on Cancer.

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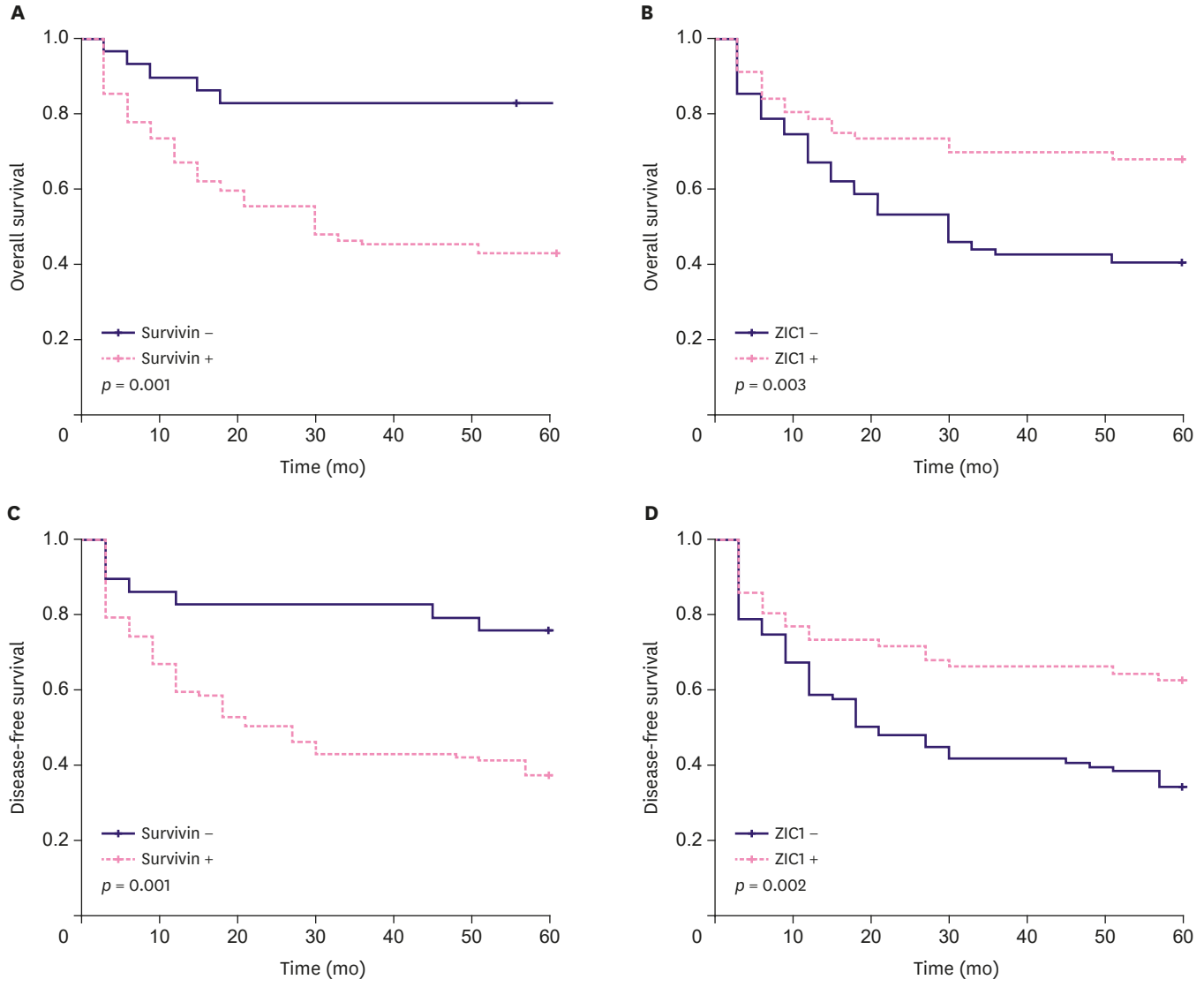

Figure 3. Kaplan-Meier survival curves of TNBC patients divided by Survivin or ZIC1 expression. (A) overall survival divided by Survivin expression; (B) overall survival divided by ZIC1 expression; (C) disease-free survival divided by Survivin expression; (D) disease-free survival divided by ZIC1 expression.
ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma.

DISCUSSION

Numerous novel biomarkers have been explored for the diagnosis of TNBC as it has high rates of metastasis and recurrence [3]. Proteins belonging to the IAP family, including Survivin and Livin, are inhibitors of apoptosis and death in breast cancer cells, which act via the inactivation of Caspase-3 and Caspase-7 and enhancement of Bcl-2 expression [15,16]. In addition, Survivin can promote the proliferation and migration of cancer cells due to the activation of the Akt/PI3K/mTOR and Wnt/β-Catenin signaling pathways [17,18]. eIF3 consists of 13 subunits and it has been proven that six of these subunits (eIF3a, eIF3c, eIF3d, eIF3e, eIF3g and eIF3h) modulate the growth, survival and transformation of breast cancer cells [6,19-21]. NME1, NME2 and NME3 are three cell migration inhibitors of breast cancer and their knockdown can rescue the Bcl3 motility phenotype [22]. ZIC family is named as such for their conspicuous wide spread expression in the cerebellar granule cells and they

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Table 4. Prognostic role of Survivin, ZIC1 expression and clinicopathological features for the overall survival of TNBC patients evaluated using univariate and multivariate analyses with Cox regression

Variables	HR	95% CI	*p*-value
Univariate analysis			
Survivin expression: high vs. low	4.16	1.68–10.33	0.002
ZIC1 expression: high vs. low	0.47	0.27–0.80	0.005
Age (yr): > 50 vs. ≤ 50	0.86	0.56–1.33	0.501
Tumor size: > 5 cm vs. ≤ 5 cm	1.14	0.61–2.15	0.678
Histologic grade: 3 vs. 1 & 2	1.28	1.11–1.47	< 0.001
Lymph node metastasis: yes vs. no	1.72	1.49–1.98	< 0.001
TNM staging: III vs. I & II	2.32	1.36–3.96	0.002
Menopausal status: premenopausal vs. postmenopausal	1.28	0.75–2.18	0.370
Multivariate analysis			
Survivin expression: high vs. low	5.33	3.13–9.09	< 0.001
ZIC1 expression: high vs. low	0.71	0.63–0.79	< 0.001
Age (yr): > 50 vs. ≤ 50	-	-	-
Tumor size: > 5 cm vs. ≤ 5 cm	-	-	-
Histologic grade: 3 vs. 1 & 2	2.81	2.51–3.15	< 0.001
Lymph node metastasis: yes vs. no	3.43	2.01–5.85	< 0.001
TNM staging: III vs. I & II	4.41	2.58–7.52	< 0.001
Menopausal status: premenopausal vs. postmenopausal	-	-	-

ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma; HR = hazard ratio; CI = confidence interval.

Table 5. Prognostic role of Survivin, ZIC1 expression and clinicopathological features for the disease-free survival of TNBC patients evaluate using univariate and multivariate analyses with Cox regression

Variables	HR	95% CI	*p*-value
Univariate analysis			
Survivin expression: high vs. low	3.35	1.54–7.27	0.002
ZIC1 expression: high vs. low	0.48	0.29–0.78	0.003
Age (yr): > 50 vs. ≤ 50	1.03	0.83–1.29	0.767
Tumor size: > 5 cm vs. ≤ 5 cm	1.17	0.94–1.45	0.172
Histologic grade: 3 vs. 1 & 2	1.42	1.14–1.77	0.002
Lymph node metastasis: yes vs. no	1.64	1.31–2.04	< 0.001
TNM staging: III vs. I & II	3.19	2.56–3.97	< 0.001
Menopausal status: premenopausal vs. postmenopausal	1.08	0.87–1.34	0.499
Multivariate analysis			
Survivin expression: high vs. low	3.52	2.83–4.39	< 0.001
ZIC1 expression: high vs. low	0.62	0.50–0.78	< 0.001
Age (yr): > 50 vs. ≤ 50	-	-	-
Tumor size: > 5 cm vs. ≤ 5 cm	-	-	-
Histologic grade: 3 vs. 1 & 2	2.08	1.67–2.59	< 0.001
Lymph node metastasis: yes vs. no	3.11	2.49–3.87	< 0.001
TNM staging: III vs. I & II	4.63	3.72–5.77	< 0.001
Menopausal status: premenopausal vs. postmenopausal	-	-	-

ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma; HR = hazard ratio; CI = confidence interval.

play an important role in the process of the growth and development of brain, nervous system, muscle cell production and in bone development [23]. In recent years, studies have shown that the abnormal expression of *ZIC* genes (*ZIC*1-5) is closely related to the occurrence and development of a variety of tumors, including myeloblastoma, endometrial cancer, mesenchymal tissue tumor and gastrointestinal cancer.

In this research, we picked out Survivin and ZIC1 as the main bio-markers for performing IHC as among all the proteins evaluated, the expression of these two proteins were significantly different in fresh-frozen TNBC than the corresponding normal tissues through RT-qPCR. Moreover, we also detected Survivin and ZIC1 expression by IHC and found that they have opposing effects on the clinicopathological diagnosis and prognostic assessment

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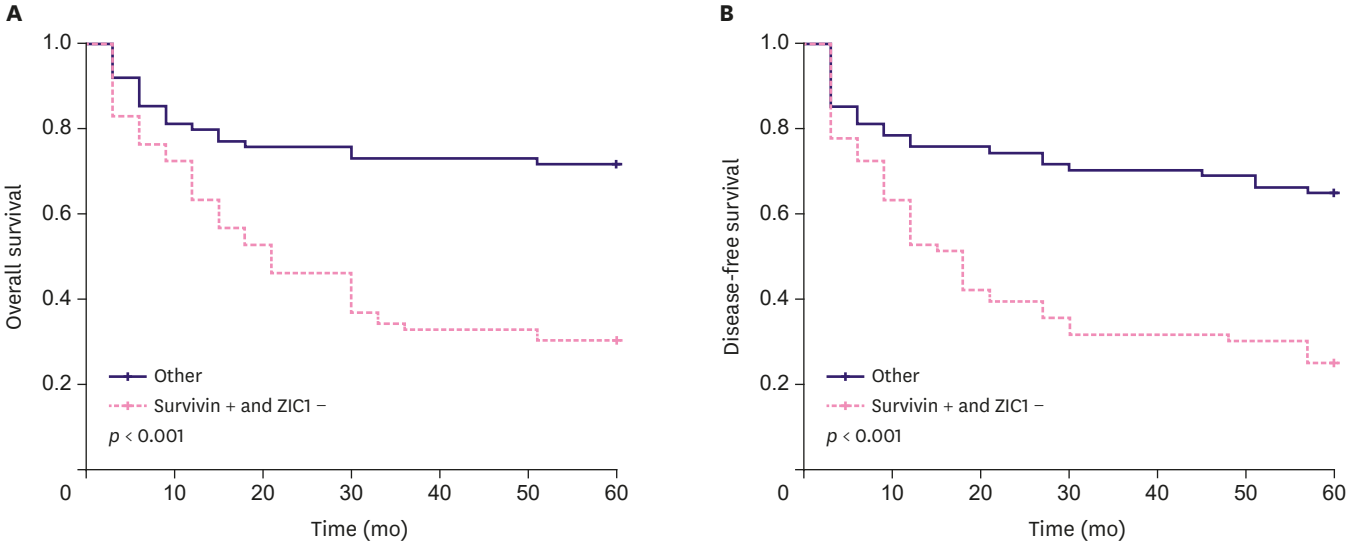

Figure 4. Kaplan-Meier survival curves of TNBC patients ("Survivin positive and ZIC1 negative expression" vs others). (A) overall survival; (B) disease-free survival.
ZIC = zinc finger of the cerebellum; TNBC = triple-negative breast carcinoma.

Table 6. Prognostic role of Survivin combined with ZIC1 expression and clinicopathological features for the OS and DFS of TNBC patients evaluated using multivariate analyses with Cox regression

Variables	HR	95% CI	*p*-value
OS			
Survivin and ZIC1 expression: Survivin + and ZIC1 - vs. other	2.81	2.26–3.50	< 0.001
Histologic grade: 3 vs. 1 & 2	1.28	1.02–1.59	0.030
Lymph node metastasis: yes vs. no	1.41	1.13–1.76	0.002
TNM staging: III vs. I & II	3.01	2.42–3.75	< 0.001
DFS			
Survivin and ZIC1 expression: Survivin + and ZIC1 - vs. other	3.14	2.52–3.91	< 0.001
Histologic grade: 3 vs. 1 & 2	1.73	1.17–2.56	0.006
Lymph node metastasis: yes vs. no	2.58	1.74–3.82	< 0.001
TNM staging: III vs. I & II	3.00	2.01–4.40	< 0.001

ZIC = zinc finger of the cerebellum; OS = overall survival; DFS= disease-free survival; TNBC = triple-negative breast carcinoma; HR = hazard ratio; CI = confidence interval.

in TNBC patients. In addition, the "Survivin positive and ZIC1 negative group" was associated with histologic grade, lymph node metastasis and TNM staging and this was also an independent prognostic factor of TNBC patients. Notably, there was a negative correlation between Survivin and ZIC1 expression, which meant that the combined detection of Survivin and ZIC1 expression could provide a better comprehensive diagnosis for TNBC patients. In addition, Survivin is considered a promising therapeutic target due to its ubiquitous over-expression in the breast neoplasm. A previous meta-analysis has reported that high Survivin expression levels are linked with unfavorable prognosis in patients with breast cancer and it was also found to be significantly associated with lymph node metastasis and stage of breast cancer [24]. On the other hand, ZIC1 is a human putative tumor suppressor gene and a cohort study revealed that of all the ZIC family proteins (ZIC2, ZIC3, ZIC4 and ZIC5), only ZIC1 was obviously down-regulated in the invasive breast carcinoma and can become a novel indicator of prognosis for invasive breast cancer patients [14].

Enhanced Survivin expression is a key mechanism of resistance to radiotherapy or chemotherapy. Stache et al. emphasized that decreasing the levels of Survivin by tyrosine kinase inhibitors combined with radiotherapy could be a promising treatment strategy [25]. Deguelin induced both apoptosis and autophagy in head and neck squamous cell carcinoma

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cells through inhibition of Akt signaling which increased the ceramide levels and activated adenosine monophosphate activated protein kinase (AMPK). This, in turn, down-regulates Survivin expression and reversed drug resistance [26]. In addition, the phosphorylation of Akt and mTOR was responsible for the up-regulation of Survivin [27]. Hypermethylation of ZIC1 was correlated with cisplatin resistance and methylation of ZIC1 promoter caused low expression of ZIC1 in malignant cells, which is a key reason for carcinogenesis [28]. In addition, its ectopic expression suppressed the growth of cancer cells by reducing Akt and Erk phosphorylation, arresting cell cycle at G1 stage and degrading Bcl-2 expression [29]. Based on these studies, we demonstrated a negative correlation between Survivin and ZIC1 expression and their opposing effects on clinicopathological diagnosis and prognostic assessment in TNBC patients. However, further research needs to be carried out to confirm the relationship between Survivin and ZIC1 and their role in the molecular mechanism. It is also important to identify if Survivin could be a potential therapeutic target for anticancer drugs. Furthermore, a recent study reported that ZIC1 was negatively correlated with Survivin in invasive breast carcinoma and elevated ZIC1 could down-regulate Survivin expression in breast cancer cells (MDA-MB-231 and SK-BR3) and tumors through the inactivation of Akt/mTOR/P70S6K pathway, which is consistent with our results [30]. Despite the lack of analysis of the mechanism of action in this research, we have found a possible negative relationship between ZIC1 and Survivin expression, which could also verify the results *in vitro* and *in vivo* in our study. In addition, compared with RT-qPCR and IHC, gene microarray is a more useful and successful method for studying the genetic differences. Further research needs to be conducted in the future to verify this negative relationship between ZIC1 and Survivin.

In conclusion, the expression of Survivin and ZIC1 in TNBC are different in TNBC tumors and normal tissues and there is a negative correlation between them. Positive Survivin expression and negative ZIC1 expression in TNBC patients were both associated with tumor histologic grade, lymph node metastasis and TNM staging and they also indicated worse overall survival and disease-free survival. Therefore, up-regulated Survivin and down-regulated ZIC1 might be two candidate biomarkers of poor prognosis for TNBC.

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