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ORIGINAL ARTICLE

Clinicopathologic and Prognostic Significance of the Zinc Finger of the Cerebellum Family in Invasive Breast Cancer

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Purpose: Five members of the zinc finger of the cerebellum (ZIC) family—ZIC1, ZIC2, ZIC3, ZIC4, and ZIC5—have been shown to be involved in various carcinomas. Here, we aimed to explore the clinicopathologic and prognostic roles of ZIC family members in invasive breast cancer patients using immunohistochemical analysis, western blotting analysis, and real-time quantitative polymerase chain reaction (RT-qPCR). **Methods:** A total of 241 female invasive breast cancer patients who underwent radical mastectomy between 2009 and 2011 were enrolled. ZIC proteins in 241 pairs of breast tumors and corresponding normal tissues were investigated using immunohistochemistry and the clinicopathologic roles of proteins were analyzed using Pearson's chi-square test. Kaplan-Meier curves and Cox regression analysis were also used to analyze the prognostic value of the ZIC proteins. In addition, 12 pairs of fresh-frozen breast tumors and matched normal tissues were used in the western blotting analysis and RT-qPCR. **Results:** Only ZIC1 expression in normal tissues was obviously higher than that in tumors ($p<0.001$). On multivariate analysis, ZIC1 expression (in overall survival analysis: hazard ratio [HR], 0.405, 95% confidence interval [CI], 0.233–0.702, $p=0.001$; in disease-free survival analysis: HR, 0.395, 95% CI, 0.234–0.669, $p=0.001$) was identified as a prognostic indicator of invasive breast cancer. **Conclusion:** ZIC1, but not the other proteins, was obviously decreased in breast tumors and associated with clinicopathologic factors. Thus, ZIC1 might be a novel indicator to predict the overall and disease-free survival of invasive breast cancer patients.

Key Words: *Breast neoplasms, Pathology, Prognosis, ZIC family*

INTRODUCTION

Invasive breast cancer, a leading cause of death among women, has received extensive attention from the international community [1]. Numerous genes and proteins have been discovered and are now considered biomarkers for more precise evaluation of the prognosis of breast cancer.

The five zinc finger of the cerebellum (ZIC) family proteins—ZIC1, ZIC2, ZIC3, ZIC4, and ZIC5—are structurally similar to each other [2]. Five Cys2His2 zinc-finger domains in each member interact with the Gli family proteins via these homologous structures and are essential for human nervous system development [3]. Currently, these five proteins play different roles in human carcinomas and have been inferred as carcinogenic or suppressor genes. ZIC1 has been found to inhibit the growth of various carcinomas, such as digestive system cancers and thyroid cancer, and has become a putative indicator of good prognosis [4-8]. In addition, the high ZIC4 methylation levels in pTa-bladder cancer patients was correlated with an elevated progression risk and became a potential poor prognostic marker in stage pTa [9,10]. However, ZIC2, ZIC3, and ZIC5 are overexpressed in lung cancer cells and can function as oncogenes by improving cell proliferation and inhibiting apoptosis [11-13]. Besides, ZIC2 expression could

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promote cell proliferation and inhibit cell apoptosis during the development of pancreatic ductal adenocarcinoma [14]. Previous studies have also demonstrated that *ZIC1* and *ZIC4* are potential suppressor genes in breast cancer [15,16]. The ZIC1 expression level was elevated in BT-549 cells after the knockdown of phosphatidylinositolglycan-class X (PIGX), reticulocalbin 1 (RCN1), or (RCN2), whereas the growth of transfected BT-549 cells was obviously inhibited [15]. Pavlova et al. [16] identified that methylated ZIC4 might be involved in breast cancer development. Without reports on ZIC2, ZIC3, or ZIC5 in breast cancer, the clinicopathologic and prognostic significance of the ZIC family proteins requires further illumination.

Here, we investigated the expression levels of the ZIC family proteins in 241 cases of invasive breast cancer using immunohistochemical analysis (IHC) and then detected the relative expression levels of these proteins in 12 pairs of breast tumors and matched normal tissues using western blotting analysis and real-time quantitative polymerase chain reaction (RT-qPCR). We also analyzed the associations between the ZIC family protein expression levels and the clinicopathological factors of breast cancer and evaluated the prognostic roles of these proteins.

METHODS

Patients and tissue samples

A total of 241 female invasive breast cancer patients (mean age, 50.53 ± 11.28 years) who underwent surgery (radical mastectomy in 45, modified radical mastectomy in 196) between 2009 and 2011 were enrolled. Each case had breast tumor and its corresponding normal tissue. None of the patients had received radiotherapy or chemotherapy before surgery. The cohort was composed of patients with complete clinicopathological data from Wujiang First People's Hospital (n = 64) and Kunshan Second People's Hospital (n = 177). Patients with stage I, II, and III disease received doxorubicin+cyclophosphamide for the first four cycles and paclitaxel for the next four cycles, while patients with stage IV disease received cyclophosphamide+doxorubicin+5-fluorouracil for six cycles. If a patient confirmed estrogen receptor (ER) or progesterone receptor (PR)-positive, she would receive tamoxifen in the premenopausal period or an aromatase inhibitor in the postmenopausal period. If a patient tested human epidermal growth factor receptor 2 (HER2)-positive, she would receive trastuzumab. Follow-up data were available for all patients for a mean duration of 54.24 ± 0.81 months (range, 3–60 months). More details of the clinicopathological data of 241 cases are listed in Table 1. In addition, 12 pairs of fresh-frozen breast tumor tissue and matched normal tissue samples (stored at −80°C) were collected from Kunshan First People's Hospital Affiliated to Jiangsu University and used for total protein extraction. Our study received ethical approval from the Kunshan First People's Hospital Ethics Committee (No. KSLL2008016). Every patient signed an informed consent form.

Table 1. Clinicopathological parameters of 241 patients with breast cancer

Parameter	No. of patients (%)
Age (yr)*	50.53 ± 11.28
Follow-up (mo)*	54.24 ± 0.81
Tumor size (cm)	
≤ 2	53 (22.0)
> 2, ≤ 5	124 (51.5)
> 5	64 (26.5)
Location	
Left	135 (56.0)
Right	106 (44.0)
Histologic grade	
1	62 (25.7)
2	133 (55.2)
3	46 (19.1)
Lymph node metastasis	
Positive	112 (46.5)
Negative	129 (53.5)
TNM staging	
I	31 (12.8)
II	114 (47.3)
III	77 (32.0)
IV	19 (7.9)
Estrogen receptor	
Positive	129 (53.5)
Negative	112 (46.5)
Progestrone receptor	
Positive	90 (37.3)
Negative	151 (62.7)
HER2 expression	
Positive	116 (48.1)
Negative	125 (51.9)
Chemotherapy	
CAF	19 (7.9)
AC-T	222 (92.1)
Hormonal treatment	
Yes	169 (70.1)
No	72 (29.9)
Targeted therapy	
Yes	116 (48.1)
No	125 (51.9)

HER2 = human epidermal growth factor receptor 2; CAF = cyclophosphamide+doxorubicin+5-fluorouracil for 6 cycles; AC-T = doxorubicin+cyclophosphamide for first 4 cycles and paclitaxel for next 4 cycles.
*Mean ± SD.

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Tissue microarray construction and immunohistochemistry

A total of 241 breast tumors and corresponding normal tissues were collected. Three representative regions of each case were selected to obtain tissue cylinders with a diameter of 0.6 mm; we then arrayed these cylinders into a recipient block using a tissue chip microarrayer. Subsequently, we cut the recipient block into 5-μm sections on pretreated slides to support sample adhesion.

Rabbit anti-human ZIC1, ZIC2, ZIC3, ZIC4, and ZIC5 polyclonal antibodies (Bioss, Beijing, China) were used as the primary antibodies diluted at 1:100 in phosphate-buffered saline. A SP Rabbit & Mouse HRP Kit (CWBIO, Beijing, China) was used for the IHC. The slides were deparaffinized, rehydrated, and then boiled in a citrate buffer solution at a concentration of 10 mmol/L. After the solution cooled to room temperature, tissue chips were treated with blocking buffers and then incubated with the primary antibodies for 12 hours each. In sequence, the slides were marked by streptavidin with horseradish peroxidase (HRP), developed by diaminobenzidine, and counterstained with hematoxylin. Finally, we dehydrated and mounted these chips for storage and evaluation of the staining results.

Evaluation of immunohistochemical staining

Two pathologists (X.J.G. and F.C.) who were blinded to the study details independently assessed ZIC family protein expression in a semi-quantitative manner combined with evaluation of the percentage of tumor cells with staining of the cytoplasm or nuclear ("0–100%" = "0–10") and the assessment of staining intensity ("faint–yellow–sepia" = "1–10"). Multiplied values, called the immunoreactivity score (IRS), were 0–100. If one protein was detected in the cytoplasm and the nucleus, we used an average score of the cytoplasm and nucleus (ZIC1–3). The samples were divided into "high-expression" (IRS >10) and "low-expression" (IRS ≤ 10) samples according to each protein's expression. This cutoff value was identified according to previous relevant studies [17,18]. ER, PR, and HER2 statuses were considered as positive if $>10\%$ of tumor cells showed staining [19]. Any disagreement of IRS was resolved by discussion or consultation with a third pathologist (H.Z.D.). The results of the agreement statistics are presented in Supplementary Figure 1 (available online) with a Bland-Altman method comparison.

Protein extraction and western blotting

Twelve pairs of fresh-frozen invasive breast cancer tumors and corresponding normal tissues were used for the western blotting analysis. We chose normal breast tissues that were >5 cm away from the tumors. In these regions, there were abundant epithelial cells of the normal mammary duct and acinar structures. We extracted total proteins from the representative tumor regions and normal breast tissues using radioimmunoprecipitation assay (RIPA) Lysis Buffer (Beyotime Biotechnology, Shanghai, China), and then collected the supernatants, whose protein concentrations were measured using a bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology). Supernatants of the samples were mixed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) Loading Buffer (Beyotime Biotechnology), boiled for 8–10 minutes, resolved on ExpressPlus PAGE Gel (Genscript, Nanjing, China), transferred to polyvinylidene fluoride membranes (Beyotime Biotechnology), and then blocked in Tris buffered saline+Tween (TBST) confining liquid with 5% nonfat dry milk for 2 hours at room temperature. Thereafter, the primary antibodies including ZIC1 (diluted at 1:400), ZIC2 (diluted at 1:400), ZIC3 (diluted at 1:400), ZIC4 (diluted at 1:400), ZIC5 (diluted at 1:400), and β-actin (mouse polyclonal antibody diluted at 1:1,000; Beyotime Biotechnology) were dissolved in TBST and used to incubate membranes in 4°C overnight. After a cleaning in TBST, corresponding secondary antibodies with HRP were used to incubate these membranes for 2 hours at 37°C and the protein bands were detected using an Enhanced Chemiluminescence Detection System (Beyotime Biotechnology). The formula of relative expression levels of proteins quantified with Image J was Gray Value (ZIC proteins)/Gray Value (β-actin). The specificity of antibodies is shown in Supplementary Tables 1, 2, and Supplementary Figure 2 (available online).

Real-time quantitative polymerase chain reaction

Twelve pairs of frozen-thawed tissues were also used to isolate the total RNA using Trizol reagent (Thermo Fisher Scientific, Waltham, USA), and then, 2 μg RNA from each sample was reverse transcribed using the SuperScript II RNase-Reverse Transcriptase System (Thermo Fisher Scientific). Circular DNA was subjected to RT-qPCR using primers specific for

Table 2. The polymerase chain reaction primers of *ZIC* family genes and GAPDH

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
ZIC1	GCGTCCTTTTGTGGATCTTTAA	AGTAATCACATCTGCTTCTGGG
ZIC2	ACACTCCTCCCAGAAGCAGAC	GCAACTGAGCAATCCCAAGAA
ZIC3	AGACTGTCCCGGATACCAAGC	CAACAGCAGCGACCGTAAGAA
ZIC4	GCCTTTTCCCAGAGGGTATTA	CCTTTCTTTCCTGATTTGTGC
ZIC5	TCCCCACTGATGAGTAACCAA	AAGAAACATTCCCATGTCCAC
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

ZIC = zinc finger of the cerebellum; GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

ZIC1, ZIC2, ZIC3, ZIC4, ZIC5, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers are shown in Table 2. The PCR cycling conditions were as follows: 94°C for 4 minutes, followed by 40 cycles at 95°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute. The amplified DNA was measured using an SYBR Premix Ex Taq™ kit (Takara Bio, Dalian, China), whereas RT-qPCR was performed using an iQ5 Real-Time PCR Detection System (Bio-Rad, Berkeley, USA). The $2^{-\Delta\Delta Ct}$ value was used to calculate the relative expression and ΔΔCt = (CtTumor-ZICn−CtTumor-GAPDH)−(CtNormal-ZICn−CtNormal-GAPDH) (n = 1–5). A higher $2^{-\Delta\Delta Ct}$ level indicated greater mRNA expression.

Table 3. Associations of ZIC family proteins expression with various clinicopathological factors of 241 patients with invasive breast cancer

Parameter	ZIC family proteins high expression															
	No.	ZIC1	χ^2	*p*-value	ZIC2	χ^2	*p*-value	ZIC3	χ^2	*p*-value	ZIC4	χ^2	*p*-value	ZIC5	χ^2	*p*-value
Total	241	120			130			108			109			93		
Age (yr)			0.209	0.648		1.651	0.199		1.341	0.247		0.272	0.602		0.010	0.920
≤50	126	63			63			52			59			49		
>50	115	57			67			56			50			44		
Tumor size (cm)			1.977	0.372		4.160	0.125		1.464	0.481		2.123	0.346		1.669	0.434
≤2	53	23			23			20			25			17		
>2, ≤5	124	67			74			59			60			48		
>5	64	30			33			29			24			28		
Location			1.200	0.273		0.094	0.759		0.017	0.897		0.256	0.613		1.709	0.191
Left	135	63			74			60			63			57		
Right	106	57			56			48			46			36		
Histologic grade			1.367	0.505		1.669	0.434		0.041	0.980		0.602	0.740		0.815	0.665
1	62	34			26			28			27			21		
2	133	66			79			60			63			53		
3	46	20			25			20			19			19		
Lymph node metastasis			5.129	0.024		3.691	0.055		0.532	0.466		0.475	0.491		0.544	0.461
Positive	112	47			53			53			48			46		
Negative	129	73			77			55			61			47		
TNM staging			10.408	0.015		5.541	0.136		3.124	0.373		5.104	0.164		4.081	0.253
I	31	14			13			10			15			7		
II	114	69			68			55			58			45		
III	77	30			42			36			31			33		
IV	19	7			7			7			5			8		
ER expression			1.827	0.177		1.969	0.161		0.096	0.757		0.185	0.667		0.544	0.461
Positive	129	59			75			59			60			47		
Negative	112	61			55			49			49			46		
PR expression			0.561	0.454		1.415	0.234		0.032	0.858		0.777	0.378		0.800	0.371
Positive	90	42			53			41			44			38		
Negative	151	78			77			67			65			55		
HER2 expression			0.334	0.563		2.890	0.089		0.612	0.434		1.337	0.247		0.993	0.319
Positive	116	60			56			55			48			41		
Negative	125	60			74			53			61			52		
Chemotherapy			1.384	0.239		2.428	0.119		0.530	0.467		2.978	0.084		0.108	0.743
CAF	19	7			7			7			5			8		
AC-T	222	113			123			101			104			85		
Hormonal treatment			2.101	0.147		1.174	0.278		0.241	0.624		0.025	0.873		0.266	0.606
Yes	169	79			95			74			77			67		
No	72	41			35			34			32			26		
Targeted therapy			0.334	0.563		2.890	0.089		0.612	0.434		1.337	0.247		0.993	0.319
Yes	116	60			56			55			48			41		
No	125	60			74			53			61			52		

ZIC = zinc finger of the cerebellum; ER = estrogen receptor; PR = progesterone receptor; HER2 = human epidermal growth factor receptor 2; CAF = cyclophosphamide+doxorubicin+5-fluorouracil for 6 cycles; AC-T = doxorubicin+cyclophosphamide for first 4 cycles and paclitaxel for next 4 cycles.

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Statistical analysis

All results were analyzed using SPSS version 20.0 software (IBM, Armonk, USA) or GraphPad Prism version 6.0 (GraphPad Software, San Diego, USA), and p-values <0.05 were considered statistically significant. Pearson's chi-square and Fisher exact tests were used to analyze the associations between ZIC protein expression and clinicopathological factors. In addition, Kaplan-Meier curves with the log-rank test and Cox univariate and multivariate regression analyses were used to evaluate the roles of every factor in overall survival (OS) and disease-free survival (DFS). Independent risk factors for survival were selected using SPSS version 20.0 software.

RESULTS

ZIC member expression in invasive breast cancer

IHC confirmed that ZIC1, ZIC2, and ZIC3 could be expressed in the nucleus or cytoplasm, whereas ZIC4 and ZIC5 were expressed only in the nucleus. The rates of high ZIC1–5 expression were 49.8%, 53.9%, 44.8%, 45.2%, and 38.6%, respectively (Table 3). Figure 1 shows the expression of ZIC proteins in tumors and matched normal tissues, while the expression score of ZIC1 protein in the normal tissues was obviously higher than that in the tumors (normal tissues vs. tumors, 32.66 ± 18.76 vs. 15.38 ± 13.37, $p<0.001$). However, no difference in ZIC2 (tumors vs. normal tissues, 16.26 ± 13.08 vs. 16.71 ± 15.38, $p>0.05$), ZIC3 (tumors vs. normal tissues, 13.49 ± 11.58 vs. 13.61 ± 9.71, $p>0.05$), ZIC4 (tumors vs. normal tissues, 14.20 ± 11.95 vs. 12.62 ± 10.38, $p>0.05$), or ZIC5 (tumors vs. normal tissues, 12.08 ± 10.23 vs. 10.81 ± 9.02, $p>0.05$) expression scores were seen between the tumor tissues and corresponding normal tissues.

We then used western blotting analysis to detect the ZIC protein expressions in the 12 pairs of tissues, and the sizes of the ZIC1–5 proteins were 48 kDa, 55 kDa, 51 kDa, 37 kDa, and 68 kDa, respectively. We found that the level of ZIC1 protein expression in the tumors was significantly lower than that

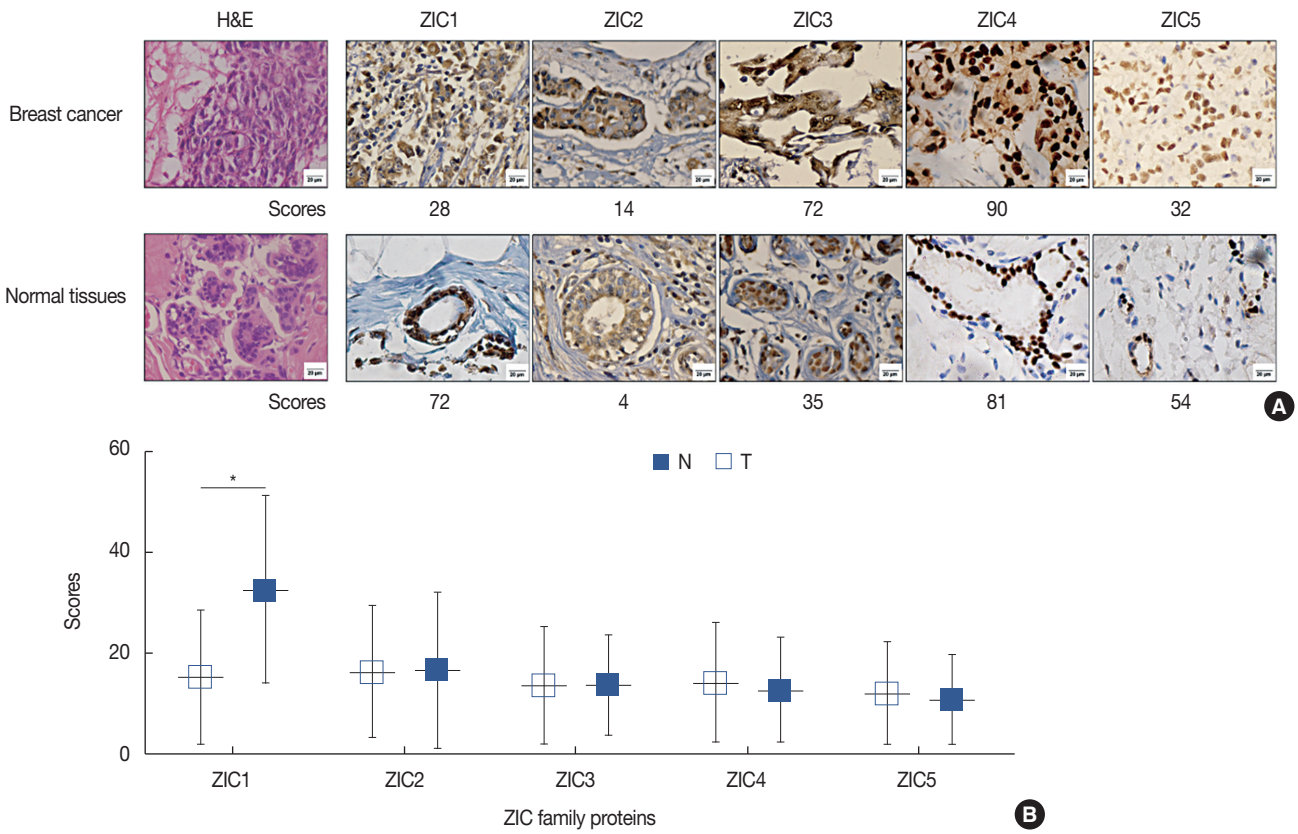

Figure 1. ZIC member expression in invasive breast cancer. (A) Hematoxylin and eosin (H&E) and immunohistochemical staining of zinc finger of the cerebellum (ZIC) family proteins in breast tumors and corresponding normal tissues. ZIC family proteins were observed in the nucleus or cytoplasm (×400 magnification). (B) There was a significant difference in ZIC1 expression, but not other proteins, between breast tumors and matched normal tissues from 241 patients.
N=normal tissue; T=breast tumor. *$p<0.001$.

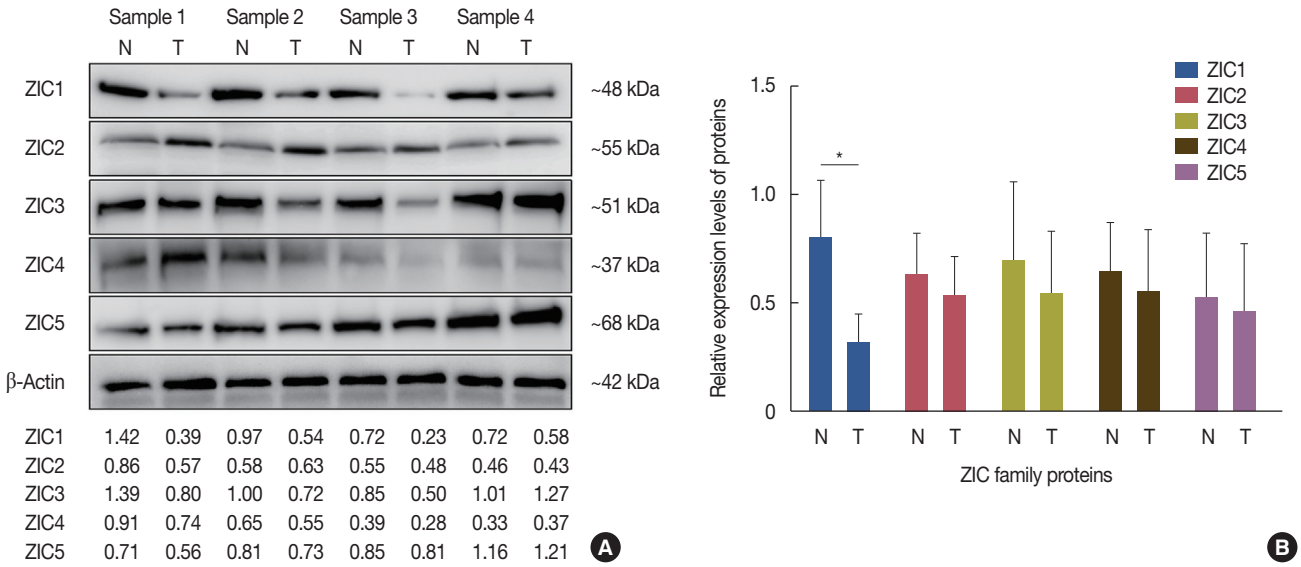

Figure 2. Western blotting analysis of zinc finger of the cerebellum (ZIC) family proteins in breast tumors and matched normal tissues. (A) Represent blots of ZIC family proteins in four samples. (B) Relative protein expression of ZIC family proteins in 12 pairs of breast tumors and matched normal tissues.
N = normal tissue; T = breast tumor. *$p < 0.001$.

in normal tissues (tumors vs. normal tissues, 0.324 ± 0.127 vs. 0.801 ± 0.261, $p < 0.001$) (Figure 2, Supplementary Figure 3). However, we found no difference in ZIC2 (normal tissues vs. tumors, 0.533 ± 0.181 vs. 0.633 ± 0.190, $p > 0.05$), ZIC3 (normal tissues vs. tumors, 0.543 ± 0.290 vs. 0.687 ± 0.365, $p > 0.05$), ZIC4 (normal tissues vs. tumors, 0.544 ± 0.294 vs. 0.641 ± 0.229, $p > 0.05$), or ZIC5 (normal tissues vs. tumors, 0.457 ± 0.317 vs. 0.522 ± 0.302, $p > 0.05$) between the tumors and matched normal tissues.

Also, in the RT-qPCR analysis, we found that the ZIC1 mRNA expression level in the tumors was significantly lower than that in the normal tissues (0.163 ± 0.139 vs. 1.197 ± 0.921, respectively, $p < 0.001$) (Figure 3).

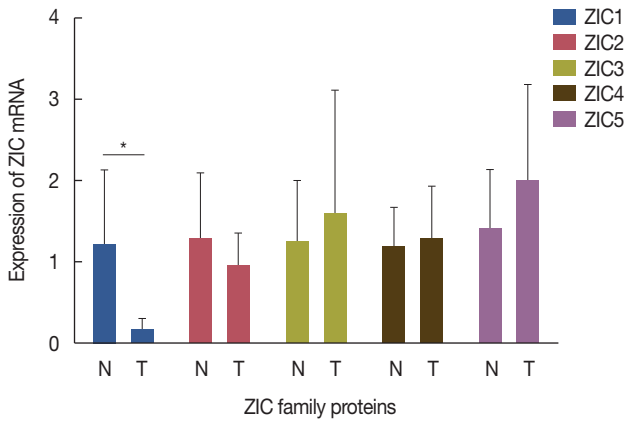

Figure 3. Real-time quantitative polymerase chain reaction analysis of zinc finger of the cerebellum (ZIC) family members in breast tumors and matched normal tissues.
N = normal tissue; T = breast tumor. *$p < 0.001$.

Associations between ZIC protein expression and clinicopathologic factors

Next, to evaluate the relationship between every ZIC protein and clinicopathologic factors, we divided all patients into two groups by cutoff values. As shown in Table 3, only the high ZIC1 expression level was negatively related to lymph node metastasis ($p = 0.024$) and TNM stage ($p = 0.015$). However, no significant relationships were observed between ZIC1 and other factors ($p > 0.05$) (Table 3).

Overall survival

The OS rate was 73.4%. We used Kaplan-Meier analysis to examine the survival rates of 241 invasive breast cancer cases with high or low ZIC protein expressions. The 5-year survival rate of patients with high ZIC1 expression was obviously higher than those with low expression (high vs. low, mean survival time, 57.08 ± 0.81 months vs. 51.42 ± 1.34 months, $p < 0.001$; 5-year survival rate, 84.2% vs. 62.8%, $p < 0.001$) (Figure 4A). However, no significant differences in survival rates were detected between the high and low ZIC2 levels (mean survival time, 54.12 ± 1.10 months vs. 54.38 ± 1.19 months, respectively, $p > 0.05$; 5-year survival rate, 73.8% vs. 73.0%, $p > 0.05$) (Figure 4B), ZIC3 (mean survival time, 52.69 ± 1.44 months vs. 55.49 ± 0.87 months, respectively, $p > 0.05$; 5-year survival rate, 72.2% vs. 74.4%, respectively,

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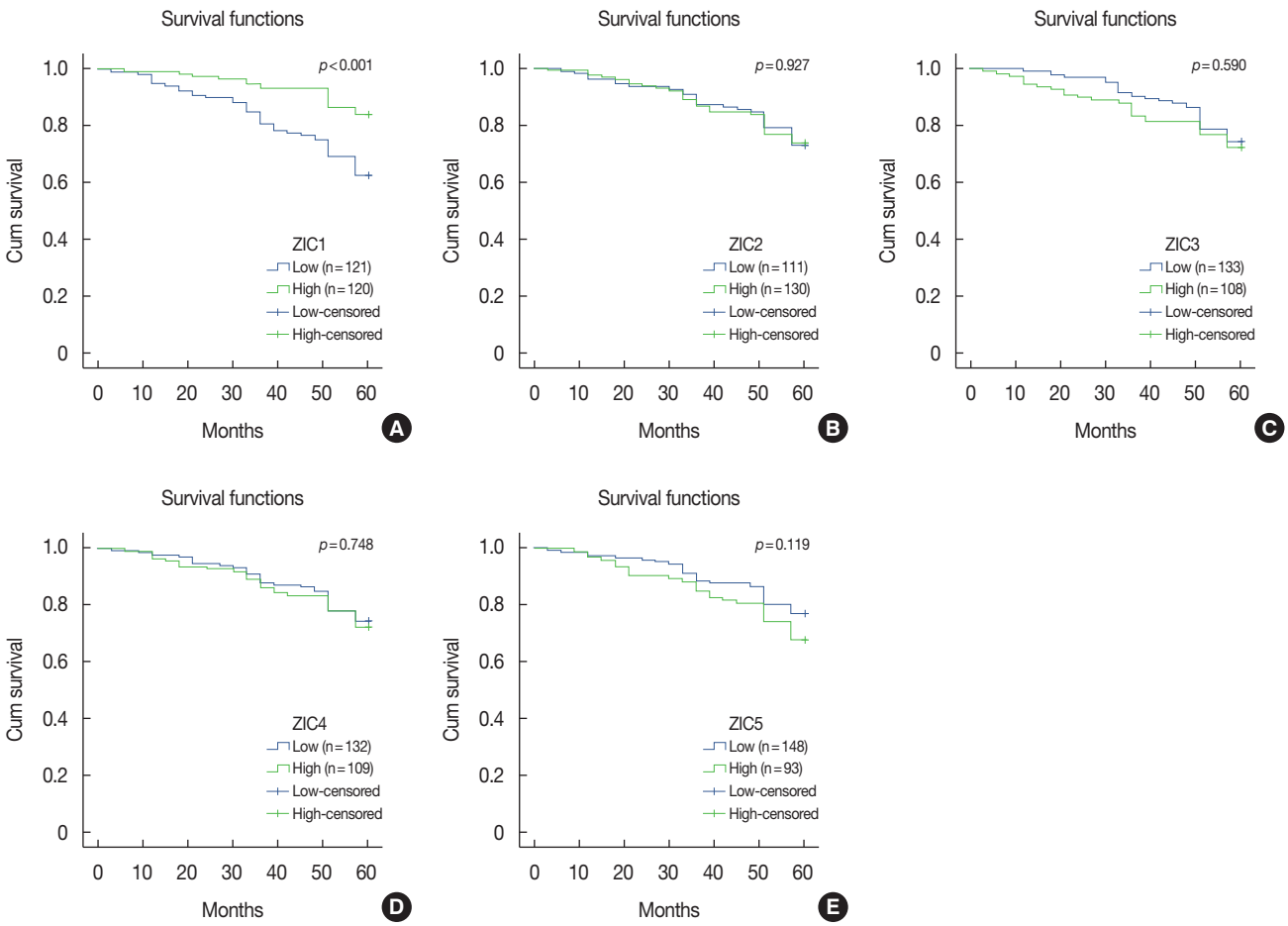

Figure 4. Kaplan-Meier survival curves of overall survival for zinc fnger of the cerebellum (ZIC) family proteins expression in invasive breast cancer. (A) ZIC1, (B) ZIC2, (C) ZIC3, (D) ZIC4, and (E) ZIC5.

$p > 0.05$) (Figure 4C), ZIC4 (mean survival time, 53.78 ± 1.27 months vs. 54.61 ± 1.04 months, respectively, $p > 0.05$; 5-year survival rate, 72.5% vs. 74.2%, $p > 0.05$) (Figure 4D), or ZIC5 (mean survival time, 52.81 ± 1.44 months vs. 55.14 ± 0.95 months, respectively, $p > 0.05$; and 5-year survival rate, 67.7% vs. 77.0%, respectively, $p > 0.05$) (Figure 4E).

In the next Cox regression analysis, we first conducted a univariate analysis and found that nine factors—ZIC1 expression, tumor size, location, histologic grade, lymph node metastasis, TNM staging, ER expression, HER2 expression, and targeted therapy—could affect the OS of invasive breast cancer patients; in the further multivariate analysis, five factors—including ZIC1 expression (hazard ratio [HR], 0.405; 95% confidence interval [CI], 0.233–0.702; $p = 0.001$), tumor size (HR, 1.762; 95% CI, 1.014–3.062; $p = 0.038$), histologic grade (HR, 2.024; 95% CI, 1.159–3.536; $p = 0.013$), TNM staging (HR, 2.606; 95% CI, 1.509–4.500; $p = 0.001$), and HER2 expression (HR, 0.535; 95% CI, 0.317–0.905; $p = 0.020$) —were identified as prognostic indicators of invasive breast cancer patients (Table 4).

Disease-free survival

The 5-year DFS rate was 71.4%. The DFS of patients with high ZIC1 expression was significantly higher than that of patients with low expression (high vs. low, mean survival time, 56.63 ± 0.88 months vs. 49.29 ± 1.46 months, respectively, $p < 0.001$; 5-year DFS rate, 82.5% vs. 60.3%, respectively, $p < 0.001$) (Figure 5A). No significant differences in DFS rates were detected between high and low levels of ZIC2, ZIC3, ZIC4, or ZIC5 ($p > 0.05$) (Figure 5). As shown in Table 5, we found that seven factors—ZIC1 expression, tumor size, histologic grade, lymph node metastasis, TNM staging, HER2 expression, and targeted therapy—were related to DFS in univariate analysis; in the further multivariate analysis, five independent factors were identified: ZIC1 expression (HR, 0.395; 95% CI, 0.234–0.669; $p = 0.001$), tumor size (HR, 1.838; 95% CI, 1.090–3.101; $p = 0.022$), histologic grade (HR, 1.936; 95% CI, 1.123–3.340; $p = 0.018$), TNM staging (HR, 2.559; 95% CI,

Table 4. Prognostic value of ZIC family proteins expression and clinicopathological factors for the overall survival by univariate and multivariate analyses with Cox regression

Variable	HR	95% CI	*p*-value	Variable	HR	95% CI	*p*-value
Univariate analysis				Multivariate analysis			
ZIC1 expression: high vs. low	0.370	0.216–0.633	<0.001	ZIC1 expression: high vs. low	0.405	0.233–0.702	0.001
ZIC2 expression: high vs. low	0.977	0.598–1.597	NS				
ZIC3 expression: high vs. low	1.145	0.701–1.870	NS				
ZIC4 expression: high vs. low	1.084	0.663–1.771	NS				
ZIC5 expression: high vs. low	1.478	0.905–2.416	NS				
Age (yr): >50 vs. ≤50	0.709	0.430–1.167	NS				
Tumor size (cm): >5 vs. ≤5	2.039	1.234–3.370	0.005	Tumor size (cm): >5 vs. ≤5	1.762	1.014–3.062	0.044
Location: right vs. left	1.568	1.086–2.264	0.016	Location: left vs. right	-	-	NS
Histologic grade: 3 vs. 1 and 2	2.127	1.254–3.610	0.005	Histologic grade: 3 vs. 1 and 2	2.024	1.159–3.536	0.013
Lymph node metastasis: yes vs. no	1.949	1.179–3.221	0.009	Lymph node metastasis: yes vs. no	-	-	NS
TNM staging: III/IV vs. I/II	3.106	1.863–5.179	<0.001	TNM staging: III/IV vs. I/II	2.606	1.509–4.500	0.001
ER expression: high vs. low	0.587	0.357–0.964	0.035	ER expression: high vs. low	-	-	NS
PR expression: high vs. low	1.342	0.819–2.199	NS				
HER2 expression: high vs. low	0.558	0.335–0.930	0.025	HER2 expression: high vs. low	0.535	0.317–0.905	0.020
Chemotherapy: CAF vs. AC-T	0.923	0.370–2.300	NS				
Hormonal treatment: yes vs. no	0.665	0.401–1.104	NS				
Targeted therapy: yes vs. no	0.558	0.335–0.930	0.025				

ZIC=zinc finger of the cerebellum; HR=hazard ratio; CI=confidence interval; NS=no significance; ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2; CAF=cyclophosphamide+doxorubicin+5-fluorouracil for 6 cycles; AC-T=doxorubicin+cyclophosphamide for first 4 cycles and paclitaxel for next 4 cycles.

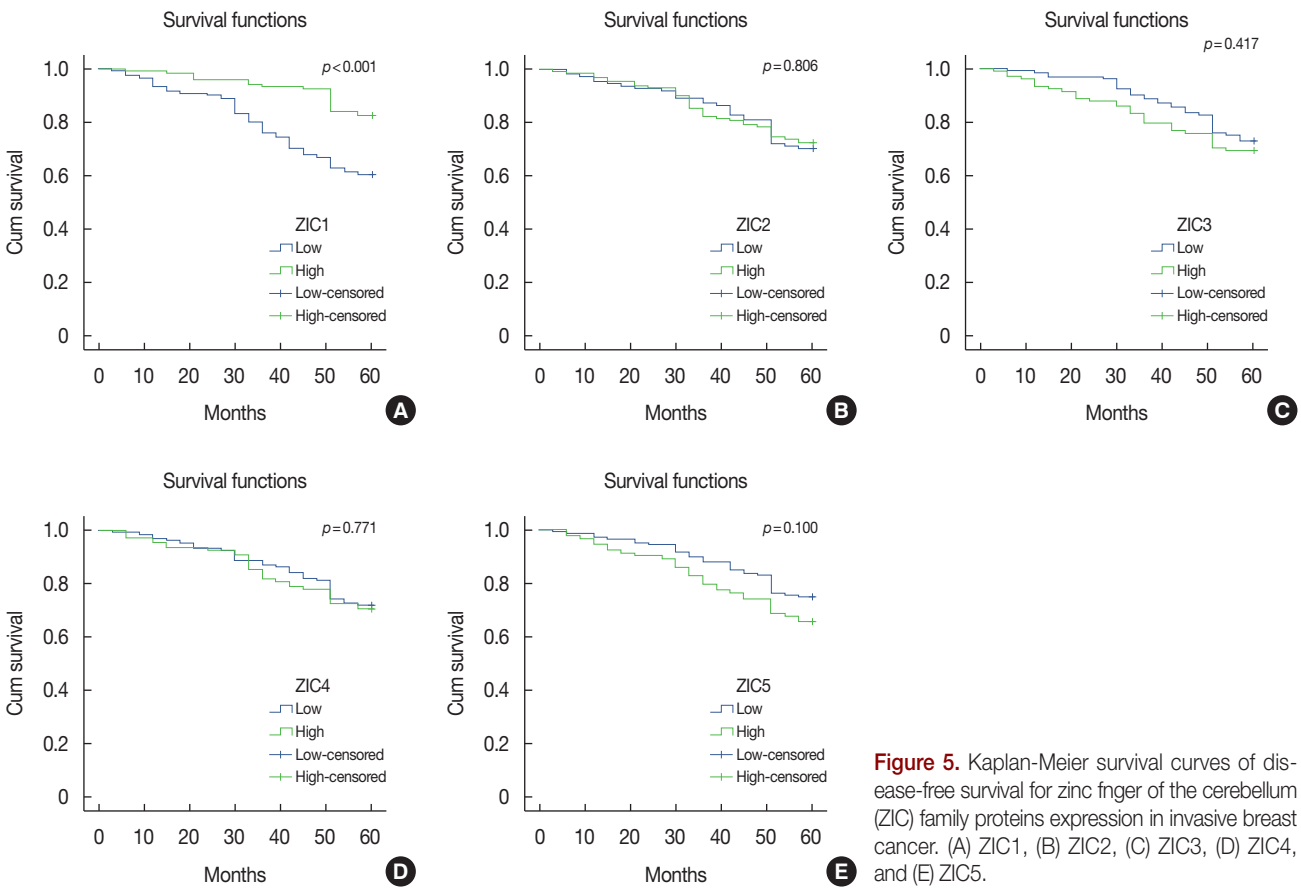

Figure 5. Kaplan-Meier survival curves of disease-free survival for zinc fnger of the cerebellum (ZIC) family proteins expression in invasive breast cancer. (A) ZIC1, (B) ZIC2, (C) ZIC3, (D) ZIC4, and (E) ZIC5.

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Table 5. Prognostic value of ZIC family proteins expression and clinicopathological factors for the disease-free survival by univariate and multivariate analyses with Cox regression

Variable	HR	95% CI	*p*-value	Variable	HR	95% CI	*p*-value
Univariate analysis				Multivariate analysis			
ZIC1 expression: high vs. low	0.368	0.220–0.616	<0.001	ZIC1 expression: high vs. low	0.395	0.234–0.669	0.001
ZIC2 expression: high vs. low	0.943	0.588–1.512	NS				
ZIC3 expression: high vs. low	1.216	0.758–1.950	NS				
ZIC4 expression: high vs. low	1.073	0.668–1.722	NS				
ZIC5 expression: high vs. low	1.488	0.927–2.388	NS				
Age (yr): >50 vs. ≤50	0.710	0.439–1.148	NS				
Tumor size (cm): >5 vs. ≤5	2.173	1.344–3.514	0.002	Tumor size (cm): >5 vs. ≤5	1.838	1.090–3.101	0.022
Location: right vs. left	1.180	0.736–1.893	NS				
Histologic grade: 3 vs. 1 and 2	1.950	1.159–3.280	0.012	Histologic grade: 3 vs. 1 and 2	1.936	1.123–3.340	0.018
Lymph node metastasis: yes vs. no	2.097	1.288–3.413	0.003	Lymph node metastasis: yes vs. no	-	-	NS
TNM staging: III/IV vs. I/II	3.099	1.896–5.067	<0.001	TNM staging: III/IV vs. I/II	2.559	1.517–4.318	<0.001
ER expression: high vs. low	0.657	0.409–1.056	NS	ER expression: high vs. low	-	-	NS
PR expression: high vs. low	1.337	0.831–2.152	NS				
HER2 expression: high vs. low	0.598	0.367–0.973	0.039	HER2 expression: high vs. low	0.580	0.352–0.955	0.032
Chemotherapy: CAF vs. AC-T	0.842	0.339–2.092	NS				
Hormonal treatment: yes vs. no	0.702	0.429–1.147	NS				
Targeted therapy: yes vs. no	0.598	0.367–0.973	0.039				

ZIC=zinc finger of the cerebellum; HR=hazard ratio; CI=confidence interval; NS=no significance; ER=estrogen receptor; PR=progesterone receptor; HER2=human epidermal growth factor receptor 2; CAF=cyclophosphamide+doxorubicin+5-fluorouracil for 6 cycles; AC-T=doxorubicin+cyclophosphamide for first 4 cycles and paclitaxel for next 4 cycles.

1.517–4.318; $p<0.001$), and HER2 expression (HR, 0.580; 95% CI, 0.352–0.955; $p=0.032$).

DISCUSSION

The development of novel effective biomarkers to assist in diagnosing clinicopathologic features and to determine the prognosis of invasive breast cancer patients has become a popular research topic. For this, in our study, we detected the protein expressions of five ZIC family members and assessed their clinicopathologic and prognostic functions. First, the distributions of the ZIC family proteins differed. ZIC1–3 proteins were distributed both in the nucleus and cytoplasm, whereas ZIC4 and ZIC5 proteins were distributed in the nucleus alone. Although the ZIC family proteins were expressed in stem cells and associated with cell differentiation, we investigated only ZIC family protein expression in breast or breast carcinoma and found that not all cancer cells within tumor tissues expressed higher ZIC protein levels [20]. In addition, only ZIC1 expression in tumors was obviously downregulated compared to that in the corresponding normal tissues, and there were no differences between tumor and normal tissues during the investigation of the other four proteins. Further comparative analyses indicated that the ZIC1 protein expression level in invasive breast cancer was negatively correlated with lymph node metastasis and TNM staging. However, only the finding of $p=0.0056$ (0.05/9) was significant according to Bonferroni correction (Table 3), which indicated that our findings (lymph node metastasis, $p=0.024$; TNM staging, $p=0.015$) may be accurate and a larger quantity of samples should be surveyed. Admittedly, there was significant heterogeneity in the cellular composition of the samples. Thus, we chose representative tumor regions and normal breast tissues >5 cm away from tumors to avoid this heterogeneity. In addition, Kaplan-Meier curves showed that invasive breast cancer patients with high ZIC1 protein expressions had higher 5-year OS rates and DFS rates than those with low expressions. Besides, other clinicopathologic factors, including tumor size, histologic grade, lymph node metastasis, TNM staging, and HER2 expression, ZIC1 expression might become an independent biomarker of OS and DFS in invasive breast cancer patients per our Cox analyses. However, the expression of other ZIC family proteins failed to assess clinicopathologic features or predict the prognosis of invasive breast cancer patients. A recent study also confirmed that only decreased ZIC1 protein expression was associated with aggressive disease progression and a poor prognosis of gastric cancer patients through an IHC analysis of 160 cases [8].

With its control of various biological processes, such as cell division, cell differentiation, myogenesis, neurogenesis, and neurodevelopment, ZIC1 is usually expressed in normal tissues [21]. Several studies demonstrated that upregulated

ZIC1, an oncogene promoting cell proliferation and invasion, was involved in the progression and development of endometrial cancer and liposarcoma [22,23]. However, accumulating evidence suggested that ZIC1 expression was significantly downregulated in various carcinomas and that overexpressed ZIC1 protein suppressed cell proliferation and induced apoptosis by interfering with the mitogen-activated protein kinase, sonic hedgehog homolog, and phosphatidylinositol 3-hydroxy kinase/protein kinase B pathways *in vitro* [4,5]. In addition, promoter hypermethylation of the *ZIC1* gene in thyroid carcinoma, digestive system neoplasms, and gynecologic malignant tumors might be responsible for ZIC1 protein downregulation [4-8,24]. In cervical scrapes, levels of methylated ZIC1 were positively correlated with cervical intraepithelial neoplasia grade [25]. Especially in breast cancer, Nakakido et al. [15] found that the phosphatidylinositol glycan anchor biosynthesis, class X-containing complex elevated BT-549 cell proliferation by inhibiting ZIC1 and then promoted breast cancer growth. Combined with these basic studies, our study showed that ZIC1 protein was a potential good prognostic marker. Despite methylated ZIC4 impelling breast cancer development in a previous study, our study failed to find any association between ZIC4 expression and clinicopathologic features or prognosis [16].

Three classic biomarkers of breast carcinoma (ER, PR, and HER2) were widely used to identify progression, predict prognosis, and select chemotherapy regimens for breast cancer patients. In our study, we also found that only HER2 was an independent biomarker of good prognosis in a Cox regression analysis. However, ZIC family member expression was not significantly associated with ER, PR, or HER2, indicating that ER, PR, and HER2 expression levels did not influence ZIC protein expression. This hypothesis requires confirmation *in vitro* and *in vivo*. In addition, further cytological studies and animal models were essential to investigate alterations of cell proliferation, apoptosis of breast cancer cells, and explore potential specific signaling pathways through lentivirus-mediated overexpression of ZIC1 protein. As a result, we could also develop new regimens of chemotherapy or targeted therapy in future studies.

In summary, using western blotting analysis and IHC evaluations, we concluded that ZIC1 was downregulated in breast tumors and could become a potential biomarker to infer the progression and predict the prognosis of invasive breast cancer patients. Further fundamental and clinical studies would be worthwhile before application of this novel marker in the clinical setting.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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