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RESEARCH ARTICLE

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Prognostic significance of long noncoding RNA Z38 as a candidate biomarker in breast cancer

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Background: Long noncoding RNA (lncRNA) Z38 has been shown to promote cell proliferation and tumorigenesis in breast cancer. However, expression pattern and prognostic value of lncRNA Z38 in breast cancer patients remain elusive.

Methods: The expression levels of SPRY4-IT1 in 110 self-paired specimens of breast cancer and adjacent normal breast tissues were measured by quantitative real-time PCR (qRT-PCR), and its correlation with overall survival of patients with breast cancer was further statistically analyzed.

Results: Compared with normal breast tissues, Z38 was upregulated in breast cancer tissues. Furthermore, of 110 breast cancer patients, high Z38 expression was significantly associated with tumor-node-metastasis stage and lymph node metastasis. Further analysis using the Cox regression model revealed that Z38 expression was an independent prognostic factor of overall survival in patients with breast cancer (hazard ratio=4.74, 95% confidence interval 2.41-9.32). The nomogram presents a good prediction of the probability of overall survival of breast cancer patients (c-index: 0.792), and its predictive efficiency was further confirmed by the calibration curve.

Conclusion: Our data highlighted the potential of lncRNA Z38 as novel candidate biomarker to identify patients with breast cancer at high risk of tumor death.

KEYWORDS
breast cancer, long noncoding RNA, prognosis, Z38

1 | INTRODUCTION

Breast cancer remains to be a major cause of cancer death in females worldwide.[1] Like several epithelial cancers, breast cancer is also a heterogeneous disease characterized by multiple pathological subtypes.[2] Despite the great development in medical science of breast cancer, the morbidity and mortality of breast cancer are still very high at present.[3] Early diagnosis and intervention treatment are essential for scientific research and clinical outcome of breast cancer. Furthermore, mounting evidence suggested that tumor biomarkers are of great clinical significance in tumor screening, diagnosis, and estimation of prognosis and treatment efficacy.[4] Thus, identification of tumor biomarkers for early diagnosis and clinical prognosis has been a hot topic of research.

Long non-coding RNAs (lncRNAs), a newly discovered class of ncRNAs with longer than 200 nucleotides in length, are widely involved in physiological and pathological processes of multiple malignancies by acting as enhancers,[5] scaffolds,[6] decoys,[7] and guides[8] in genome regulation. As previously reported, many lncRNAs are generally deregulated

Abbreviations: lncRNA, long noncoding RNA; HOTAIR, HOX transcript antisense RNA; GAS5, growth arrest-specific 5; MALAT1, metastasis associated lung adenocarcinoma transcript 1; CLDND1, claudin domain containing 1; QRT-PCR, quantitative real-time PCR; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor-2; AUC, area under receiver operating characteristic curve; OS, overall survival; HR, hazard ratio; CI, confidence interval; TNM, tumor-node-metastasis.

ZLN and YSW are contributed equally to this work.

during carcinogenesis and tumor metastasis in a variety of tumors.[9-12] A series of lncRNAs have been demonstrated as oncogenes and/or tumor suppressor genes.[13,14] Furthermore, a number of recent studies suggest the involvement of lncRNAs in cancer development, including in breast cancer.[15-17] For example, HOX transcript antisense RNA (HOTAIR) is overexpressed in patients with breast cancer and inhibits the expression of metastasis-associated suppressor genes to promote metastasis.[16] Additionally, growth arrest-specific 5 (GAS5), a cell cycle regulator, is decreased in breast cancer specimens [18836484]. Some of the candidate lncRNAs that are shown to be deregulated in breast cancer have the potential to be used as prognostic biomarkers.[18,19] Aberrant expression of metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is associated with the progression of breast cancer and is a positive prognostic factor in patients with breast cancer.[20] BC200 lncRNA plays an oncogenic role in estrogen-dependent breast cancer by targeting for attenuating deregulated cell proliferation and possibly serves as a prognostic marker.[21]

Z38 is a long (762 bp), highly conserved lncRNA, which derives from an important protein coding sequence (isoform a) of claudin domain containing 1 (CLDND1) mRNAs and was overexpressed in breast cancer.[22] A series of in vitro and in vivo experiments demonstrated that knockdown of Z38 markedly inhibited cell proliferation and tumor growth in breast cancer.[22] However, its prognostic value in breast cancer needs to be further investigated. Herein, we assessed the expression profile of Z38 in breast cancer tissues by quantitative real-time PCR (QRT-PCR). Next, we investigated its associations with clinical features to identify its clinical value in breast cancer. In addition, we established a predictive model to predict clinical outcome according to lncRNA Z38 expression in patients with breast cancer.

2 | MATERIALS AND METHODS

2.1 | Study subjects

A total of 110 breast cancer patients were enrolled in the present study. All the patients were histologically diagnosed as breast cancer and underwent surgical resections at The Second People's Hospital of Lianyungang and Nanjing First Hospital. None of the patients had received chemotherapy or radiotherapy before surgery. Clinical characteristics of individuals including age, family history, tumor differentiation, tumor-node-metastasis (TNM) stage, lymph node status, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (Her2) were collected and summarized in Table 1. All breast cancer tissues were obtained after surgical resection and stored immediately in liquid nitrogen. Each subject was followed up with a median follow-up period of 51 months. Written consent was obtained from each individual, and the study protocol was approved by the Medical Ethics Committee of Nanjing First Hospital.

2.2 | Total RNA extraction and cDNA synthesis

Total RNA was extracted from tumor tissues using the TRIzol reagent (Invitrogen, CA, USA) following the manufacturer protocol and stored immediately at −80°C. The RNA purity were further detected with its $OD_{260/280}$ ranged from 1.8 to 2.0 to perform the subsequent experiments, and the cDNA was synthetized from 1 μg RNA template in a volume of 20 μL using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Dalian, China).

2.3 | Quantitative real-time PCR

The Z38 expression was measured using SYBR Premix Ex TagTM II (Takara, Dalian, China) according to the manufacturer instruction via ABI 7500 System (Applied Biosystems, Carlsbad, CA, USA). The processes of QRT-PCR reaction were conducted in a volume of 20 μL mixed with 30 ng cDNA templates and gene-specific primers for Z38 (forward primer AGTGGGATTGTGGAGACGGTGT, reverse primer AGGTAAAAGGAACTGGCAACGC) and internal control GAPDH (forward primer AACGGATTTGGTCGTATTGGG, reverse primer CCTGGAAGATGGTGATGGGAT) for each well. PCR cycling protocol was as follows: initiate hold at 95°C for 10 min, followed by 40 amplification cycles of melting at 95°C for 15 seconds, annealing and extension at 60°C. The relative expression was calculated as fold changes by the comparative Ct (ΔΔCt) method.

2.4 | Statistical analysis

A paired Wilcoxon signed-rank test was employed to evaluate Z38 expression in breast cancer tissues versus adjacent normal tissues. Correlations between Z38 expression and clinical features were performed by a Person's χ^2 test. Data were expressed as number (percentage) and mean±SD if necessary. The optimal cut-off value of Z38 in tumor/normal was determined by a receiver operating characteristic (ROC) curve analysis. The Kaplan–Meier and Cox proportional hazards model were used to analyze whether Z38 expression impacted prognosis. A prognostic model was established according to significant factors in univariate COX regression analysis by R version 3.2.2 software (Institute for Statistics and Mathematics, Vienna, Austria), and its predictive efficiency was examined by a Harrell's concordance index (c-index). All statistical analyses were performed using SPSS version 20.0 software (IBM, Carlsbad, CA, USA). Values of $P<.05$ were considered significant.

3 | RESULTS

3.1 | Upregulation of Z38 in breast cancer specimens

To investigate expression of Z38 in tumor tissues, we used a QRT-PCR to detect the expression levels of Z38 from 110 breast cancer and corresponding normal breast tissue samples. The results showed that Z38 was highly expressed in breast cancer as compared with corresponding normal breast tissues ($P<.001$, Figure 1). Furthermore, lncRNA Z38 was a good candidate to discriminate tumor specimens from corresponding normal specimens (sensitivity: 78%, specificity: 70%) by ROC curve analysis. Additionally, the optimal cutoff value of Z38 expression (2.86-fold) in tumor/normal was determined by

TABLE 1 Association between lncRNA Z38 and baseline characteristics

Factors	No. of patients	Z38 expression		*P* value[a]
		Low (n=53)	High (n=57)	
Age (y)				
<62	55 (50.0)	30 (56.6)	25 (43.9)	.182
≥62	55 (50.0)	23 (43.4)	32 (56.1)	
Family history				
Absent	102 (92.7)	50 (94.3)	52 (91.2)	.530
Present	8 (7.3)	3 (5.7)	5 (8.8)	
Tumor grade				
I-II	86 (78.2)	42 (79.2)	44 (77.2)	.795
III	24 (21.8)	11 (20.8)	13 (22.8)	
TNM stage				
I	26 (23.6)	18 (34.0)	8 (14.0)	.002
II	37 (33.6)	22 (41.5)	15 (26.3)	
III	30 (27.3)	8 (15.1)	22 (38.6)	
IV	17 (15.5)	5 (9.4)	12 (21.1)	
Lymph node metastasis				
Absent	53 (48.2)	19 (35.8)	34 (59.6)	.013
Present	57 (51.8)	34 (64.2)	23 (40.4)	
ER status				
Negative	60 (54.5)	29 (54.7)	31 (54.4)	.972
Positive	50 (45.5)	24 (45.3)	26 (45.6)	
PR status				
Negative	13 (11.8)	5 (9.4)	8 (14.0)	.455
Positive	97 (88.2)	48 (90.6)	49 (86.0)	
Her2 status				
Negative	32 (29.1)	16 (30.2)	16 (28.1)	.807
Positive	78 (70.9)	37 (69.8)	41 (71.9)	

lncRNA, long noncoding RNA; TNM, tumor-node-metastasis; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor type 2.
[a]Person's χ^2 test.

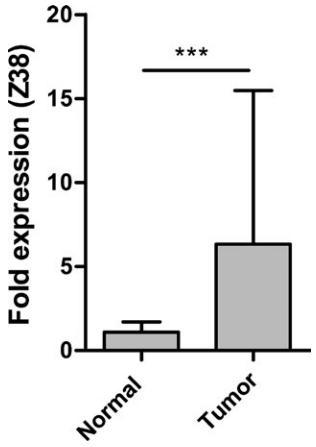

FIGURE 1 Relative levels of Z38 in breast cancer tissues samples. quantitative real-time PCR analyses to determine the relative levels of Z38 in a total of 110 breast cancer patients, a paired Wilcoxon signed-rank test was performed, *** indicate *P* <.001.

corresponding largest Youden's index. The area under ROC curve is 0.758 (95% confidence interval [CI]: 0.67-0.85, *P*<.001; Figure 2). To further explore the association of Z38 expression with clinical characteristics, breast cancer patients were categorized as high or low expression group according to the optimal cut-off value of fold expression of Z38.

3.2 | Association between lncRNA Z38 expression and clinical characteristics in breast cancer

To identify the clinical relevance of Z38 expression in breast cancer, the association between Z38 expression and baseline characteristics was analyzed in 110 breast cancer tissues. The association of Z38 expression with baseline characteristics was summarized in Table 1. High Z38 expression was remarkably correlated with TNM stage (*P*=.002) and lymph node metastasis (*P*=.013), but not correlated with patient's age, family history, and tumor grade, as well as ER, PR, and Her2 status

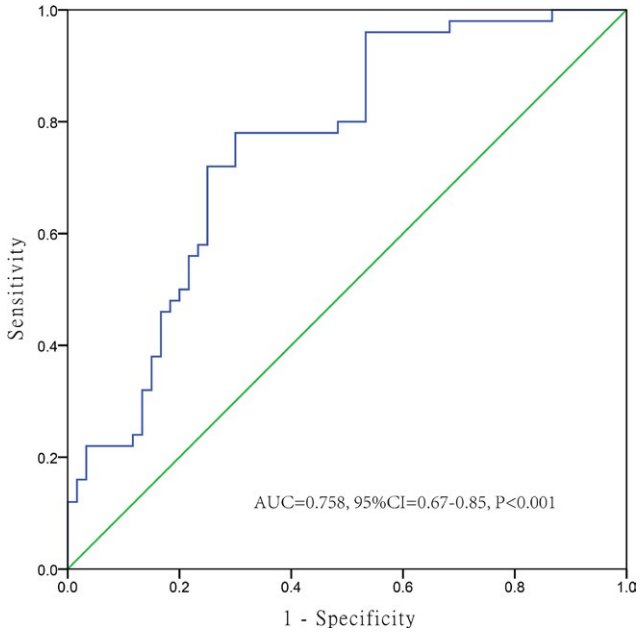

FIGURE 2 Optimal cutoff value of Z38 expression determined by receiver operating characteristic curve for overall survival.

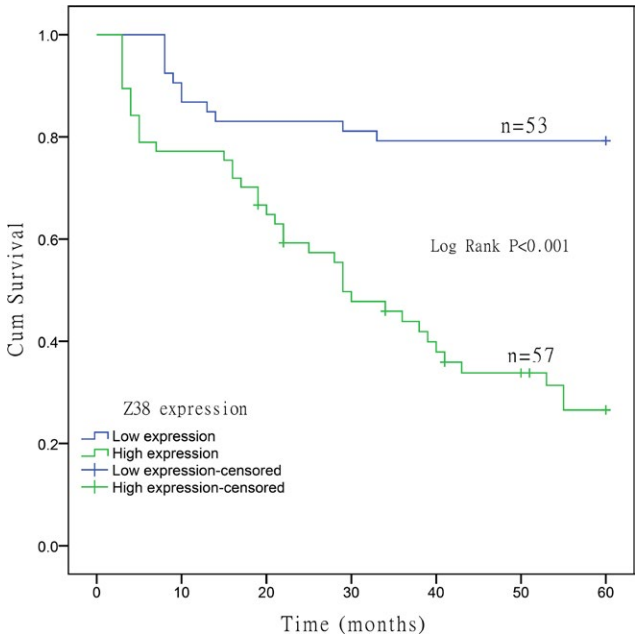

FIGURE 3 Survival analysis in breast cancer patients based on Z38 expression. Overall survival based on high Z38 expression versus low Z38 expression in patients with breast cancer.

(*P*>.05). Collectively, these results suggest that high Z38 expression was associated with the development and progression of breast cancer.

3.3 | Prognostic value of Z38 in breast cancer

In 110 patients with breast cancer, the median follow-up time was 51 months. Fifty cases (45.5%) died (39 patients died of breast cancer, eight patients died of other malignancies, and three patients died of other diseases). At the cut-off value of Z38 expression, the 5-year overall survival (OS) rates of the high expression group versus the low expression group were 20.8% and 68.4% respectively (*P*<.001; ure 3 and Table 2). Significant factors in univariate analyses (Z38 expression, tumor grade, TNM stage, and lymph node metastasis) were further included in a multivariate Cox regression analysis. As shown in Table 3, Z38 expression was an independent prognostic factor of OS in breast cancer patients (Hazard ratio=4.74, 95% CI=2.41-9.32, *P*<.001; Table 3).

3.4 | A nomogram for predicting overall survival

To accurately predict overall survival of breast cancer patients, a predictive model was constructed using the significant factors identified in univariate analyses (Table 3). This model was performed through summing the points projected on the top scale for Z38 expression, tumor grade, TNM stage, and lymph node metastasis. The sum of point scores was identified on the total points scale to know the probability of 3- and 5-year overall survival (Figure 4). The c-index for the nomogram was 0.792 on the basis of fitted multivariate Cox regression analysis on a total of 110 breast cancer patients. To further investigate its predictive efficacy, a calibration curve was plotted. As shown in Figure 5, the dashed line presented a performance of an ideal model, in which the predicted findings perfectly matched with the actual findings.

4 | DISCUSSION

It is widely accepted that lncRNAs play a critical role of cancer origination and progression. Emerging studies started to focus on the regulatory roles of lncRNAs in cancer. For example, UCA1 acted as an endogenous sponge through recruiting miR-216b to upregulate fibroblast growth factor receptor 1 expression and activate ERK signaling pathway.[23] The small nucleolar RNA host gene 20 acted as a lncRNA to regulate cell proliferation, invasion and migration, and cell cycle progression in colorectal cancer cells.[24] Similarly, lncRNA Z38 could contribute to cell proliferation and tumorigenesis in breast cancer.[22] However, clinical relevance of lncRNA Z38 in breast cancer remains unknown. In the present study, our results indicated that Z38 expression was increased in breast cancer tissues compared with corresponding normal breast tissues. These data combined with a previous study showed that Z38 was overexpressed in breast cancer tissues and promoted cell proliferation and tumorigenesis of breast cancer cells.[22] We firstly explored the clinical implication of Z38 expression in breast cancer patients, providing a novel perspective on prognostic predication of breast cancer. Furthermore, the constructed nomogram could conduct to predict clinical outcomes in breast cancer patients by its c-index and the calibration curve (Figure 5). These findings reveal that Z38 may play an important role in the progression and prognosis of breast cancer, and it may be considered as a potential prognostic biomarker for breast cancer.

As we all know, breast cancer is a heterogeneous disease at the clinical characteristics and molecular levels. Traditional clinical factors,

TABLE 2 Association of clinical characteristics with overall survival in patients with breast cancer

Characteristics		Total n (%) 110	Overall survival n (%) of patients with death 50	*P* value[a]
Age (y)	<62	55 (50.0)	22	.174
	≥62	55 (50.0)	28	
Family history	Absent	102 (92.7)	45	.490
	Present	8 (7.3)	5	
Tumor grade	I-II	86 (78.2)	34	.029
	III	24 (21.8)	16	
TNM stage	I-II	63 (57.3)	22	.002
	III-IV	47 (42.7)	28	
Lymph node metastasis	Absent	53 (48.2)	18	.012
	Present	57 (51.8)	32	
ER status	Negative	60 (54.5)	30	.212
	Positive	50 (45.5)	20	
PR status	Negative	13 (11.8)	7	.501
	Positive	97 (88.2)	43	
Her2 status	Negative	32 (29.1)	17	.417
	Positive	78 (70.9)	33	
Z38 expression	Low	53 (48.2)	11	<.001
	High	57 (51.8)	39	

TNM, tumor-node-metastasis; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor type 2.
[a]Log rank test.

TABLE 3 Univariate and multivariate analysis of clinical characteristics of overall survival by Cox regression model

Characteristics	Univariate analysis HR (95% CI)	*P*	Multivariate analysis HR (95% CI)	*P*
Age	1.47 (0.84-2.56)	.180	1.30 (0.74-2.29)	.360
Family history	1.38 (0.55-3.48)	.495		
Tumor grade	1.91 (1.05-3.46)	.033	1.72 (0.95-3.13)	.073
TNM stage	2.36 (1.35-4.14)	.003	2.25 (1.28-3.96)	.005
Lymph node metastasis	2.05 (1.15-3.66)	.015	1.28 (0.42-3.91)	.660
ER status	0.70 (0.40-1.23)	.218		
PR status	0.76 (0.34-1.70)	.506		
Her2 status	0.79 (0.44-1.41)	.422		
Z38 expression	4.89 (2.47-9.56)	<.001	4.74 (2.41-9.32)	<.001

TNM, tumor-node-metastasis; ER, estrogen receptor; PR, progesterone receptor; Her2, human epidermal growth factor receptor type 2; HR, hazard ratio; CI, confidence interval.

including TNM stage, tumor differentiation, lymph node status, seem not to be sufficient for predicting overall survival of breast cancer patients. With the development of epigenetics, lncRNAs, a recently found class of ncRNAs, have been involved in the tumorigenesis and development of cancer.[25] Although a series of lncRNAs have been correlated with cancer progression, the expression profile and prognostic significance of lncRNAs in breast cancer have not been systematically explored due to the lack of available samples and prognostic information of breast cancer. The lncRNA Z38 has been identified to promote cell proliferation and tumor growth through a series of in vitro and in vivo experiments.[22] In this study, lncRNA Z38 expression was significantly correlated with TNM stage and lymph node metastasis, but not correlated with ER status. However, previous investigations reported that several lncRNAs were associated with ER-positive tumors in breast cancer. For example, lncRNA BC200 expression is obviously lower in ER-negative tumors than in ER-positive tumors.[21] Concurrently, MALAT1 was also overexpressed in ER-positive breast cancer specimens.[26] The contradictory results indicate the spatial

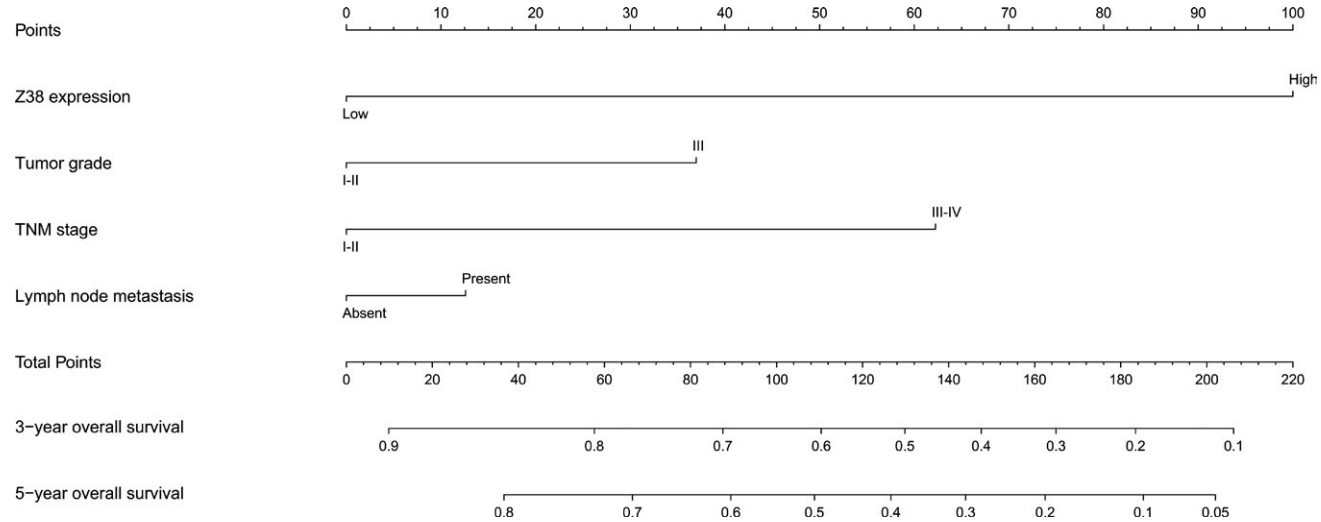

FIGURE 4 Nomogram conveyed significant clinical characteristics and Z38 expression to predict overall survival of breast cancer patients. Nomogram is interpreted by summing up the points assigned to each factor, which is indicated at the top of scale. The total points can be converted to predicted 3-y and 5-y probability of death for a patient in the lowest scale. The Harrell's c-indexes for overall survival prediction were 0.792. TNM, tumor-node-metastasis.

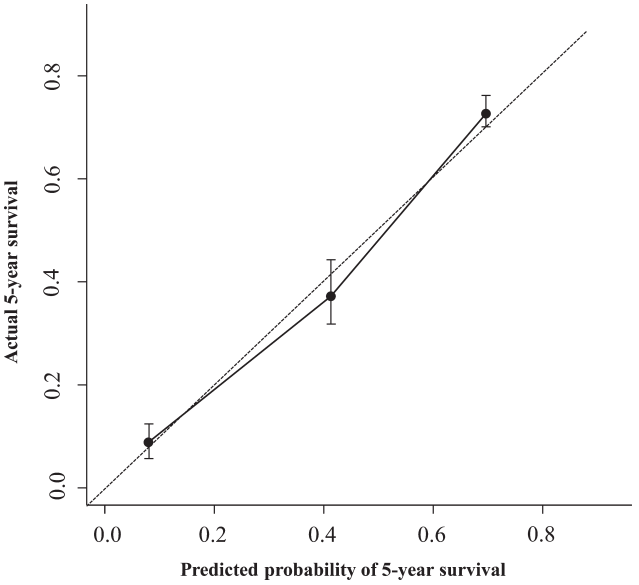

FIGURE 5 Calibration curve for 5-y overall survival. The *x*-axis is its predicted probability of 5-y survival and *y*-axis is actual survival. The dashed line is 45 degree and presents perfect calibration.

specificity of lncRNAs. Furthermore, we demonstrated that lncRNA Z38 was an independent predictor of poor OS in breast cancer patients.

More interestingly, we revealed that Z38 expression was used to predict clinical outcomes in patients with breast cancer through the predictive model. It is well-known that a good model can facilitate physicians to identify high-risk patients to improve their therapeutic strategies. The nomogram has been developed to evaluate the clinical prognosis in patients with various types of cancers.[27,28] We have also constructed a predictive nomogram to predict the probability of 3-year or 5-year overall survival for patients with breast cancer according to Z38 expression and the significant variables in univariate analysis. The predictive efficiency of this nomogram was further identified by the c-index and calibration curve. The nomogram aims to calculate some of the heterogeneity within TNM stage and provide an accurate predictor for clinical outcome.

This study has several limitations: (1) The expression of lncRNA Z38 was only analyzed in tissues, and we will collect serum samples of breast cancer to further confirm our findings. (2) This study lacked of an independent cohort to identify the predictive value of this lncRNA signature. Thus, the findings of this study should be further confirmed for a larger sample in a multicenter, randomized, controlled, and prospective study.

In summary, we have shown a distinct expression pattern of lncRNA Z38 in breast cancer tissues compared with corresponding normal breast tissues. The lncRNA Z38 overexpression is markedly associated with shorter OS in patients with breast cancer. Therefore, Z38 is a potential biomarker in breast cancer patients. In the future, following studies will be essential to explore the molecular mechanism of Z38 in breast cancer.

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AUTHORS' CONTRIBUTION

ZLN and SKW conceived and designed the project, ZLN, YSW, YPM, XL, and GXZ acquired the data, HLS and YLW analyzed and interpreted the data, ZLN and YSW wrote the paper.

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