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ORIGINAL RESEARCH

Nuclear expression of XBP1s is correlated with breast cancer survival: a retrospective analysis based on tissue microarray

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Abstract: An alternatively spliced transcription factor that participates in the unfolded protein response, XBP1 is a novel protein involved in cancer progression and outcome. This study aimed to investigate the relationship of spliced XBP1 (XBP1s) with the clinicopathological characteristics and prognosis of breast cancer by using tissue-microarray analysis. A consecutive series of 170 patients with breast cancer diagnosed between 2001 and 2004 in hospitals in eastern and southern China were included. Immunohistochemical staining for XBP1s was performed, and the expression of XBP1s was separately examined in nuclei and cytoplasm. We found that a higher expression of XBP1s in nuclei strongly correlated with poorer survival (46.7% versus 75%, P=0.018); however, the expression of XBP1s in the cytoplasm had no relationship with survival. Multivariate Cox regression analysis indicated that the expression of XBP1s was not an independent prognostic factor (RR 2.074, 95% CI 0.909–4.736; P=0.083). None of the other clinicopathological characteristics – age, pathology grade, T stage, N stage, TNM stage, estrogen receptor, progesterone receptor, or HER2 status – was found to be correlated with XBP1s expression in the nuclei. In conclusion, independently of other clinicopathological factors, the nuclear expression of XBP1s is correlated with shorter breast cancer survival; however, whether nuclear XBP1s is an independent prognostic biomarker needs to be confirmed by further studies with larger samples and detailed sample stratification.

Keywords: breast cancer, tissue microarray, XBP1s, endoplasmic reticulum stress, biomarker

Introduction

Breast cancer (BC) is a heterogeneous disease sustained by complex growth pathways. Chemotherapy, hormone therapy, and molecular target therapy have had a significant impact on the survival of BC patients;[1,2] however, adjuvant treatments do not always guarantee optimal results.[3–5] In the past few decades, a better understanding of the molecular pathways involved in BC growth and progression allowed the identification of molecular targets that could be selectively inhibited by targeting agents.[6,7] Nowadays, we are going through a bottleneck period of identifying new targets, allowing the possibility to develop novel approaches to further increasing the survival of BC patients.

XBP1, a key molecule in the unfolded protein response (UPR), was discovered to have a close relationship with cancer progression.[8–10] The alternatively spliced form of XBP1 (XBP1s) is a major active form that as a transcriptional factor translocates into the nucleus and regulates gene expression, which helps resolve ER stress and support cell survival during the UPR.[11–14] Evidence has emerged that XBP also plays a role in BC development.[15–19] Serial analysis of gene expression showed *XBP1* to be

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highly expressed in cancerous mammary epithelial cells.[16] Immunohistochemistry analysis demonstrated expression of XBP1 in 90% of breast tumors, and in vitro UPR activation induced resistance to some chemotherapy drugs.[17] An analysis of independent cohorts of patients with triple-negative BC revealed a specific *XBP1* gene-expression signature that was strongly associated with poor prognosis.[18] Recent work has revealed that increasing expression of XBP1 is associated with BC progression and that the XBP1 protein is significantly overexpressed in metastatic tumors.[19]

In our previous study,[20] we demonstrated that XBP1s may play a role in estrogen-therapy resistance. Increased expression of both XBP1s mRNA and protein was discovered in tamoxifen-resistant MCF7 cells compared with normal MCF7 cells. Moreover, the sensitivity of XBP1s MCF7-TAMR cells to tamoxifen can be reestablished in vitro by a novel XBP1s inhibitor called STF083010. Furthermore, in an analysis of 170 BC patients' samples, XBP1s expression was discovered to be highly correlated with overall survival of ER^+ BC patients, strongly suggesting potential therapeutic value of XBP1 inhibitors in BC treatment.

The purpose of this study was to investigate the effect of XBP1s expression and subcellular localization on survival of BC patients through immunohistochemistry of a tissue microarray containing 170 patients with invasive BC. Kaplan–Meier survival analysis indicated a significant correlation between nuclear XBP1s (XBP1s-N) expression and overall survival. In addition, correlations among XBP1s expression and clinicopathological variables was explored to determine its potential value in BC classification and prognosis.

Materials and methods

Ethics statement

Ethical approval was given by the medical ethics committee of Tongji Medical College, Huazhong Science and Technology University (S025), and was approved for a project of the National Natural Science Foundation of China (81202094). This study was a part of this project. Patients provided written informed consent for the use of their tissue for future research prior to treatment.

Patients and tissue

A consecutive series of 170 patients diagnosed with invasive BC who underwent surgery between 2001 and 2004 in hospitals in Jiangsu, Zhejiang, and Shanghai were selected. Their information was obtained from the tissue bank of the Shanghai Biochip Center. Tissues were collected immediately after surgical resection and snap-frozen in liquid nitrogen, then stored in the tissue bank until later use. After exclusion of patients lost to follow-up or lacking full data, 160 patients were included. Detailed information on histopathological variables, clinical data, and long-term follow-up was available for these patients and registered in a database. The median follow-up of these patients was 9–12.5 years.

Tissue-microarray preparation

Formalin-fixed paraffin-embedded tumor samples of patients were obtained from the Shanghai Biochip Center. Histopathological characteristics of these samples are shown in Table 1. Three morphologically representative tumor regions were chosen from each of the 160 samples. Three corresponding normal adjacent tissue samples were obtained simultaneously. Cylindrical core-tissue specimens (5×15×15 mm) were acquired from each tumor sample and then arrayed into a newly designed recipient paraffin block using a custom-built precision instrument (Outdo Biotech, Shanghai, China). After being heated at 52°C, the core tissues were melted and closely fitted into the paraffin block.

Immunohistochemistry

All tissue-microarray samples were cut with a microtome into 4 μm sections that were mounted on poly-L-lysine-coated glass slides. Immunohistochemical staining for XBP1s was recorded separately for the cytoplasm and nuclei of the 160 BC samples and ten normal adjacent tissue samples. ER, PR, and HER2 staining had been performed previously, and results were recorded after the initial surgery was done. After a second incubation with biotinylated antigoat antibodies, slides were incubated with peroxidase-labeled streptavidin. Reaction products were visualized by immersing the slides in diaminobenzidine tetrachloride and counterstaining with Harris hematoxylin. Staining for XBP1s was considered positive only if a minimum of 10% definite tumor cells showed a positive reaction.

Statistical analysis

All analyses were completed using SPSS 16.0 software. Survival curves were calculated using the Kaplan–Meier method, with significance evaluated using the Mantel–Cox log-rank test. The prognostic significance of parameters was assessed using the Cox proportional-hazard model with overall survival as an end point. A multivariate analysis was performed using a Cox model; previously identified prognostic factors for BC were included in the model. Relationships between XBP1s expression and clinicopathological parameters were calculated using nonparametric Kruskal–Wallis and Mann–Whitney methods using Spearman's correlation

Table 1 Patient characteristics

Patient characteristics	n (%)
Age (years)	
<35	10 (6.25)
35–50	66 (41.25)
>50	84 (52.5)
Lost*	0
Tumor size	
<2 cm	13 (8.125)
2–4 cm	112 (70)
>4 cm	33 (20.625)
Lost	2 (1.25)
Pathological grade	
I	18 (11.25)
II	136 (85)
III	6 (3.75)
Lost	0
T stage	
T1	36 (22.5)
T2	107 (66.875)
T3	15 (9.375)
T4	0
Lost	2 (1.25)
N stage	
N0	63 (39.375)
N1	48 (30)
N2	37 (23.125)
N3	8 (5)
Lost	4 (2.5)
TNM clinical stage	
TNM1	13 (8.125)
TNM2	94 (58.75)
TNM3	48 (30)
TNM4	0
Lost	5 (3.125)
ER	
Negative	48 (30)
Positive	108 (67.5)
Lost	4 (2.5)
PR	
Negative	60 (37.5)
Positive	95 (59.375)
Lost	5 (3.125)
HER2	
Negative	105 (65.625)
Positive	52 (32.5)
Lost	3 (1.875)

Note: *Lost to follow-up.

analysis. $P<0.05$ was considered statistically significant. Correlations with numerical variables were analyzed by the Mann–Whitney *U* test.

Results

Patient and tumor characteristics

Data of the patients and tumor characteristics are outlined in Table 1. The median age of the study group was 51 years (range 29–83 years). The majority of tumors (95.1%) were categorized as invasive ductal carcinoma; other presentations included infiltrative lobular carcinoma (3.7%) and a mixture of both (1.2%). Most tumors were histological grade 2 (85%), and 11.25% presented with grade 1. We identified T1, T2, T3, and T4 tumors in 11.3%, 66.9%, 9.4%, and 0 patients based on the sixth American Joint Committee on Cancer TNM staging system. Axillary nodal metastases were identified in 58.1% of patients. TNM stage 2 and 3 were the most common stages (58.8% and 30%), followed by 8.1% cases of stage 1 and none of stage 4. There were more hormone receptor (ER/PR)-positive cases than negative cases (ER 67.5% versus 30%, PR 59% versus 38%). Only 32.5% cases were HER2-positive and 65.6% HER2-negative.

Evaluation of XBP1s expression by immunohistochemistry

For each spot, the regions of most intense and/or predominant staining pattern were scored by eye. Nuclear and cytoplasmic staining intensity and positive percentage were determined separately for each specimen. Staining intensity was graded on a scale of 0–3. Positive percentage was classified into five categories: 0 (negative), 1 (1%–25%), 2 (26%–50%), 3 (51%–75%), and 4 (76%–100%). Total scores were calculated from the product of staining intensity and positive percentage that was used to divide all specimens into two groups: a low-expression group (score 0–5) and a high-expression group (score 6–12). For specimens that were uninterpretable or not infiltrating carcinoma, a designation of "not applicable" was given. A sample of immunohistochemistry results is displayed in Figure 1. According to the evaluation standard of immunohistochemical staining, 9.4% and 4.4% of cells showed high XBP1s expression in the nucleus and cytoplasm (XBP1s-C).

Survival analysis

By July 2013, the median survival of all 160 patients was 101 months (2–131 months). Kaplan–Meier survival analysis was performed with respect to clinical stage. Survival rates from stage 1 to stage 3 were 88.9%, 79.4%, and 59.2%, respectively, and differences were statistically significant (log-rank $P=0.005$) (Figure 2).

Further survival analyses was performed for XBP1s-C and XBP1s-N, as well as other clinicopathological parameters: age, pathology grade, T stage, N stage, TNM stage, ER, PR, HER2, and *HER2* gene amplification (*HER2* fluorescence in situ hybridization). The statistical significance of differences was tested via log-rank analysis (Table 2). In the XBP1s-N-expression group, survival rates in patients with low and high XBP1s expression were 75% (108 of 144) and

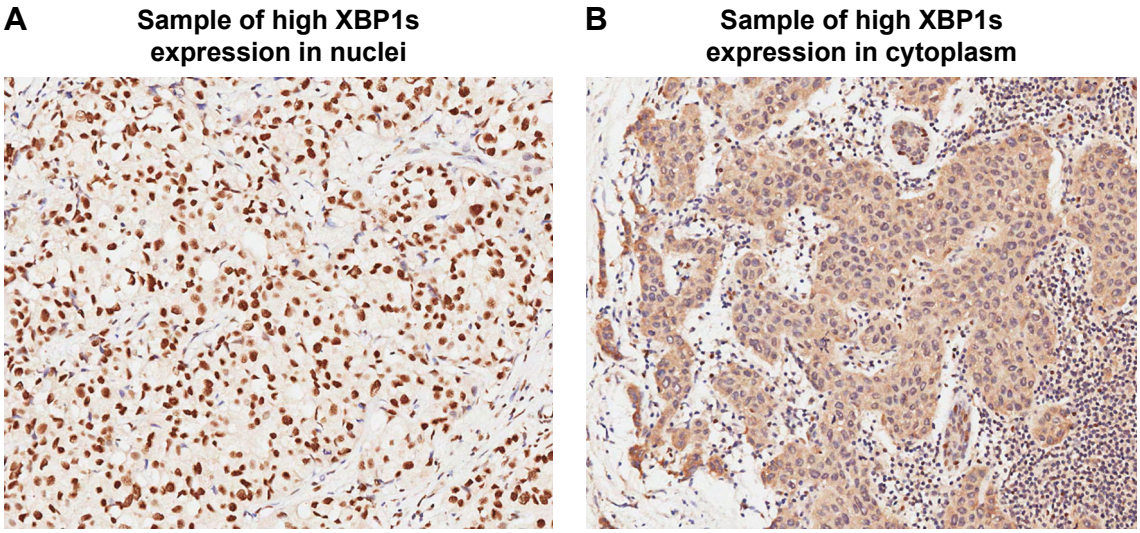

Figure 1 Immunohistochemical staining data.
Notes: (**A**) 9.4% of nuclei highly expressed XBP1s; (**B**) 4.4% of cytoplasm highly expressed XBP1s. Magnification: 20×.

46.7% (eight of 15), respectively. The log-rank test showed that the difference between survival rates had statistical significance (P=0.018), ie, higher XBP1s-N expression corresponded strongly to poorer survival of patients with BC. In contrast, in the XBP1s-C-expression group, 71.7% (109 of 152) patients with low XBP1s expression and 85.7% (six of seven) patients with high XBP1s expression survived; however, the difference was not significant (P=0.471). Survival curves are shown in Figure 3. Other factors significantly affecting survival included pathological grade (P=0), clinical N stage (P=0.042), TNM stage (P=0.012), ER status (P=0.004), and PR status (P=0.009), correlations that have been widely verified by prior researchers.

Correlation of XBP1s-N expression with various clinicopathological parameters

Because XBP1s-N expression had a significant effect on survival, but was not an independent prognostic factor, we examined the correlation of XBP1s-N expression with other clinicopathological parameters. Under Kruskal–Wallis and Mann–Whitney analyses, XBP1s-N-expression levels showed no significant differences among age–groups <35,

A Kaplan–Meier survival analysis of clinical stage

Clinical stage	Censored		
	Total	Survival (n)	Percentage
Stage 1	18	16	88.9
Stage 2	97	77	79.4
Stage 3	49	29	59.2
Overall	164	122	74.4
	Overall comparisons		
	χ^2	*df*	*P*-value
Log-rank	10.682	2	0.005

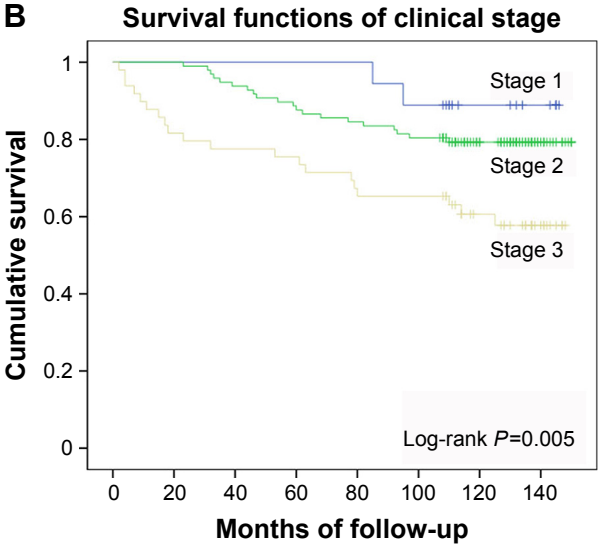

Figure 2 Kaplan–Meier survival analysis categorized by clinical stage.
Notes: (**A**) Survival rates of stage 1, stage 2, and stage 3 were 88.9%, 79.4%, and 59.2%, respectively, and differences were statistically significant (log-rank P=0.005); (**B**) survival curves of clinical stage 1–3 (using the 6th American Joint Committee on Cancer TNM staging system as the criterion).

Table 2 Kaplan–Meier survival analysis and log-rank test

Groups	Total	Survived		Log-rank *P*-value
		n	Percentage	
XBP1s-C				0.471
Low	152	109	71.7	
High	7	6	85.7	
Overall	159	115	72.3	
XBP1s-N				0.018
Low	144	108	75	
High	15	7	46.7	
Overall	159	115	72.3	
Age, years				0.243
<35	10	8	80	
35–50	66	52	78.8	
>50	83	55	66.3	
Overall	159	115	72.3	
Pathology grade				0
1	18	12	66.7	
2	135	103	76.3	
3	6	0	0	
Overall	159	115	72.3	
T stage				0.286
T1	36	27	75.0	
T2	106	78	73.6	
T3	15	9	60.0	
Overall	157	114	72.6	
N stage				0.042
N0	62	47	75.8	
N1	48	39	81.3	
N2	37	21	56.8	
N3	8	5	62.5	
Overall	155	112	72.3	
TNM stage				0.012
TNM1	13	11	84.6	
TNM2	93	73	78.5	
TNM3	48	28	58.3	
Overall	154	112	72.7	
ER				0.004
–	48	28	58.3	
+	107	84	78.5	
Overall	155	112	72.3	
PR				0.009
–	60	37	61.7	
+	94	74	78.7	
Overall	154	111	72.1	
HER2				0.938
–	104	74	71.2	
+	52	38	73.1	
Overall	156	112	71.8	

Notes: XBP1s-C, cytoplasmic XBP1s; XBP1s-N, nuclear XBP1s.

35–50, or >50 years (P=0.477). No differences were seen for pathology grades 1–3, T stages 1–3, N stages 0–3, TNM stages 1–4 (P=0.112, 0.144, 0.071, and 0.241, respectively), for ER status, PR status, HER2 status, or the luminal subgroup (0.718, 0.315, and 0.578, respectively). The same results were obtained from Spearman's correlation analysis. We can conclude that expression of XBP1s-N was not correlated with any of the clinicopathological parameters analyzed in this study. Detailed data are shown in Table 3.

Multivariate Cox regression analysis

Multivariate Cox regression analyses were performed with factors that have previously been proven to affect survival. The results are shown in Table 4. In the Cox model, the P-value of XBP1s-N expression was 0.083, and RR was 2.074 (95% CI 0.909–4.736), indicating that XBP1s-N expression was not an independent prognostic factor. P-values for pathological level and TNM stage were 0.247 and 0.155, respectively. We concluded from these results that pathological grade and TNM stage are not independent prognostic factors of BC. Clinical N-stage, ER-status, and PR-status correlation coefficients were negative (–0.079, –0.409, and –0.607, respectively); however, P-values were all greater than 0.05 (0.799, 0.36, and 0.177, respectively), indicating no statistical significance. Therefore, N stage, ER status, and PR status were not independent prognostic factors either in the present study.

Discussion

Our survival analysis showed that elevated expression of XBP1s-N is associated with worse clinical outcome. The overall survival rate of patients with low XBP1s-N expression was 1.6 times higher than patients with high XBP1s-N expression. However, the same was not true for the XBP1s-C-expression group. XBP1s is an important participant in the UPR, and has been studied as a novel protein involved in BC in recent years. Researchers have observed that the expression level of XBP1s in BC tissue is correlated with prognosis; however, these studies were mostly performed in the context of gene expression.[12,21–25] In this study, using tissue-microarray methods, we showed that a higher expression of XBP1s protein in nuclei corresponded to poorer survival in BC. This finding suggested that XBP1s affects survival in patients via its role as a nuclear transcription factor.

Scientists have explored the mechanism of XBP1s in tumor progression. The low sugar, anoxic, and acidic microenvironment in malignant tumors can lead to an increase in proteins unfolding/misfolding, causing higher ER stress.[21,26,27] In response to ER-stress signals, IRE1 in the endoplasmic reticulum membrane dimerizes and becomes autophosphorylated, resulting in its activation and the unconventional splicing of XBP1 pre-mRNA. XBP1-splicing creates transcriptionally active XBP1s.[8] XBP1s enters the nucleus and activates a variety of genes involved in protein

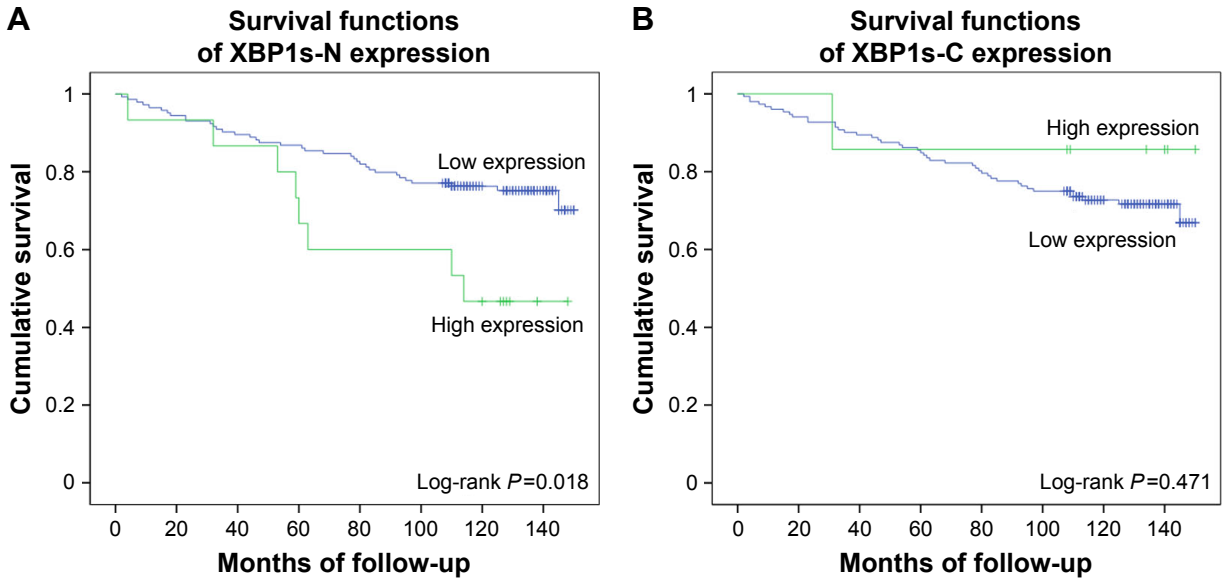

Figure 3 Survival curves of (**A**) nuclear XBP1s (XBP1s-N) expression and (**B**) cytoplasmic XBP1s (XBP1s-C) expression.
Notes: (**A**) In the XBP1s-N group, the survival rate of patients with low XBP1s expression was 75% and that of patients with high XBP1s expression 46.7% (*P*=0.018). (**B**) In the XBP1s-C group, 71.7% of patients with low XBP1s expression survived and 85.7% with high XBP1s expression survived; however, this difference was not significant (*P*=0.471). Reproduced from Ming J, Ruan S, Wang M, et al. A novel chemical, STF-083010, reverses tamoxifen-related drug resistance in breast cancer by inhibiting IRE1/XBP1. *Oncotarget*. 2015;6(38):40692–40703.[20]

maturation, degradation, or ER expansion, which enables cells to produce and secrete proteins efficiently, helping to resolve ER stress and promote cell survival.[28–30] However, if the UP level exceeds a threshold, affected cells are committed to cell death.[31,32] This mechanism gives us a rough understanding of why XBP1s expression in nuclei affects BC survival. Similarly to the discoveries about hormone receptors and consequent endocrine treatment in BC,[7] XBP1s may have potential value in prognostication and targeted therapy in the future.[10]

Our multivariate Cox regression analysis showed that it is too early to conclude that XBP1s is an independent prognostic factor. Other factors, such as pathology grade, N stage, TNM stage, ER, and PR, had no significance in our model either. The generally accepted biomarker HER2, which has been shown to have a detrimental effect on relapse-free survival and risk of death,[6,33] likewise showed no effect on survival in this study. This result is not so surprising when considering that the sample was not very large and the majority of our samples were from middle- to late-stage patients. More importantly, ER status was reported to be related to the induction of PR.[34,35] This may be the main reason that even though ER and PR are both important biomarkers that obviously affect BC survival,[36–38] they do not always show significance in multivariate-regression models,[39] which is consistent with our results. It is noteworthy that the *P*-value of XBP1s-N expression in our model was 0.083, which was the lowest *P*-value and very close to 0.05. Further studies with a larger samples and detailed sample stratification are needed to revise our conclusion.

In further exploration of the correlation of XBP1s-N expression with other clinicopathological parameters, we found that it had no significant correlation with age, pathology grade, T stage, N stage, TNM stage, ER, PR, HER2 status, or luminal subtype. From this, we can preliminarily conclude that XBP1s-N expression is independent of these clinicopathological factors. Interestingly, XBP1 has been reported to interact with ER in a ligand-independent manner and can also induce transcription from estrogen-responsive elements containing a luciferase-reporter gene, even in the absence of estrogen.[40] Further research found large-scale chromatin unfolding associated with XBP1-mediated increases in ER-transcriptional activity.[41] Newer studies on ER- and PR-associated genes in BC found a high degree of correlation between ER levels and expression of XBP1.[42] These findings seem contradictory with ours; it is unclear whether XBP1s expression is correlated with ER expression. We can only temporarily speculate that XBP1 may not be related to the hormone-signal pathway on a macroscopic level. The relationship of XBP1s with the ER is a largely unclear issue that needs to be further studied.

Conclusion

Our study indicates that XBP1s is an important biomarker in nuclei that is correlated with an adverse effect on BC survival. Although it is too early to say XBP1s is an independent

Table 3 Correlation of nuclear XBP1s expression with various clinicopathological parameters

Groups	XBP1s expressed in nuclei			*P*-value
	n (160)	Low	High	
Age (years)				0.477
<35	10	10	0	
35–50	66	62	4	
>50	84	74	10	
Pathological grade				0.112
1	18	17	1	
2	136	124	12	
3	6	4	2	
T stage				0.144
T1	36	35	1	
T2	107	96	11	
T3	15	12	3	
T4	0	0	0	
N stage				0.071
N0	63	61	2	
N1	48	41	7	
N2	37	31	6	
N3	8	8	0	
Clinical stage				0.241
TNM1	13	13	0	
TNM2	94	86	8	
TNM3	48	41	7	
TNM4	0	0	0	
ER				0.718
–	48	44	4	
+	108	97	11	
PR				0.315
–	60	56	4	
+	95	84	11	
HER2				0.578
–	105	94	11	
+	52	48	4	
Luminal subtype				0.717
Luminal A	13	12	1	
Luminal B	61	54	7	
HER2-rich	20	19	1	
Triple-negative	19	16	3	

prognostic factor, its prognostic value requires further research. Moreover, XBP1s may be a new prognostic and therapeutic target, which may potentially improve the survival of patients.[10,28]

Table 4 Multivariate Cox regression analysis

Factors	CC	SE	*P*-value	RR	95% CI	
					Lower	Upper
XBP1s-N	0.73	0.421	0.083	2.074	0.909	4.736
Pathology	0.621	0.537	0.247	1.862	0.65	5.331
N stage	–0.079	0.311	0.799	0.924	0.502	1.701
TNM stage	0.752	0.529	0.155	2.122	0.752	5.99
ER	–0.409	0.447	0.36	0.664	0.276	1.596
PR	–0.607	0.45	0.177	0.545	0.226	1.316

Abbreviations: CC, correlation coefficient; RR, relative risk; XBP1s-N, nuclear XBP1s.

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Disclosure

The authors report no conflicts of interest in this work.

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