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ORIGINAL RESEARCH

Expression of CPEB4 in invasive ductal breast carcinoma and its prognostic significance

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Aims: Cytoplasmic polyadenylation element binding proteins (CPEBs) are RNA-binding proteins that regulate translation by inducing cytoplasmic polyadenylation. CPEB4 has been reported in association with tumor growth, vascularization, and invasion in several cancers. To date, the expression of CPEB4 with clinical prognosis of breast cancer was never reported before. We aim to investigate the expression of CPEB4 and its prognostic significance in invasive ductal breast carcinoma.

Methods: Immunohistochemical staining of CPEB4 and estrogen receptor, progesterone receptor, and human epidermal growth factor receptor was performed in 107 invasive ductal carcinoma (IDC) samples, and prognostic significance was evaluated.

Results: High expression of CPEB4 was observed in 48.6% of IDC samples. Elevated CPEB4 expression was possibly related to increased histological grading (P=0.037) and N stage (P<0.001). Patients with high expression of CPEB4 showed shorter overall survival (P=0.001). High CPEB4 expression was an independent prognostic factor for overall survival (P=0.022, hazard ratio =4.344, 95% confidence interval =1.235–15.283).

Conclusion: High CPEB4 expression is associated with increased histological grading and N stage, and it can serve as an independent prognostic factor in IDC.

Keywords: cytoplasmic polyadenylation element binding protein 4, invasive ductal carcinoma, immunohistochemistry, prognosis

Introduction

Invasive breast cancer is the most frequent cancer and the leading cause of cancer death in young women, in which the invasive ductal carcinoma (IDC) is the most common pathologic type.[1,2] Several biomarkers have been routinely tested clinically to evaluate the prognosis and establish the treatment strategy. For example, estrogen receptor (ER) and progesterone receptor (PR) have served as predictors to patient's suitability for endocrine therapy.[3,4] The human epidermal growth factor receptor-2 (HER2/neu) has also been used as a valuable prognostic and treatment biomarker.[5,6] Trastuzumab and lapatinib have been included in clinical practice for HER2-postive breast cancer patients.[7–9] However, drug resistance is quite common,[10,11] and the clinical outcome remains hard to predict for individual patients. Therefore, there is a continual drive to find new biomarkers as reliable prognostic indicators and treatment targets.[12]

Cytoplasmic polyadenylation element binding protein (CPEB) is a combination of a sequence-specific RNA-binding protein with a RNA-recognition motif and a zinc-finger.[13,14] CPEBs specifically target a sequence with a cis-acting sequence in their 3′-untranslated region (UTR) and contribute to polyadenylation, resulting in translation termination.[15–17] CPEB4, one of the most important subtypes that affects cell proliferation and differentiation, can bind to a distinct loop-forming U-rich motif.[18,19] Till now, the

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specific CPEB4 binding sequence overlapped with the cytoplasmic polyadenylation element is unclear.[18,20,21] According to previous studies, elevated CPEB4 expression seems to contribute to tumor growth, vascularization, migration, invasion, and metastasis.[22–24] However, the expression characteristics of CPEB4 in breast cancer have not been reported yet.

In this study, we performed an immunohistochemical study on 107 cases of IDC. The aim of this study was to investigate the clinicopathologic significance of CPEB4 expression in IDC and evaluate its potential value when served as a prognostic indicator.

Materials and methods

Patient population and clinical data

One hundred and seven patients with primary IDC underwent curative surgery at the Huashan Hospital of Fudan University between January 1999 and December 2002. None of the patients in this study received preoperative neoadjuvant chemotherapy and/or radiotherapy, and all patients received four cycles of cyclophosphamide, methotrexate, and 5-fluorouracil after surgery. All patients were women aging from 34 years to 87 years, with a mean age of 53 years. In addition, as controls, normal breast tissues were taken from randomly selected tissues of breast IDC patients who received operation over the same period. The study was approved by the Ethical Committee for Clinical Research of Fudan University, and informed consent was obtained from all subjects.

All pathologic slides were reevaluated by two independent pathologists. The pathologic diagnosis was made according to the WHO classification of breast tumors, and histological grading was assessed according to the Nottingham modification of the Bloom and Richardson grading criteria.[25] The American Joint Committee on Cancer (AJCC)/International Union for Cancer Control (UICC) tumor, node, metastasis (TNM) classification and stage grouping system was used to evaluate the clinical stage.[26] Patients' characteristics are listed in Table 1.

The follow-up started postoperatively and ended on December 31, 2008. The follow-up time ranged from 3.5 months to 119.6 months, with a median time of 81.6 months. At the end of the follow-up period, 89 patients were still alive and 18 patients had died of the disease.

Table 1 Clinicopathological characteristics and follow-up data of 107 IDC patients

Characteristics	Number of patients/total number (%)
Age (years)	
<55	63/107 (58.9)
≥55	44/107 (41.1)
Histological grade	
I	10/107 (9.3)
II	80/107 (74.8)
III	17/107 (15.9)
T	
1	59/107 (55.1)
2	48/107 (44.9)
N	
0	58/107 (54.2)
1	27/107 (25.2)
2	22/107 (20.6)
M	
0	103/107 (96.3)
1	4/107 (3.7)
Clinical TNM stage	
I	38/107 (35.5)
II	46/107 (43.0)
III–IV	23/107 (21.5)
Menstrual status	
Premenopausal	45/107 (57.9)
Postmenopausal	62/107 (42.1)

Abbreviations: IDC, invasive ductal carcinoma; TNM, tumor, node, metastasis.

Antibodies

Polyclonal anti-CPEB4 antibody was purchased from Abcam (Cat ab83009, Cambridge, UK); mouse monoclonal antibodies anti-HER2/neu/c-erbb-2 (Cat M-0196), ER (Cat M-00241), and PR (Cat M-0448) were all purchased from Shanghai Long Island Biotech Co., Ltd. (Shanghai, People's Republic of China).

Immunohistochemistry

Tissue samples were fixed in 10% formalin, embedded with paraffin, and cut into sections of 4–5 μm. After that, all slides were dehydrated with xylene and graded alcohol/water mixtures. Antigen retrieval was performed with 0.01 M citrate buffer (pH =6.0) at 95°C for 20 minutes. Then slides were incubated with diluted primary antibodies (anti-CPEB4, 1:200 dilution; anti-ER, anti-PR, and anti-HER2, 1:100 dilution) at 4°C for 12 hours, followed by incubations with biotinylated secondary antibody for 1 hour and peroxidase-labeled streptavidin (Shanghai Long Island Biotech Co., Ltd.) for 15 minutes. The color was developed by reacting with 3,3-diaminobenzidine for 1 minute. Slides were again counterstained with Mayer's hematoxylin. The primary antibody was omitted as a negative control, being replaced by phosphate-buffered solution. The reproducibility of CPEB4 staining was examined by two independent pathologists.

Semi-quantitative analysis

The immunohistochemical results were evaluated by two pathologists. Ten visual fields at a high power (×400) were

observed in each slide by a light microscope (Carl Zeiss Meditec AG, Jena, Germany). For CPEB4 expression, the staining intensity was observed (score 0, negative staining; score 1, pale yellow; score 2, dark yellow; score 3, brown), and the percentage of positive cells was calculated (score 0, <25% positive cells/field; score 1, 25%–50% positive cells/field; score 2, 50%–75% positive cells/field; and score 3, >75% positive cells/field). Based on the product of the two scores, the staining grades were classified into low (<4) and high (≥4).

Scoring of ER, PR, and HER2 expressions was also reevaluated by two independent pathologists. Based on the percentage of positive cells and the intensity of the staining, if there were no reactivity or nuclear (ER or PR)/membranous (HER2) reactivity in <1% of tumor cells, the samples will be regarded as negative stains, otherwise positive stains.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences, Version 20 (IBM Corporation, Armonk, NY, USA) and Prism® 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Pearson's correlation coefficients were used to determine the relationship between the CPEB4 expression and the clinicopathologic parameters, including ER, PR, and HER2 expressions. Kaplan–Meier survival analysis was used to estimate the prognostic value of CPEB4, and the log-rank test was used to assess the survival differences between different groups. Univariate and multivariate Cox regression analyses were performed to evaluate differences of all possible factors in the risk of death. For all tests, a *P*-value <0.05 was defined as statistically significant.

Results

Expression of CPEB4 in normal breast and IDC tissues

We analyzed CPEB4 expression in 107 primary IDC samples. Immunoreactivity of CPEB4 was detected only in cytoplasm. In normal breast tissues, CPEB4 was negative in all adipocytes and myoepithelial cells but sometimes positive in ductal epithelium (Figure 1). High expression of CPEB4 expression was observed in 48.6% (52/107) of IDC samples.

Expressions of ER, PR, and HER2 in IDC tissues

For ER, PR, and HER2, typical patterns of positive and negative immunohistochemical staining are shown in Figure 2. ER and PR were stained brown in the nucleus, while HER2 in the membrane. Positive rates for ER, PR, and HER2 were 49.5%, 42.1%, and 64.5%, respectively, in our 107 IDCs.

Correlations between CPEB4, ER, PR, and HER2 expressions and clinicopathologic parameters

Statistical analysis showed that CPEB4 expression was positively correlated with the histological grading (*P*=0.037) and N stage (lymph node status, *P*<0.001) of IDC, and it was not statistically related to patients' age, T stage (tumor size), M stage (metastasis), TNM stage, or menopausal status. PR expression was significantly associated with age (*P*=0.028) and menopausal status (*P*=0.005), while no significant association was found between ER and menstrual status. No significant relationship was found between HER2 expression and age, histological grading, T stage, N stage, M stage, TNM stage, and menstrual status. Detailed information is listed in Table 2.

Correlations between CPEB4 and ER, PR, and HER2 expressions

The correlations between CPEB4 and ER, PR, and HER2 expressions were evaluated. No significant relationship was observed between CPEB4 and ER (*r*=–0.028, *P*=0.770), PR (*r*=0.043, *P*=0.658), and HER2 (*r*=0.096, *P*=0.319) (Table 3).

Survival analysis

The average follow-up time for the 107 IDC cases was 83.6 months. Event was defined as death from any disease. No patient was excluded from the analysis. A total of 18 patients died (16.8%) during the follow-up period.

Correlation between higher CPEB4 expression and shorter overall survival times was revealed by Kaplan–Meier survival analysis (*P*=0.001, log-rank test) (Figure 3A). High HER2 expression and advanced TNM stage were both negatively correlated to survival time (*P*=0.020, *P*<0.001, log-rank test) (Figure 3B and C).

Univariate analysis regarding age, menstrual status, histological grading, TNM stage, ER, PR, HER2, and CPEB4 expression showed that the positive HER2 expression (*P*=0.036), high TNM stage (*P*<0.001), and high CPEB4 expression (*P*=0.005) were risk factors for IDC. Multivariate analysis using the Cox model demonstrated that the HER2 expression (*P*=0.026, hazard ratio [HR] =5.439, 95% confidence interval [CI] =1.227–24.114) was an independent risk factor, and high CPEB4 expression (*P*=0.022, HR =4.344,

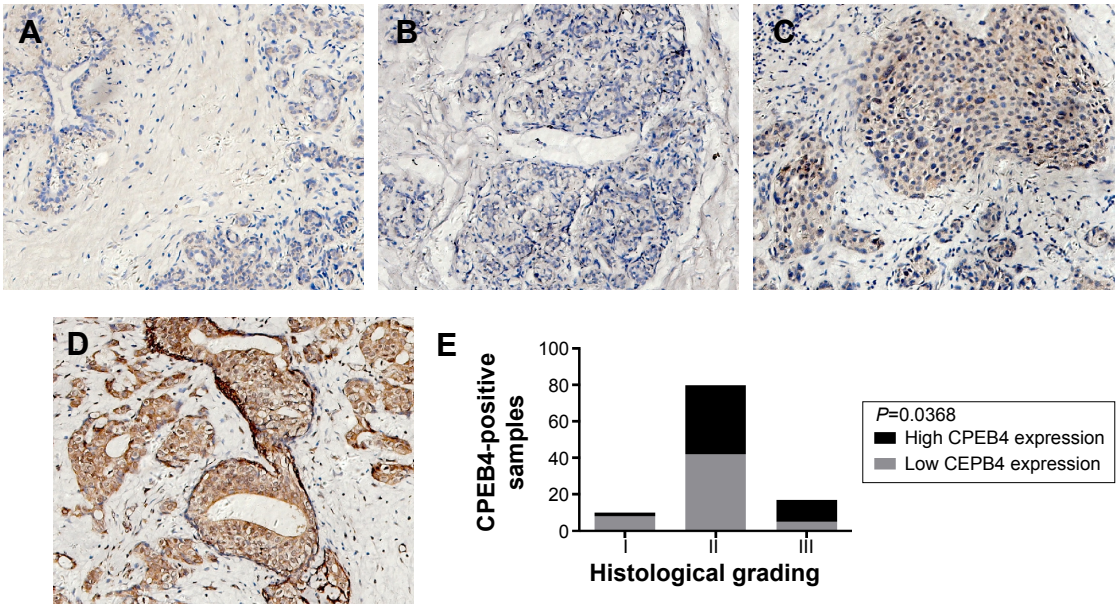

Figure 1 Representative photographs of CPEB4 staining in normal and malignant breast tissues.
Notes: (**A**) In normal breast tissues, CPEB4 was negative in all adipocytes and myoepithelial cells but sometimes positive in ductal epithelium. (**B**) In Grade I IDC, no staining of CPEB4 was observed. (**C**) Cytoplasm of Grade II IDC cells was stained weakly positive. (**D**) High expression in cytoplasm of Grade III IDC. (**E**) Quantification of CPEB4 expression in IDC tissue samples according to histology. The stacked bars show the percent contribution of high and low CPEB4-positive samples. All representative images are taken on power of ×400.
Abbreviations: CPEB4, cytoplasmic polyadenylation element binding protein 4; IDC, invasive ductal carcinoma.

95% CI =1.235–15.283) and high TNM stage ($P<0.001$, HR =13.804, 95% CI =4.769–39.935) were also independent risk factors (Table 4).

Discussion

In this study, we analyzed CPEB4 expression in 107 IDC tissues and evaluated its value as a potential prognostic indicator when compared with commonly used biomarkers: ER, PR, and HER2. Our data showed that high CPEB4 overexpression was observed in 48.6% of IDC samples, and its expression level was related to the histological grading and N stage. Patients with higher CPEB4 expression appeared to have poorer prognosis. Multivariate analysis showed that high CPEB4 expression was an independent prognostic factor for overall survival.

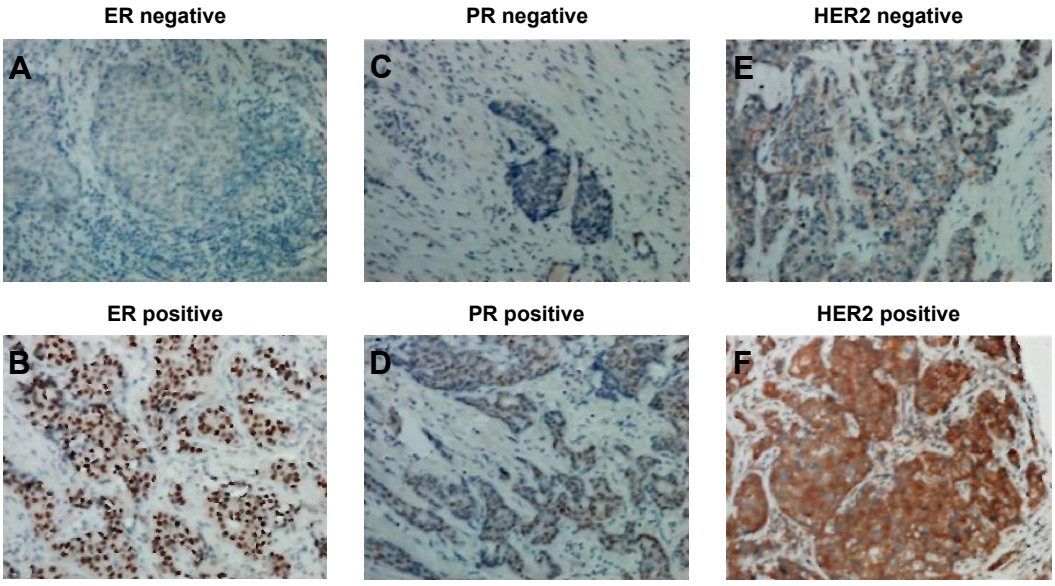

Figure 2 Representative photographs of ER, PR, and HER2 staining in IDC tissues.
Notes: ER and PR are stained brown in the nucleus, while HER2 in the membrane. If <10% cells are stained or the staining is weak, the sample is regarded as negative. (**A**) Negative ER staining in IDC tissues. (**B**) Positive ER staining in IDC tissues. (**C**) Negative PR staining in IDC tissues. (**D**) Positive PR staining in IDC tissues. (**E**) Negative HER2 staining in IDC tissues. (**F**) Positive HER2 staining in IDC tissues.
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; IDC, invasive ductal carcinoma.

Table 2 Correlation of CPEB4 expression and the clinicopathological characteristics in invasive ductal breast cancer (n=107)

Clinicopathological parameters	n	CPEB4		ER		PR		HER2	
		Expression (%)	*P*-value	Expression (%)	*P*-value	Expression (%)	*P*-value	Expression (%)	*P*-value
Age (years)			0.349		0.755		0.028*		0.573
<55	63	33 (52.4)		32 (50.8)		32 (50.8)		42 (66.7)	
≧55	44	19 (43.2)		21 (47.7)		13 (29.5)		27 (61.4)	
Histological grading			0.037*		0.062		0.224		0.927
I	10	2 (20.0)		5 (50.0)		4 (40.0)		7 (70.0)	
II	80	38 (47.5)		44 (55.0)		37 (46.2)		51 (63.7)	
III	17	12 (70.6)		4 (23.5)		4 (23.5)		11 (64.7)	
T stage			0.899		0.763		0.64		0.671
1	59	29 (49.2)		30 (50.8)		26 (44.1)		37 (62.7)	
2	48	23 (47.9)		23 (47.9)		19 (39.6)		32 (66.7)	
N stage			<0.001*		0.597		0.402		0.381
0	56	17 (29.3)		27 (46.6)		23 (39.7)		34 (58.6)	
1	37	19 (70.4)		13 (48.1)		10 (37.0)		19 (70.4)	
2	14	16 (48.6)		13 (59.1)		12 (54.5)		16 (72.7)	
M stage			0.354		0.618		0.743		0.654
0	103	49 (47.6)		52 (50.5)		43 (41.7)		66 (64.1)	
1	4	3 (75.0)		1 (25.0)		2 (50.0)		3 (75.0)	
TNM Stage			0.075		0.152		0.152		0.767
I	38	16 (42.1)		17 (44.7)		17 (44.7)		23 (60.5)	
II	46	20 (43.5)		15 (32.6)		15 (32.6)		30 (65.2)	
III–IV	23	16 (69.6)		13 (56.5)		13 (56.5)		16 (69.6)	
Menstrual status			0.733		0.065		0.005*		0.994
Premenopausal	45	21 (46.7)		27 (60.0)		26 (57.8)		29 (64.4)	
Postmenopausal	62	31 (50.0)		26 (41.9)		19 (30.6)		40 (64.5)	

Notes: *Signficant variables; $P<0.05$.

Abbreviations: CPEB4, cytoplasmic polyadenylation element binding protein 4; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; TNM, tumor, node, metastasis.

CPEB4 protein is overexpressed in a large variety of tumors (17 out of a total of 20 tumor types listed at www.proteinatlas.org/ENSG00000113742-CPEB4/cancer/tissue), and CPEB4 mRNA has been also confirmed in many tumor cells.[27] Rhodes et al carried out a meta-analysis of 42 studies comparing global gene expression in 92 human cancers with matched normal tissue using oncomine.[28] At this cutoff of 1.5-fold and a *P*-value of <0.05, 90 of 245 analyses showed a change in CPEB4. In the IDC, the expression of CPEB4 mRNA was upregulated with the score of 2.2, which was consistent with our result.

The relationship between CPEB4 expression and IDC progression together with poor survival has never been reported before, although it was demonstrated in other cancers. Tian et al showed that CPEB4 was commonly suppressed in hepatocellular carcinoma (HCC), and its expression was correlated with HCC prognosis. CPEB4 was directly targeted by miR-550a, which was frequently upregulated in HCC and facilitated HCC cell migration and invasion.[24] Ortiz-Zapater et al demonstrated that the overexpression of CPEB4 regulated tPA expression to contributing tumor growth and angiogenesis in pancreatic ductal

Table 3 Relationship between CPEB4 expression and ER, PR, and HER2 expression in IDC

Clinicopathological parameters	n	High CPEB4 expression (%)	*R*	*P*-value
ER			−0.028	0.77
−	54	27 (50.0)		
+	53	25 (47.2)		
PR			0.043	0.658
−	62	29 (46.8)		
+	45	23 (51.1)		
HER2			0.096	0.319
−	38	16 (42.1)		
+	69	36 (52.2)		

Abbreviations: CPEB4, cytoplasmic polyadenylation element binding protein 4; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; IDC, invasive ductal carcinoma.

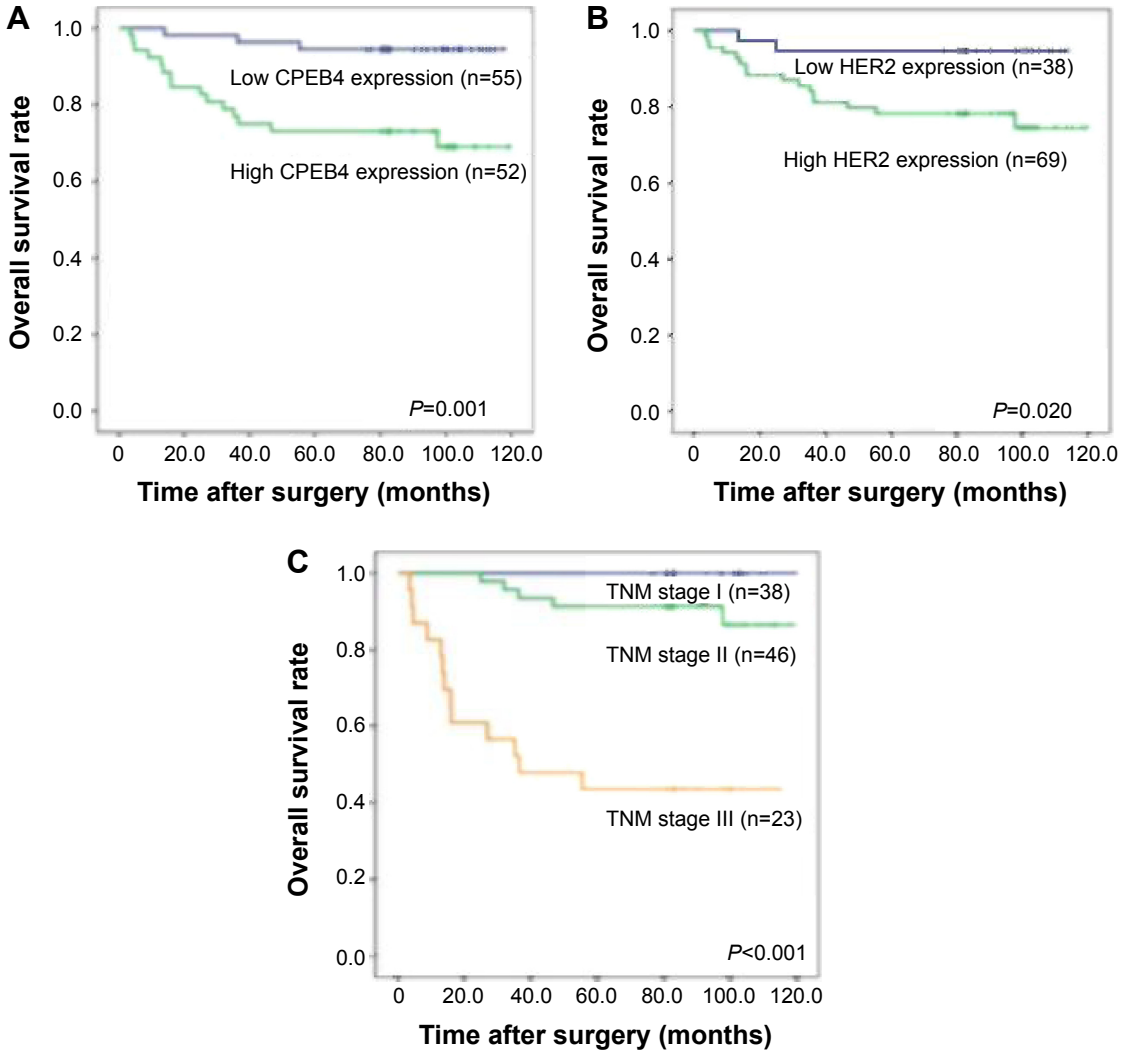

Figure 3 Kaplan–Meier survival curves of overall survival of 107 IDC patients according to CPEB4 expression (**A**), HER2 expression (**B**), and TNM stage (**C**) were demonstrated.

Abbreviations: IDC, invasive ductal carcinoma; CPEB4, cytoplasmic polyadenylation element binding protein 4; HER2, human epidermal growth factor receptor-2; TNM, tumor, node, metastasis.

Table 4 Univariate and multivariate survival analyses of influencing factors in IDC patients

Characteristics	Category	Overall survival		
		HR	95% CI	*P*-value
Univariate analysis				
Age (years)	≥55 vs <55	1.171	0.454 to 3.022	0.743
Menstrual status	Premenopausal vs postmenopausal	0.386	0.127 to 1.174	0.094
Histological grading	I vs III	0.448	0.050 to 4.016	0.473
	II vs III	0.731	0.238 to 2.248	0.584
ER expression	+ vs –	1.027	0.407 to 2.591	0.955
PR expression	+ vs –	1.459	0.578 to 3.682	0.424
HER2 expression	+ vs –	4.826	1.109 to 20.996	0.036
CPEB4 expression	High vs low	6.036	1.746 to 20.863	0.005
TNM stage	III, IV vs II, I	14.428	5.115 to 40.700	<0.001
Multivariate analysis				
HER2 expression	+ vs –	5.439	1.227 to 24.114	0.026
CPEB4 expression	High vs low	4.344	1.235 to 15.283	0.022
TNM stage	III, IV vs II, I	13.804	4.769 to 39.935	<0.001

Abbreviations: IDC, invasive ductal carcinoma; HR, hazard ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor-2; CPEB4, cytoplasmic polyadenylation element binding protein 4; TNM, tumor, node, metastasis.

adenocarcinoma and glioblastomas.[22] Xu and Liu suggested that CPEB4 was a candidate biomarker for defining metastatic cancers and promoted invasion and metastasis through TGF-beta signaling pathway.[23]

It is well established that the function of a protein depends on its location and is affected by normal or abnormal expression.[29–31] We found that immunoreactivity of CPEB4 was detected only in cytoplasm. It supports the opinion that CPEB4 is associated with specific sequences in mRNA 3′-UTR, influencing translation by inducing cytoplasmic polyadenylation,[32] which may help to explain how CPEB4 functions in the development of IDC. The present data supported that CPEB4-mediated regulation of gene expression might be a more general mechanism in cancer. It is obvious that factors in translation can influence cancer development. The relationship between CPEB4 expression and many clinical prognosis of cancer still needs further investigation.

In the present study, ER, PR, and HER2-positive rates were 49.5%, 42.1%, and 64.5%, respectively. PR expression was correlated with age and menopausal status (P=0.005). Multivariate Cox regression analysis showed that HER2 was associated with poor prognosis (P=0.026), correspondent with previous studies.[27,28,33] By comparing commonly used biomarkers to CPEB4, no correlations between CPEB4 and ER, PR, and HER2 were observed.

The current study has several limitations. First, our findings should be replicated in other populations and larger cohorts to further validate our results. Second, further studies are needed to delineate the mechanisms behind this association between CPEB4 and IDC. Third, how to make best use of CPEB4 to stratify cancer patients for personalized treatment remains a critical goal.

In conclusion, high CPEB4 expression is associated with increased histological grading and N stage. Our study suggested that CPEB4 could serve as a useful prognostic indicator for IDC.

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Disclosure

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