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p-CREB-1 at Ser 133 is a potential marker for breast cancer

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Abstract. – OBJECTIVE: Dysregulation of numerous oncogenes and their downstream signaling pathways, among others in the signaling transduction molecule p-CREB-1 (p-cAMP responsive element binding protein-1), is an essential feature of different types of cancer. To investigate whether p-CREB-1 is also pivotal in tumorigenesis and metastogenesis of breast cancer, we conducted a prospective study with long-term follow-up on 96 patients with breast cancer.

PATIENTS AND METHODS: Pathway array and tissue microarray (TMA) were used to detect the differential expression of CREB (cAMP-responsive element binding protein) and p-CREB-1 in breast cancer cells, breast cancer stem cells (BCSCs), human breast cancer tissues (BCTs), and adjacent normal tissues (ANTs). The associations between p-CREB-1 expression, clinicopathological variables, and survival rates of the patients were analyzed and calculated.

RESULTS: Our results revealed that p-CREB-1 and CREB expression in cancerous cell lines and tissues were significantly upregulated compared with non-cancerous cell lines and tissues. Most statistically significant overexpression was detected in BCSCs ($p<0.01$). In TMA and immunohistochemical analyses, BCTs exhibited significantly higher expression of p-CREB-1 and CREB than ANTs ($p<0.001$). Clinicopathological variable and survival analysis revealed a correlation between high expression (++/+++) of p-CREB-1 and the presence of axillary lymph node metastasis ($p<0.05$) and poorer disease-free and overall survival.

CONCLUSIONS: p-CREB-1 is a potential predictive and prognostic biomarker and a promising therapeutic target in breast cancer.

Key Words:
Breast cancer, Phosphorylated cyclic AMP responsive element-binding protein-1, Carcinogenesis, Tumor marker, Biomarker, Tissue microarray, Pathway array, Axillary lymph node metastasis.

Introduction

Breast cancer is the most prevalent solid cancer and the second-ranked major cause of malignancy-related mortality in women globally[1]. Studies have revealed that mutations or dysregulated expression of specific genes (e.g., BRCA1/BRCA2, human epidermal growth factor receptor 2 [HER-2], p53) can cause breast carcinogenesis[2,3]. Although substantial progress has been made in diagnosis and early treatment, there is still an essential lack of reliable early prevention and prediction methods to allow detection of a novel or recurrent disease early enough to enable effective treatment. Therefore, it is imperative to identify the novel biomarkers of breast cancer, which would also facilitate further understanding of molecular oncogenetic processes and identification of potential molecular targets for treatment.

Cyclic AMP (cAMP) responsive element-binding protein (CREB) is a leucine zipper domain transcription factor that activates target genes via cAMP responsive elements[4] and mediates signals from different physiological stimuli, which renders it imperative for a variety of regulatory cellular response processes[5]. cAMP is activated by signaling pathway components, including extracellular signal-regulated kinase (ERK), calcium, pro-inflammatory cytokines (e.g., interleukin [IL]-4, IL-10, IL-13), kinases (e.g., protein kinase A [PKA], mitogen-activated protein kinase (MAPK), and different growth factors, hormones, and prostaglandins[6]. CREB is phosphorylated by a number of protein kinases. Once phosphorylated, CREB (p-CREB) induces transcription in response to hormonal stimulation of the cAMP

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pathway and performs important functions in cell differentiation, survival, proliferation, and development[4,6].

The earliest reports of CREB focused on cognition, memory, and stress[7]. The first evidence of CREB in malignancies was from a study by Schuller and Cekanova[8], which reported that nicotine-derived nitrosamine ketone (NNK) induces lung adenocarcinomas in mammals through overexpression of β2-adrenergic and epidermal growth factor receptor (EGFR) signaling paths[8]. Cekanova et al[9] verified that small airway-derived pulmonary adenocarcinomas, induced by NNK, overexpress Raf-1 and p-CREB. NNK triggers β-adrenergic receptor activation, while the resulting cell proliferation activates CREB via the adenylyl cyclase/cAMP/PKA/CREB pathway *in vitro*[10,11]. Wang et al[12] showed involvement of CREB in the regulation of B-cell lymphoma-2 expression in apoptotic HeLa cells. CREB was therefore proposed as a therapeutic target. Several agents have been used to verify the participation of p-CREB in the oncogenesis of diversiform carcinomas. The PKA activator, 6-Bnz-cAMP, has been identified as a factor activating p-CREB expression in prostate cancer cells in a dose-dependent manner[13,14], associating the cAMP/PKA/p-CREB pathway with prostate tumorigenesis. Poon et al[15] applied the pharmacological inhibitor PD98059 to block EGF-induced ERK1/2 signaling and examined the status of p-CREB, demonstrating that PD98059 pre-treatment attenuated EGF-induced phosphorylation of CREB.

The precise function of p-CREB-1 in mammary cancer development and its potential clinical application have not been elucidated. Our study was the first to investigate p-CREB in breast cancer cells and adjacent normal tissues (ANTs) with regard to molecular pathways, patient characteristics, and clinical outcomes.

Patients and Methods

Cell Line and Fluorescence-Activated Cell Sorting (FACS) for Side Population (SP) Cells

Human breast cancer cells (MDA-MB-231, MCF-7, AU565, and T47D) and a non-tumorigenic human mammary epithelial cell line (MCF10A) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium+105 fetal bovine serum (DMEM+105FBS) fluid nutrient medium at 37°C in a 5% CO_2 incubator. The MCF-7 cells were collected and labeled with 5.0 μg/mL Hoechst 33342 dye and an additional 50 μg/mL verapamil at 37°C for 90 minutes. After resuspension and washing, cells were incubated with 3 μg/mL propidium iodide (PI) for 15 minutes at 37°C to eliminate dead cells. SP cell analysis and a sorting procedure were performed on the FACSVantage SE (BD Biosciences, Franklin Lakes, NJ, USA). Based on the fluorescent character of the DNA-binding Hoechst 33342 dye effluxion, the treated MCF-7 cells were separated into SP and non-SP cells.

Clinical Tissue Samples

Ninety-six patients with pathologically confirmed breast cancer who had undergone modified radical mastectomies at Shanghai Zhongshan Hospital from January 1, 2010 to December 31, 2013 were prospectively enrolled. Of the 96 patients, 20 were randomly selected and entered into the study for pathway array analysis. Signed, written informed consent was obtained from the enrolled patients a priori, and tumor tissues and ANTs (each with 3x3x5 mm minimum dimensions) were collected. None of the enrolled patients had received neoadjuvant therapy or had distant metastasis before the surgery. Clinicopathological variables, including age, menopausal status, tumor size, type of pathology, histological grade, presence of axillary lymph node metastasis, expression status of estrogen receptor (ER), progesterone receptor (PR), HER-2, and Ki-67 were carefully reviewed from clinical records and pathology data. Breast cancer was staged according to the American Joint Committee on Cancer staging system for breast cancer[16]. This investigation was approved by the Ethics Committee of Shanghai Zhongshan Hospital, Fudan University (Shanghai, China). The Ethics Approval Number was B2019-099.

Western Blot Assay

NP-40 lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China) was used to extract total protein from breast tissues and different breast cells, including SP and non-SP cells. After three sonication treatments, the lysate was centrifuged (11,000 × g for 30 minutes at 4°C). The concentration of proteins was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, sodium dodecyl sulfate-polyacryl-

amide gel electrophoresis (SDS-PAGE) (10% separated gel and 5% concentrated gel) was performed to separate the proteins. There were 10 μg protein per lane. Then, the proteins were electrophoretically transferred to a nitrocellulose membrane and blockade in 5% skim milk at room temperature for one hour.

Next, the Mini-PROTEAN II Multiscreen apparatus (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to clamp the membrane, which separated 20 channels across the blotted membrane. After the addition of different sets of antibodies, including monoclonal CREB antibody (dilution, 1:1,000; rabbit. no. 9197; Cell Signaling Technology, Inc., Danvers, MA, USA), monoclonal p-CREB-1 antibody (dilution, 1:1,000; rabbit. no. 9198; Cell Signaling Technology, Inc., Danvers, MA, USA), and monoclonal β-Actin antibody (dilution, 1:1,000; rabbit. no. 12620; Cell Signaling Technology, Inc., Danvers, MA, USA) to the channels of each membrane, overnight incubation was performed at 4°C for hybridization. After the reaction, the blot was washed before undergoing another hybridization at room temperature for one hour with secondary horseradish peroxidase(HRP)-conjugated antibodies (dilution, 1:1,000; anti-rabbit. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA). The enhanced chemiluminescence (ECL) Western Blotting Kit (No. E412-01/02; Vazyme, Inc., Nanjing, China) and ChemiDoc XRS system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to stain the protein bands and evaluate the differential protein expression levels by capturing chemiluminescence signals at different intensities.

Tissue Microarray (TMA) Construction

TMAs of breast cancer and ANT specimens were constructed as previously described[18]. In total, 96 resected breast cancer tissue specimens and 60 corresponding ANTs were collected. Thirty-six ANT samples were not used in the study due to insufficient breast epithelial tissue. Briefly, all the resected samples were fixed in 10% formalin at room temperature (<24 hours) and embedded in paraffin blocks. Cores with a 1.0 mm diameter from non-necrotic and morphologically representative areas were punched out of the paraffin block and arrayed into a freshly constructed recipient block using a tissue arrayer (Beecher Instruments, Woodland, CA, USA). Each specimen was represented by two tissue cores.

Immunohistochemistry (IHC) and Evaluation

Immunohistochemical staining using primary monoclonal p-CREB-1 antibody (dilution, 1:400; rabbit. No. 9198; Cell Signaling Technology, Inc., Danvers, MA, USA) was performed on consecutive 5-mm sections of each electrostatically charged glass slide (US Biomax Inc., Derwood, MD, USA). In brief, the slides were routinely dewaxed, rehydrated, and immersed in 10 mM sodium citrate buffer (pH 6.0) for 20 minutes at 95°C for antigen retrieval. Endogenous peroxidases were quenched by incubation with methanol containing 3% H_2O_2 for five minutes. After blockade with non-immune goat serum (goat. No. SP-9000, ZSGB-bio, Inc., Beijing, China) at room temperature for 20 minutes, the slides were incubated with primary p-CREB-1 (Ser 133) antibody at 4°C overnight. Then, the sections were incubated with secondary antibody from the EnVision+ HRP Immunohistochemical Detection System (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) for 30 minutes and counterstained with hematoxylin at room temperature for three minutes (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA) before analysis. The chromogenic signal and pattern of subcellular expression were assessed using a Zeiss microscopy system (Zeiss AG, Oberkochen, Germany).

Areas of infiltrating lesion and solid tumor for cancer tissues and morphologically typical breast epithelia for ANTs were selected for evaluation. Staining proportion and intensity of stained cells were evaluated for p-CREB-1, as described previously[17]. The intensity of staining was graded using the following standards: zero, no staining; one, weak staining; two, moderate staining; and three, strong staining. Intensity scores were based on the preponderant staining areas if the staining profile was heterogeneous. The proportion of staining was the percentage of cells with positive p-CREB-1 expression in all cells in the field: zero, negative; one (1%-25%) positive; two (26%-50%) positive; three (51%-75%) positive; and four (76%-100%) positive. The final score was obtained by multiplying the intensity and percentage scores. According to the final score, we divided the p-CREB-1 tissue expression into score zero to two negative expression (-), score three to four low expression (+), score six to eight intermediate expression (++), and score nine to 12 high expression (+++). Lastly, we classified negative expression (-) and low expression (+) tissues into a low-expression group and interme-

diate expression (++) and high expression (+++) into a high-expression group. The sections were semi-quantitatively assessed by two pathologists using a double-blind observation procedure. If the pathologists disagreed, a third independent reviewer of the slides reached a consensus.

Statistical Analysis

For pathway array analysis, one-way analysis of variance (ANOVA) followed by Post Hoc Test (Least Significant Difference) was used to evaluate the statistical significance of differential p-CREB-1 expression across the groups of different cell lines, and a paired *t*-test was applied to compare the mean expression level of p-CREB-1 between cancer tissues and ANTs. A c^2-test was adopted to compare the differences between expression of p-CREB-1 and the clinicopathological parameters. Overall survival (OS) was calculated as the period from the day of surgery to mortality from any cause or the date of the last follow up. Disease-free survival (DFS) was measured from the day of surgery to the day of breast cancer recurrence. The Kaplan-Meier survival curve was used to compare the OS and DFS between groups of differential p-CREB-1 expression, and discrepancies in survival were estimated using the log-rank test. $p<0.05$ was considered statistically significant. Statistical analyses were performed with the Statistical Product and Service Solutions (SPSS) 22.0 software package for Windows (IBM Corp., Armonk, NY, USA).

Results

Comparison of Differential Expression of p-CREB-1 in Breast Cancer Cells, Cancer Stem Cells, Tumor Tissues, and ANTs

Pathway array analysis was adopted to identify the differential expression of CREB and p-CREB-1 in different breast cell lines, including MCF-7, MDA-MB-231, AU565, and T47D, and the non-tumorigenic cell line MCF10A (Figure 1). Compared with the MCF10A cell line, MDA-MB-231 ($p<0.01$; Figure 1A), AU565 ($p<0.01$;

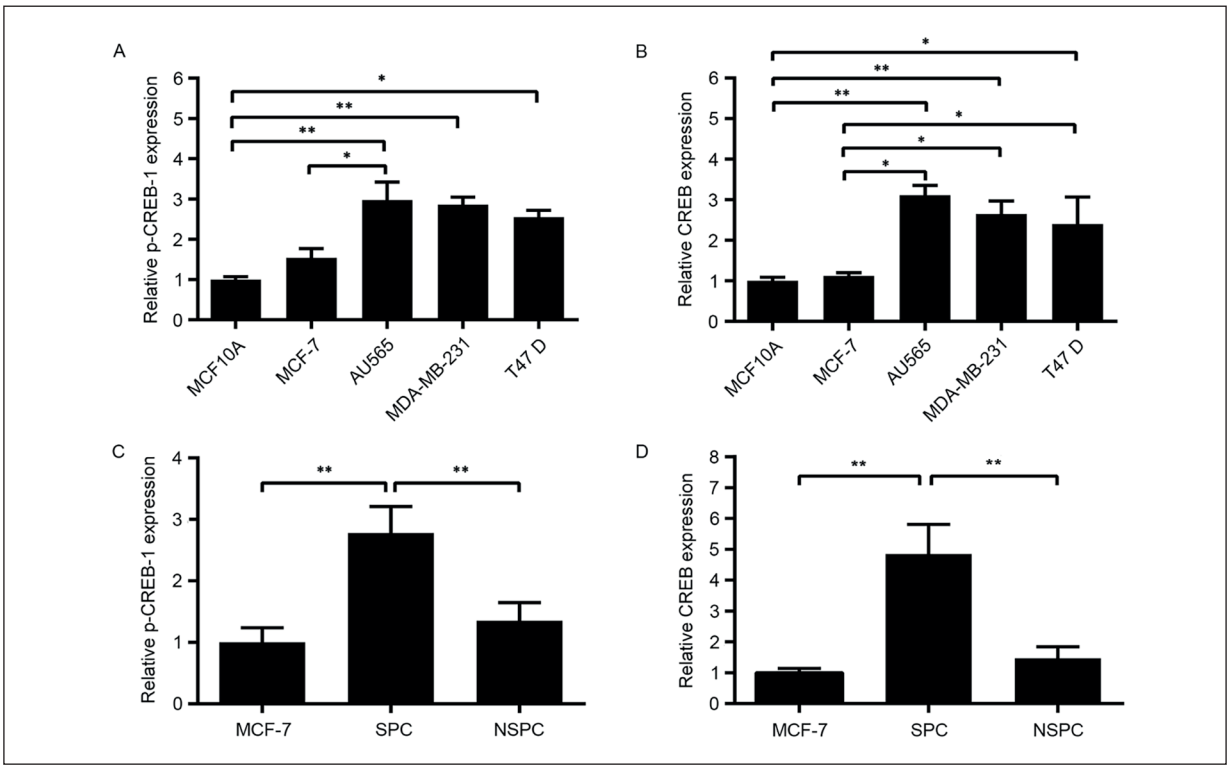

Figure 1. Differential expression of p-CREB-1 and CREB in different cell lines. Relative expression level was measured by pathway array. Quantitative measurements were performed ≥3 times. **A,** p-CREB-1 and **B,** CREB expression in MCF-10A, MCF-7, AU565, MDA-MB-231, and T47D cells. **C,** p-CREB-1 and **D,** CREB expression in the MCF-7 cell line, SPCs, and NSPCs. Data are presented as the mean ± standard error of the mean. p-CREB-1, phosphorylated cAMP response element-binding protein-1; SPC, side population cells; NSPC, non-side population cells. *$p<0.05$, †$p<0.01$.

Figure 1A) and T47D ($p<0.05$; Figure 1A) cell lines exhibited significant upregulation of CREB and p-CREB-1. In MCF-7 cells, statistical significance was not reached (Figures 1A and 1B). Intergroup analysis of p-CREB-1 expression in all cancer cell lines exhibited a significant difference in expression between MCF-7 and AU565 cells ($p<0.05$; Figure 1A). Similarly, intergroup analysis of CREB expression in cancer cell lines showed that expression in the AU565, MDA-MB-231, and T47D cell lines had at least a two-fold upregulation compared with the MCF-7 cell line ($p<0.05$; Figure 1B).

The relationship between p-CREB-1 expression and the stemness of breast cancer cells may result from the aberrant expression of p-CREB-1 in mammary cancer cells due to the fact that stem cells are propagators, as well as initiators, of cancer[18]. To validate this hypothesis, a pathway array was used to detect the level of CREB and p-CREB-1 expression in breast cancer cells with different degrees of stemness. MCF-7 cells were separated into non-SP and SP cells. Comparison of protein expression in unsorted MCF-7, SP, and non-SP cells showed a higher expression of CREB and p-CREB-1 in the SP fraction than in the non-SP and unsorted MCF-7 cells ($p<0.01$; Figure 1C and 1D). Thus, our pathway array demonstrated distinct overexpression of the signaling proteins p-CREB-1 and CREB in diverse breast tumorigenic cells, including cancer stem cells, which suggested latent participation of abnormal expression of p-CREB-1 in breast cancer oncogenesis.

To further validate the oncogenic function of p-CREB-1, expression of p-CREB-1 in cancer tissues and ANTs was observed and quantified. Twenty patients with invasive breast carcinoma were enrolled, and the expression of p-CREB-1 and CREB was compared between tumor tissues and ANTs. Tumor tissues exhibited a 3.7-fold increase in p-CREB-1 gene expression compared with ANTs ($p<0.001$; Figure 2A). The expression of CREB in tumor tissues exhibited a more prominent difference, with a 6.8-fold higher expression in cancer tissues compared with ANTs ($p<0.001$; Figure 2B). Taken together, these results suggest that p-CREB-1 upregulation is a cause of carcinogenesis of breast cancer.

IHC for Detection of p-CREB-1 Expression in a Paraffin-Embedded TMA

Tumorous and non-tumorous immunostaining of p-CREB-1 in resected tissues is demonstrated in Figure 3, and the positive rates of p-CREB-1 expression are presented in Table I. Immunopositivity for p-CREB-1 was predominant in the cytoplasm of breast cancer cells and normal breast epithelia. The positively stained breast cancer cells were atypical to a different extent and were distributed in clusters, with an increased nuclear-to-cytoplasmic ratio. Compared with the weak staining of p-CREB-1 in normal breast epithelia (Figure 3A and 3B), a much higher positivity of p-CREB-1 staining in breast cancer tissues was observed (Figure 3C and 3D). The immunohistochemical assessment was performed as previously mentioned. The positive expression profile exhibited discrepancies between the two types of breast tissues. Of the ANTs, 26.7% (16/60) were of + expression grade, 20.0% (12/60) were of ++ expression grade, and 36.7% (22/60) were of +++ expression grade. Breast cancer tissues exhibited more prominent expression of p-CREB-1, with corresponding rates of 6.25% (6/96; +), 19.8%

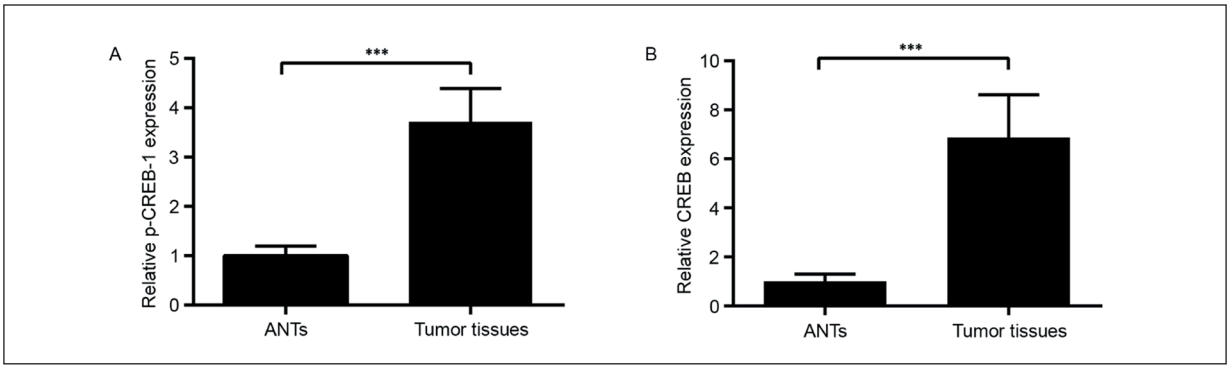

Figure 2. Comparison of p-CREB-1 and CREB expression in breast cancer tissues and ANTs. Relative expression levels were measured using pathway array. Quantitative measurements were repeated ≥3 times. **A,** p-CREB-1 and **B,** CREB expression in tumor tissue and ANTs. Data are presented as the mean ± standard error of the mean. p-CREB-1, phosphorylated cAMP response element-binding protein-1; ANTs, adjacent normal tissues. ‡$p<0.001$.

Table I. p-CREB-1 expression in breast cancer and ANTs.

p-CREB-1 expression	Tumor tissues, n (%)	ANTs, n (%)	*p*-value
Expression grade			
-	12 (12.5)	10 (16.7)	0.001[a]
+	6 (6.2)	16 (26.7)	
++	19 (19.8)	12 (20.0)	
+++	59 (61.5)	22 (36.7)	
High/low expression			
Low expression (-/+)	18 (18.8)	26 (43.3)	0.001[a]
High expression (++/+++)	78 (81.2)	34 (56.7)	

[a]$p < 0.01$. p-CREB-1, phosphorylated cyclic AMP response element-binding protein 1; ANTs, adjacent normal tissues.

(19/96; ++), and 61.5% (59/96; +++), respectively ($p<0.01$; Table I). There was markedly lower p-CREB-1 expression in the ANTs than in the breast carcinoma tissues ($p<0.01$; Table I).

Association between p-CREB-1 Overexpression and Clinicopathological Parameters

The median age of all 96 enrolled patients was 55.8 years (range, 32-86 years). Thirty-eight of the 96 patients (39.6%) were premenopausal. The percentages of pT1, pT2, pT3, and pT4 patients in this cohort were 39.6%, 54.1%, 5.2%, and 1.0%, respectively. Most patients had histological grade II and III tumors (52.1% and 46.9%, respectively). Only one patient had grade I disease. Axillary lymph node (ALN) metastases were identified in 33.3% (32/96) of the patients. The proportion of pN1, pN2, and pN3 patients in this cohort was 17.7%, 12.5%, and 5.2%, respectively. Overall, 75 of the 96 patients presented with infiltrating ductal carcinoma, nine with infiltrating lobular carcinoma, seven with mucinous adenocarcinoma, three with

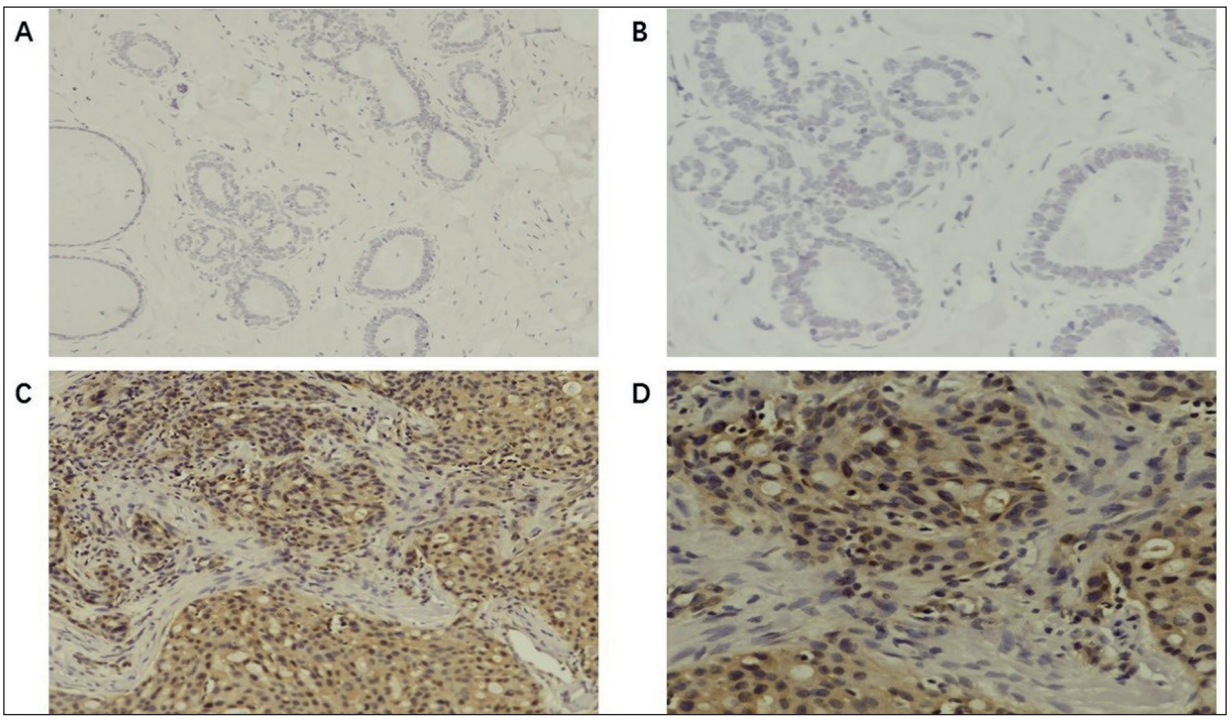

Figure 3. Representative expression of p-CREB-1 in breast cancer tissues and ANTs using TMA and IHC. IHC revealed weak, scattered staining of p-CREB-1 in ANTs at **A,** (magnification: 200×) **B,** (magnification: 400×) and diffuse, strong staining of p-CREB-1 in breast cancer tissues at **C,** (magnification: 200×) **D,** (magnification: 400×) p-CREB-1, phosphorylated cAMP response element-binding protein-1; TMA, tissue microarray; IHC, immunohistochemistry; ANTs, adjacent normal tissues.

mixed cancer types, and two with medullary carcinoma and inflammatory carcinoma. Luminal B breast cancer predominated (54/96) in different molecular types of resected samples.

The patients were classified into high- and low-expression groups. As shown in Table II, p-CREB-1 expression and the presence of ALN metastases (p=0.027; Table II) were significantly associated. However, the relationship between p-CREB-1 expression and pN stage was not statistically significant (p=0.255; Table II). No statistically significant association was observed between level of p-CREB-1 expression and age, menopausal status, tumor location, pathological type, histological grade, pT stage, pathological Tumor-Node-Metastasis stage, or expression levels of ER, PR, HER-2, or Ki-67.

Survival Analysis of p-CREB-1 Protein Expression

To further determine the prognostic value of p-CREB-1 in breast cancer, a Kaplan-Meier survival curve and log-rank test were adopted. With a median follow-up of 65.1 months (range, five to 98 months), five-year DFS was 71.5% and 78.3% for the high- and low-expression groups, respectively. The Kaplan-Meier survival curve suggested that overexpression of p-CREB-1 was predictive of poor DFS in the long term, approaching statistical significance in the log-rank test (p=0.559) (Figure 4A). Five-year OS for the high- and low-expression groups was 75.8% and 88.3%, respectively, with no significant differences in the Kaplan-Meier curve between the two groups (p=0.444) (Figure 4B). These data suggest that p-CREB-1 expression might have prognostic potential in breast cancer patients, but it requires validation in a larger cohort.

Discussion

Breast cancer is a pivotal burden in both developed and developing countries. Studies have identified associations between breast cancer and genetic, environmental, and individual factors contributing to carcinogenesis. The rapid progress of molecular and nano-precision medicine has allowed further detection of cellular and molecular pathways involved in the etiology, tumorigenesis, and progression of breast cancer. CREB has been regarded as a multifunctional signaling protein that mediates diverse biological processes. CREB overexpression is relevant to reduced apoptosis, enhanced cell proliferation, and migration[4-6].

In this study, pathway array, a potent high throughput detection technology for analyzing differential expression of intracellular proteins and phosphorylation status in a cell signal transduction pathway, was conducted to identify the expression profile of CREB and p-CREB-1 in breast cancer cell lines and tumor tissues. Comparisons of p-CREB-1 and CREB expression levels revealed that both proteins are overexpressed in cancer cell lines and cancer tissues. As expected, the upregulation of p-CREB-1 in MCF-7 cells showed no statistical significance compared with the upregulation in MCF10A cells. The MCF-7 cell line, distinguished by low invasiveness, has often been used as a noninvasive model in breast cancer research[19,20]. This suggests that overexpression of p-CREB-1 and CREB is sensitive and especially suitable for patients with invasive tumors, which are particularly problematic because most are detected at a late stage and are more aggressive. In addition, the results allow us to hypothesize that the upregulation of p-CREB-1 is associated with increased potential for invasion, which facilitates the metastasis of breast cancer.

Based on the current knowledge that phosphorylation confers elevated transcriptional activity to CREB in carcinomas, it is reasonable to deduce that the phosphorylation of CREB may promote tumorigenesis in breast carcinoma. Naqvi et al[21] concluded that the phosphorylation of CREB at Ser 133 promotes recruitment of the co-activator protein CREB-binding protein (CBP) and increases the transcription of CREB-dependent genes via the cAMP/PKA/MAPK signaling pathway. Some studies[22,23] have demonstrated that CREB phosphorylation at Ser 133 is associated with tumorigenesis and progression in different types of carcinoma. However, the exact function of CREB phosphorylation at Ser 133 in normal epithelial and breast cancer cells has not been established.

Our results indicated that the expression of non-phosphorylated CREB was consistent with that of p-CREB-1 and that it exhibited an evident overexpression in breast cancer cells and tumor tissues compared with normal mammary epithelial cells and tissues. To further investigate the role of p-CREB-1 overexpression in breast cancer tissues, IHC in a paraffin-embedded microarray was performed. The analysis revealed low expression of p-CREB-1 in ANTs

Table II. Association between p-CREB-1 expression and clinicopathological variables in breast cancer.

Variable	p-CREB-1 expression		*p*-value
	Low (-/+)	High (++/+++)	
Age, years			
<50	8	25	0.318
≥50	10	53	
Menopause			
Pre	6	32	0.547
Post	12	46	
Location			
Left	10	42	0.896
Right	8	36	
Pathological type			
IDC	14	61	0.574
ILC	1	8	
MA	2	5	
Mixed	0	3	
Others	1	1	
Histological grade			
I	1	0	0.280
II	9	41	
III	8	37	
pT stage			
T1	7	31	0.315
T2	9	43	
T3	1	4	
T4	1	0	
Lymph node metastasis			
Negative	16	48	0.027[a]
Positive	2	30	
pN stage			
N0	16	48	0.255
N1	1	16	
N2	1	11	
N3	0	3	
pTNM stage			
I	5	27	0.542
II	8	38	
III	5	13	
ER			
Negative	9	26	0.185
Positive	9	52	
PR			
Negative	9	30	0.391
Positive	9	48	
HER-2			
Negative	11	55	0.438
Positive	7	23	
Ki-67			
< 15% +	3	11	1.000
≥ 15% +	15	67	
Molecular type			
Luminal A	2	9	0.893
Luminal B	9	45	
HER-2+	3	11	
TNBC	4	13	

[a]$p<0.05$. p-CREB-1, phosphorylated cyclic AMP response element-binding protein 1; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma; MA, mucinous adenocarcinoma; pT, pN, pTNM, pathological Tumor-Node-Metastasis; ER, estrogen receptor; PR, progesterone receptor; HER-2, human epidermal growth factor receptor 2; TNBC, triple-negative breast cancer.

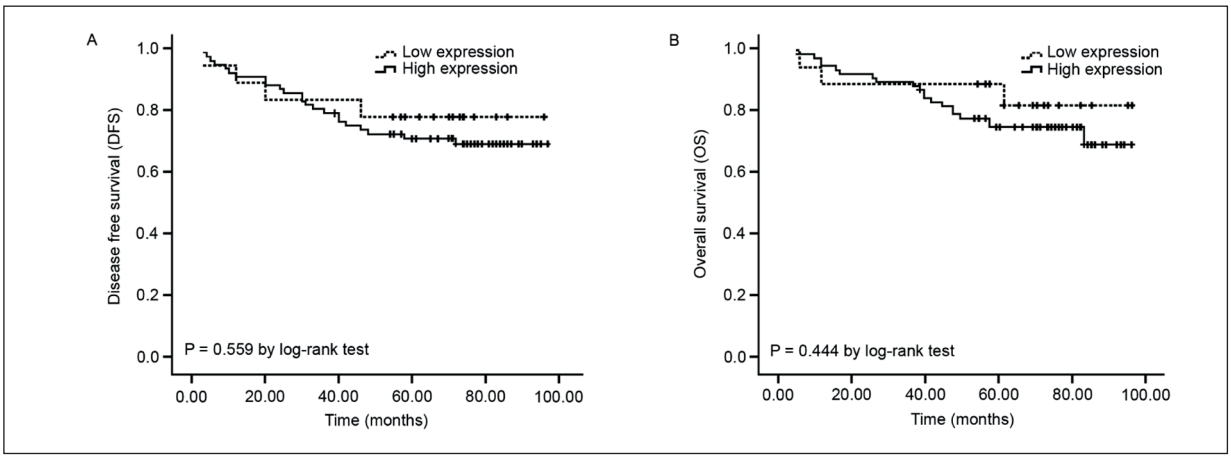

Figure 4. Kaplan-Meier survival curve for OS and DFS grouped by p-CREB-1 expression. **A,** Kaplan-Meier curves demonstrating the DFS of the high p-CREB-1 expression group versus the low p-CREB-1 expression group. The differences between the two groups were not statistically significant, as determined by the log-rank test (p=0.559). **B,** Kaplan-Meier curves demonstrating the OS of the high p-CREB-1 expression group versus the low p-CREB-1 expression group. The differences between the two groups were not statistically significant, as determined by the log-rank test (p=0.444). OS, overall survival; DFS, disease-free survival; p-CREB-1, phosphorylated cAMP response element-binding protein-1.

and significantly higher expression in breast cancer tissues. Thus, we suggest that the expression level of p-CREB-1 can be a suitable additional marker for early breast cancer diagnosis and early detection of metastases.

Increasing evidence demonstrates the involvement of CREB in self-renewal and decreased chemotherapeutic sensitivity of cancer cells[24]. He et al[25] reported that cancer cells acquire drug resistance and enhanced carcinogenesis via the phosphoinositide 3-kinase/protein kinase B/β-catenin/CBP pathway. Sandoval et al[26] showed that the cooperation of sex-determining region Y-box 4 and CREB increases the survival and self-renewal of mouse bone marrow cells in myeloid transformation. Furthermore, several neurotransmitters activate cancer stem cells in non-small cell lung cancer via cAMP-mediated pathways that involve p-CREB[27]. Therefore, we decided to investigate the correlation between p-CREB expression in breast cancer stem cells and non-stem-cell breast cancer cells (SP and non-SP). To the best of our knowledge, no previous studies have determined this association. The level of p-CREB-1 expression was compared in different cell lines, including SP and non-SP. The results revealed notable upregulation of p-CREB-1 and CREB in SP cells compared with other cancer cells, indicating the potential association between p-CREB-1 expression and stemness in breast carcinoma. Although the mechanism of this upregulated expression remains unknown, our results indicate that CREB and p-CREB are promising markers of predicting high developmental and tumor formation potency in patients with breast cancer.

MAPK cascade activation, the subsequent phosphorylation and translocation of ERK1/2 activates transcription factors, including nuclear factor-κB, hypoxia-inducible factor-1 and CREB, results in an increase in the transcription of pro-invasive genes (e.g., vascular endothelial growth factor [VEGF], cyclooxygenase-2)[28]. VEGF-mediated vessel formation, the most potent angiogenic process, is crucial for solid tumor growth and progression, including lymphatic metastasis[29]. Barresi et al[22] reported that high expression of p-CREB is related to angiogenesis and an increased risk of recurrence in human meningiomas. Liu et al[30] observed that the oncoprotein hepatitis B X-interacting protein upregulates fibroblast growth factor 8 through activation of CREB, promoting tumor angiogenesis and growth. In line with these findings, we revealed an association between high p-CREB-1 expression and ALN metastasis, implying the latent development-promoting role of this signaling protein in breast neoplasms.

Kaplan-Meier survival curves revealed that high expression of p-CREB-1 is highly predictive of worse DFS and OS. Despite the log-rank test not showing a statistical significance, the protein still shows a high promise, and further validations on larger cohorts are needed.

It is worth noting that using p-CREB-1 in an unselected cohort has several limitations, as there may be a different expression level in different types of breast cancer (i.e., more aggressive types, including triple-negative breast cancer, likely have a higher p-CREB-1 expression level).

Conclusions

In summary, our results suggest that high p-CREB-1 expression, even in early-stage breast cancer, is associated with carcinogenesis and tumor growth. Increased p-CREB-1 expression may facilitate tumor invasion, including lymph node metastasis, rendering the protein a potential predictive and prognostic biomarker in patients with breast cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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