The Novel Transcription Factor CREB3L4 Contributes to the Progression of Human Breast Carcinoma

Qian Pu [1,2] · Li Lu [3] · Ke Dong [4] · Wen-wen Geng [2] · Yan-rong Lv [1] · Hai-dong Gao [1,2]

Abstract

Breast carcinoma(BC)is the most common cancer type among females globally. Understanding the molecular pathways that trigger the development of BC is crucial for both prevention and treatment. As such, the role of transcription factors (TFs) in the development of BC is a focal point in this field. CREB3s play a critical role in initiating the unfolded protein response (UPR); however, the role of CREB3 family members in breast cancer development remains largely unknown. Here, we mined the ONCOMINE database for the transcriptional data of CREB3s in patients with BC. Then, the regulatory functions of a novel TF, CREB3L4, were investigated. CREB3L4 knockdown in MDA-MB-231 and MCF-7 cells suppressed proliferation and promoted apoptosis and cell cycle arrest. ChIP assays confirmed that CREB3L4 can directly bind to the PCNA promoter region, suggesting that the PCNA protein may be functionally downstream of CREB3L4. Additionally, the expression level of CREB3L4 was assessed using our cohort. CREB3L4 is upregulated in breast cancer tissues and is significantly associated with histological grade and tumour size ($P = 0.001$ and $P < 0.001$, respectively). Furthermore, PCNA expression was upregulated in breast cancer tissues and positively correlated with CREB3L4. In summary, CREB3L4 may play an important role in the progression of human BC and may serve as a therapeutic target.

Keywords Breast carcinoma · Transcription factors · CREB3L4 · PCNA

Introduction

According to the latest GLOBOCAN statistics, breast carcinoma (BC) is the most common invasive cancer in women and the second leading cause of cancer-related death worldwide [1]. The latest hypothesis is that

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appropriately inhibiting oncogenic transcription factors (TFs) is a target for BC treatment [2]. TFs are often the ultimate regulators of several central processes, such as proliferation, survival, self-renewal and invasion. Under different pathological conditions, TFs may aberrantly initiate the transcription of cancer-related genes, which increases cell proliferation, differentiation, and angiogenesis and suppresses apoptosis or cell-cell adhesions, all of which consequently lead to cancer development [3].

Cyclic adenosine monophosphate (cAMP) response element binding (CREB) [4], a phosphorylation-dependent TF, is overexpressed and constitutively phosphorylated in numerous human cancers [5] and plays a critical role in disease pathogenesis and prognosis [6] and cancer cell migration [7]. CREB belongs to the basic leucine zipper (bZIP)-containing TF superfamily, which includes mammalian C/EBP, c-Jun, c-Fos and c-Myc [8]. Among these bZIP factors, CREBs, activating transcription factors(ATFs), and cAMP response element modulator (CREM) constitute a subgroup named ATF/CREB based on the extent of their homology at the bZIP region [9]. Among the members of this subgroup, the CREB3 proteins and ATF6 are substrates for S1P/S2P [10]. These proteins possess a transmembrane segment, localize to

the endoplasmic reticulum (ER) and are activated by regulated intramembranous proteolysis, suggesting that they could play a critical role in initiating the unfolded protein response (UPR) [11], which is widely considered a mechanism of tumour progression [12].

The distinctive CREB3 subfamily consists of five members [11, 13]: CREB3 (LZIP/Luman) [14], CREB3L1 (OASIS) [15], CREB3L2 (BBF2H7) [16], CREB3L3 (CREB-H) [17] and CREB3L4 (Tisp40, AIbZIP, or ATCE1) [18]. CREB3L4, a novel human CREB gene, is localized on chromosome 1q21.3 and encodes a protein comprising 395 amino acid residues. As is typical of other ATF/CREB proteins, CREB3L4 contains a bZIP domain, which is located in its central region (amino acids 222–280). In humans, CREB3L4 is highly expressed in a variety of cancer types, including prostate carcinomas [11, 19, 20], hepatocellular carcinomas [21] and endometrial cancers [13]. Recently, it was revealed that the high messenger RNA (mRNA) expression of CREB3L4 was associated with reduced relapse-free survival (RFS) and disease-specific survival (DSS) in BC [22]. However, further studies investigating the mRNA expression of all CREB3 members in BC are necessary. In addition, the mechanism(s) responsible for CREB3L4 overexpression also need to be investigated, and it is unclear whether CREB3L4 overexpression is a consequence of or a contributing factor to cancer development.

The purpose of the current study was twofold. The first objective was to focus on the importance and relevance of the CREB3 family in BC and to identify which family member is most involved in the development of breast cancer. The second objective was to identify a possible target gene of CREB3L4. Data pertaining to all of these factors should considerably increase our understanding of the function of CREB3L4.

Materials and Methods

ONCOMINE Database Analysis

Gene expression array datasets from ONCOMINE (http://www.oncomine.org), a publicly online database containing 715 datasets and 86,733 samples [23], were searched to examine the transcription levels of the different CREB3s subfamily members in different cancers. The mRNA expression of these genes (log2-transformed) in clinical cancer specimens was compared with that in normal controls samples using Student's t test to generate a P value. To identify the CREB3 proteins with the most significant expression, the following parameters were defined: $P < 0.001$, fold change >2 and gene ranking in the top 10%.

Cells and Tissues

Four human breast cancer cell lines, MDA-MB-231, MDA-MB-468, MCF-7 and T-47D, were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (HyClone, USA) under a 5% CO2 atmosphere. MCF10A cells were cultured in DMEM-F12 (Hyclone, China) supplemented with 10 μg/ml insulin (Gibco), 0.5 μg/ml hydrocortisone (Sigma), 15 ng/ml epidermal growth factor (Sigma), 5% horse serum (Gibco), and 100 ng/ml cholera toxin (Sigma) and incubated at 37 °C in a humidified incubator with 5% CO2. This study was approved by the Human Ethics Review Committee of Qilu Hospital of Shandong University (Qingdao). The use of 80 pairs of breast cancer tumour and matched adjacent normal tissues was approved by the Department of Pathology of Qilu Hospital of Shandong University (Qingdao). All patients underwent surgical resection at Qilu Hospital of Shandong University (Qingdao). Informed consent was obtained from all subjects or their relatives. All of the tissue samples were snap-frozen and stored at −80 °C in liquid nitrogen.

Lentivirus Infection

Lentiviruses carrying a short hairpin RNA (shRNA) target sequence for shCREB3L4 (5'-CGGAGTGACTTCCA GAAATAT-3′) were obtained from GeneChem. The viruses produced were used in the presence of polybrene to infect MCF7 and MDA-MB-231 cells. Forty-eight hours later, MCF7 and MDA-MB-231 cells were cultured in medium containing puromycin for the selection of clones with stable CREB3L4 knock down, which were identified and verified by Western blotting using the indicated primary antibodies.

Real-Time PCR

Total RNA was extracted using TRIzol reagent (TaKaRa) following the manufacturer's protocol. The miScript Reverse Transcription Kit (TaKaRa) was used for quantitative real-time polymerase chain reaction (qRT-PCR). SYBR green PCR Master Mix (TaKaRa) was used for the detection of mRNA expression following the manufacturer's protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as an endogenous control. The 2-$\Delta\Delta$CT method was used to analyse the relative fold changes in expression. The primers were designed by Sangon Biotech (Shanghai, China). Their sequences are as follows: CREB3L4, forward: 5'-CAGACGCTAATTGCTCAAAC TTC-3′ and reverse 5'-CCACTTGGGTCTCCAGATTT TCT-3′; PCNA, forward: 5'-CCTGCTGGGATATTAGCTCC A-3′ and reverse 5'-CAGCGGTAGGTGTCGAAGC-3′; and GAPDH, forward 5'-GGAGCGAGATCCCTCCAAAAT-3′ and reverse 5'-GGCTGTTGTCATACTTCTCATGG-3′.

Immunoblot Assay

Cells were lysed with RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA), and the resulting protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) through a 10% gel and transferred onto PVDF membranes (Millipore). The membranes were blocked with 5% nonfat milk at room temperature for 2 h before they were incubated overnight with primary antibodies at 4 °C. After they were washed with TBST three times for 15 min each, the membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature with gentle shaking. GAPDH was used as the loading control. Immunoreactive bands were visualized using SuperSignal West Pico Chemiluminescent Substrates (Thermo, USA). The primary antibodies used in this study were anti-CREB3L4 (Thermo Fisher, PA5–18028), anti-PCNA (Thermo Fisher, MA5–11358), and anti-GAPDH (Abcam, ab181602).

Proliferation Assay

Cells were assessed for proliferation by measuring 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt Sigma (MTS) reduction. Cells were seeded into 96-well plates (100 µl cell suspensions, 1×10^3 cells/ml). Twenty-four hours later, 0.05 mg ml^{-1} MTS reagent (Promega) was added to each well and incubated at 37 °C for 4 h followed by measurement of the absorbance at 490 nm. The values were standardized to those of wells containing media alone.

Apoptosis Assay

MCF7 and MDA-MB-231 cells were transfected with shCtrl and shCREB3L4. Forty-eight hours later, transfected cells were treated with cisplatin (10 µM, 24 h, Sigma). The apoptotic cells were then washed with phosphate-buffered saline (PBS) and stained with fluorescein isothiocyanate-annexin V and propidium iodide (PI) according to the manufacturer's protocol (Beijing Biosea Biotechnology Annexin V Kit). Apoptotic cells (annexin V-positive, PI-negative) were then determined by flow cytometry.

Cell Cycle Assay

Cell cycle analysis was performed by monitoring the DNA content at various time points with PI staining. Cells synchronized at S phase with a double thymidine (Sigma, 2 mM) block were released, cultured in fresh medium for 12 h, fixed in 70% cold ethanol, incubated on ice for 2 h, washed once in PBS containing 0.1% glucose, and incubated for 30 min at 37 °C in PBS containing 0.1% glucose, 1 mg of RNase A (Thermo) per ml, and 20 µg of PI per ml. DNA content was determined by flow cytometry.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using an iDeal ChIP-seq Kit for Transcription Factors (Diagenode, C01010170) with antibodies against CREB3L4 and PCNA. A lysate of 5×10^6 cells was used for each ChIP reaction. Precipitated DNA samples were analysed by quantitative PCR with primer pairs specific to the promoters of the PCNA and CREB3L4 genes. qRT-PCR was used to quantify gene expression as previously described. The ChIP primers used were as follows: forward, 5'-ATGGACACGATTGGCCCTAAA-3'; reverse, 5'-CTACGCCTGCAACCGTTTAAT-3'.

Luciferase Reporter Assay

PCNA promoter cloning: The region of the PCNA promoter was cloned by PCR from normal human breast cDNA. Primers were listed in the followed table. The sequenced fragments were inserted into the to the promoter less plasmid pGL2-enhancer control vector using SacI and NarI enzyme.

PCR	Primers	Sequence (5′-3′)
Promoter cloning	Upstream	ATTATGTTTTGATATTGAGATGTATGCAA
	Downstream	CAAGCGCGCGCTCTCACCCTGCGCCGCGT

Immunohistochemical (IHC) Staining

Haematoxylin and eosin (H&E) staining was performed to differentiate cancer and normal breast tissues in the same section. Immunohistochemistry was performed by using the avidin-biotin complex method (ABC; Vector Laboratories, Burlingame, CA), which included heat-induced antigen retrieval procedures. Incubation with antibodies against CREB3L4 (PA5–58254, 1:500 dilution, Thermo Fisher) and PCNA (MA5–11358, 1:400 dilution, Thermo Fisher) was carried out at 4 °C for 18 h. All staining was assessed by pathologists blinded to the diagnosis of the subjects and their outcomes. The widely accepted German semiquantitative scoring system that considers the staining intensity and extent of staining was used. Each specimen was assigned a score according to the intensity of the nucleic, cytoplasmic, and/or membrane staining (no staining = 0; weak staining = 1, moderate staining = 2, strong staining = 3) and the percentage of stained cells (0% = 0, 1–24% = 1, 25–49% = 2, 50–74% = 3, 75–100% = 4). The final immunoreactive score was determined by multiplying the intensity score by the percentage score, which ranged from 0 (the minimum score) to 12 (the maximum score). A score ≥ 5 was considered positive staining, and a score ≤ 4 was considered negative staining.

Analysis type by cancer	Cancer vs. Normal CREB3		Cancer vs. Normal CREB3L1		Cancer vs. Normal CREB3L2		Cancer vs. Normal CREB3L3		Cancer vs. Normal CREB3L4	
Bladder cancer				2						
Brain and CNS cancer					1					
Breast cancer			4					3	8	
Cervical cancer										
Colorectal cancer				2				2		
Esophageal cancer	1		3							
Gastric cancer										
Head and neck cancer			1							
Kidney cancer				3						
Leukemia	1				2				3	
Liver cancer										
Lung cancer				2					1	
Lymphoma				1						
Melanoma				2						
Myeloma								2		
Other cancer			1	1						
Ovarian cancer				1					1	
Pancreatic cancer										
Prostrate cancer			1		1					
Sarcoma			3	1				1		
Significant Unique Analyses		2	13	14	4	0		8	13	
Total Unique Analyses	**408**		**412**		**346**		**251**		**278**	
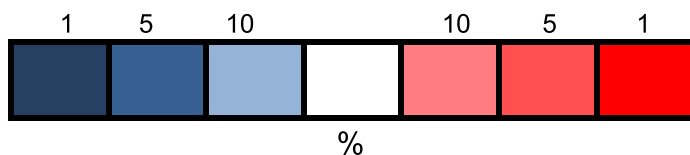
Fig. 1 The mRNA expression levels of CREB3s subfamily members in ONCOMINE datasets of different types of human cancers were analysed. These results highlight the numbers of datasets with statistically significant (p < 0.01) mRNA overexpression (red) or underexpression (blue) of the target gene (cancer vs. normal tissue). Cell colour was determined by the best gene rank percentile for the analyses within the cell. The gene rank was analysed by the percentile of target genes in the top of all genes measured in each experiment. The p value threshold was 0.001, and the fold change was defined as 2

Table 1 The significant changes of CREB3s expression at the transcription level between different types of breast carcinoma and normal tissues (ONCOMINE database)

	Subtype of BC vs. Normal	Fold change	P value	Rank(%)	Sample	t-test	Refs
CREB3L4	Invasive ductal and lobular BC	4.927	5.48E-4	6	64	8.245	TCGA
	Mixed lobular and ductal BC	4.663	1.73E-6	1	68	8.001	
	Invasive lobular BC	3.379	6.83E-14	2	99	8.657	
	Invasive ductal BC	2.594	3.62E-13	16	450	8.656	
	Invasive BC	3.391	0.006	14	158	4.759	Gluck(24)
	Tubular BC	2.638	7.2E-32	1	211	15.933	Curtis(25)
	Mucinous BC	2.162	1.23E-14	4	190	9.752	
	Invasive ductal BC	2.045	1.01E-49	8	1700	20.289	
CREB3L1	Invasive ductal and lobular BC	2.524	1.96E-4	5	64	9.804	TCGA
	Mixed lobular and ductal BC	1.606	4.77E-4	8	68	4.378	
	Invasive lobular BC	2.837	2.85E-12	3	99	8.795	
	Invasive ductal BC	2.162	4.81E-22	7	450	11.368	
	Invasive BC	2.135	0.026	24	158	2.987	Gluck(24)
	Tubular BC	1.075	1.30E-8	19	211	5.886	Curtis(25)
	Mucinous BC	1.088	3.14E-6	19	190	4.889	
	Invasive ductal BC	1.050	2.93E-9	35	1700	6.123	

Statistical Analysis

The immunohistochemistry results were analysed by the χ^2 test, and all other results are expressed as the mean ± standard deviation (SD) derived from three independent experiments unless stated otherwise. Student's t test was used for statistical comparisons between means where applicable. Differences among more than two groups in the above assays were estimated using analysis of variance (ANOVA). A correlation analysis was performed with Spearman's rank correlation coefficient. All statistical analyses were performed with GraphPad Prism 5 and SPSS 18.0 software. All statistical tests were two-sided, and P values <0.05 were considered statistically significant.

Results

The Transcription Levels of CREB3 Subfamily Members in BC

As mentioned before, five CREB3 subfamily members have been identified [13]. First, we analysed the differences in the transcription levels of these genes between tumour and normal tissues in multiple types of cancer obtained from the online ONCOMINE database. As shown in Fig. 1, the ONCOMINE database contained a total of 408, 412, 346, 251 and 278 unique analyses for CREB3, CREB3L1, CREB3L2, CREB3L3 and CREB3L4, respectively. CREB3L4 was

observed in 4 human cancer types, including haematological malignancies and solid tumours. The mRNA expression level of CREB3L4 was significantly increased in BC samples in 8 studies. In The Cancer Genome Atlas (TCGA) dataset, CREB3L4 overexpression was observed in all types of BC compared with normal tissue, including invasive ductal and lobular BC (fold change = 4.927), mixed lobular and ductal BC (fold change = 4.663), invasive lobular BC (fold change = 3.379), and invasive ductal BC (fold change = 2.594). In the Gluck [24] dataset, CREB3L4overexpression was observed in invasive BC compared to normal tissue (fold change =3.391). In the Curtis [25] dataset, CREB3L4 overexpression was observed in tubular BC (fold change = 2.638), mucinous BC (fold change = 2.162), and invasive ductal BC (fold change = 2.045) compared to the respective normal tissues (Table 1).

Another gene showing increased mRNA expression according to the 4 datasets was CREB3L1. In the TCGA dataset, CREB3L1 overexpression was found in invasive ductal and lobular BC (fold change = 2.524), mixed lobular and ductal BC (fold change = 1.606), invasive lobular BC (fold change = 2.837), and invasive ductal BC (fold change = 2.162). In the Gluck dataset, CREB3L1 overexpression was observed in invasive BC compared to normal tissues (fold change =2.135) (Table 1). However, neither CREB3 nor CREB3L2 mRNA was detected in breast cancer tissue samples. In addition, the mRNA expression of CREB3L3 was decreased in BC according to 3 datasets (Fig. 1). These results suggest that CREB3L4 may be an oncogenic factor and play a specific role in the occurrence and development of BC.

Fig. 2 CREB3L4 is upregulated in breast cancer cells. (a) Analysis of CREB3L4 mRNA and protein expression in the BC cell lines MDA-MB-231, MDA-MB-468, MCF-7 and T-47D by RT-PCR and western blot. The highest CREB3L4 expression among the breast cancer cell lines was observed in MCF-7 and MDA-MB-231 cells ($p < 0.05$). (b) The mRNA level of CREB3L4 in MCF-7 ($p < 0.01$) and MDA-MB-231 ($p < 0.05$) cells transfected with CREB3L4 shRNA was significantly reduced compared to that in nontransfected cells. (c) The protein expression of CREB3L4 in MCF-7 and MDA-MB-231 cells transfected with CREB3L4 shRNA was detected with immunoblotting. The data are presented as the means ± SDs of three independent experiments. $*p < 0.05$ and $**p < 0.01$. PCR: polymerase chain reaction; IB: immunoblot
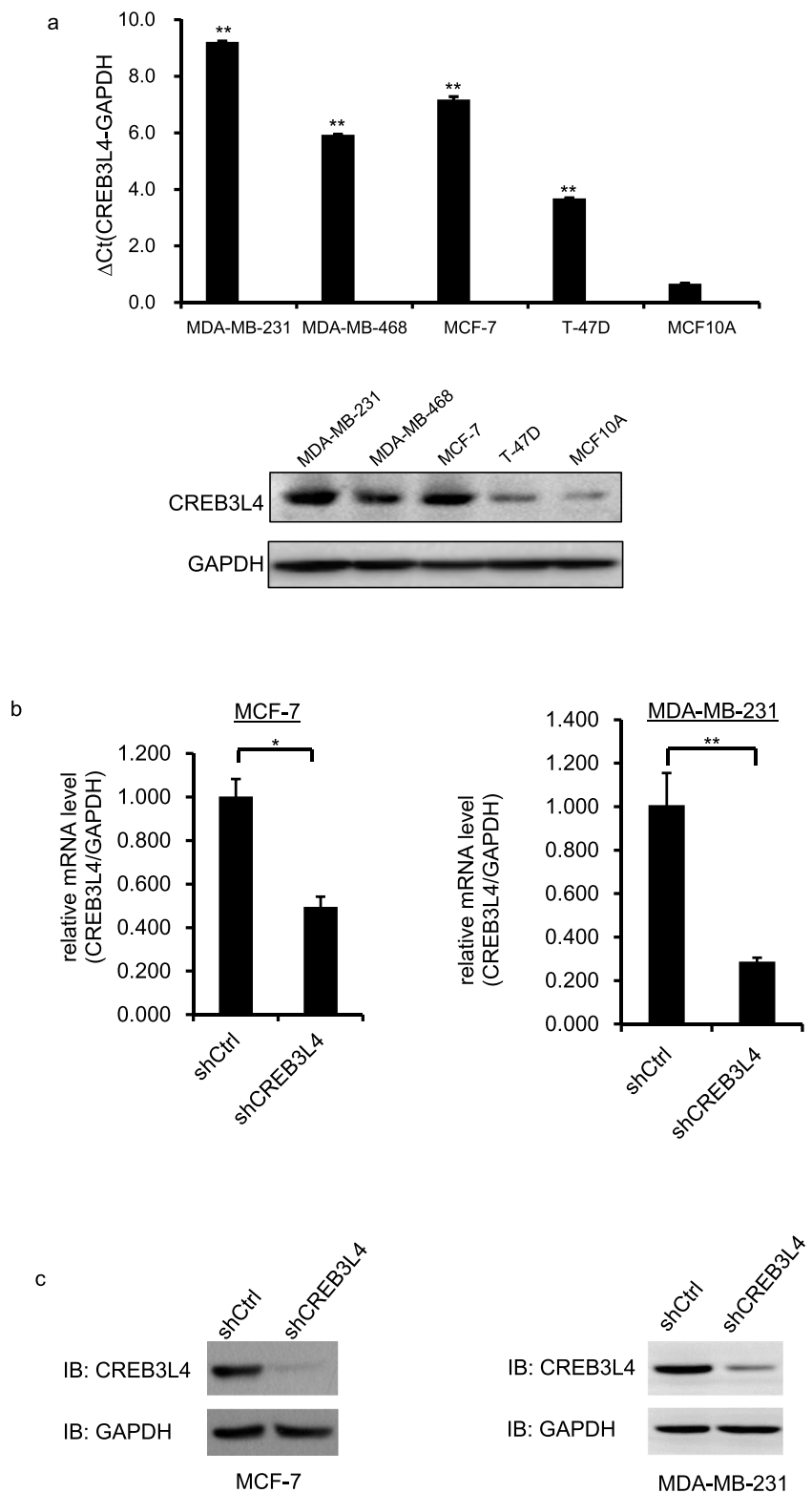
## CREB3L4 Is Upregulated in Breast Cancer Cells

On the basis of Northern blot analyses, full-length CREB3L4 mRNA is expressed only in the human prostate as well as in breast and prostate cancer cell lines [19]. Therefore, RT-qPCR

and Western blot analyses were performed to detect CREB3L4 expression in BC cells. As shown in Fig. 2a, CREB3L4 was upregulated not only in oestrogen receptor-positive MCF-7 and T-47D breast cancer cells but also in the triple-negative breast cancer (TNBC) cell lines MDA-MB-
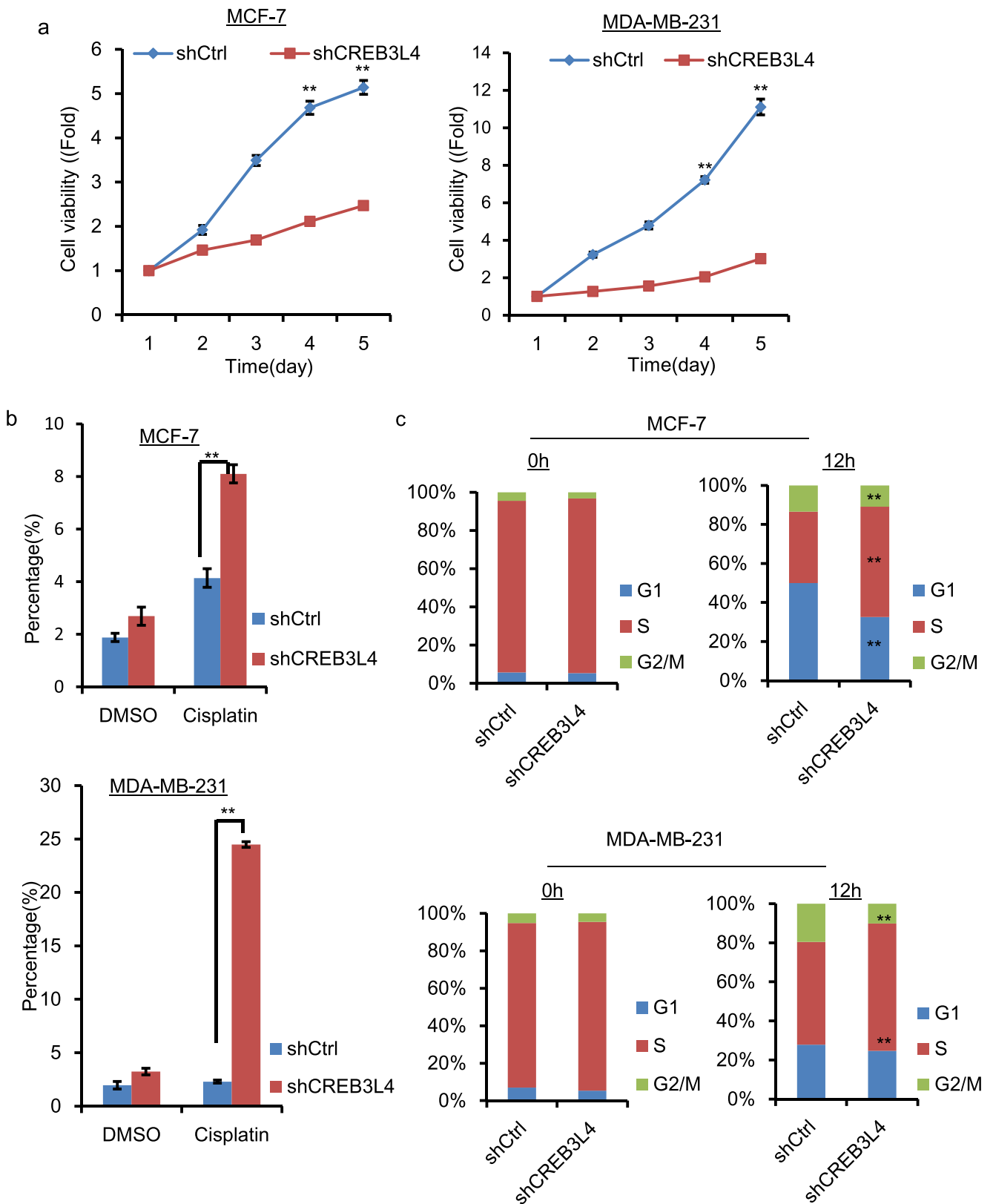
Fig. 3 The function of CREB3L4 in BC cell proliferation, apoptosis and cell cycle arrest. (a) The proliferation activities of MCF-7 and MDA-MB-231 cells transfected with shCtrl and shCREB3L4 were determined by the MTS assay. (b) The percentages of apoptotic cells in MCF-7 and MDA-MB-231 cells transfected with shCtrl and shCREB3L4 were evaluated by flow cytometry. (c) The percentages of PI-stained cells at G1, S and G2/M phases in MCF-7 and MDA-MB-231 cells transfected with shCtrl and shCREB3L4 were determined by flow cytometry. The data are presented as the means ± SDs of three independent experiments. $*p < 0.05$, $**p < 0.01$
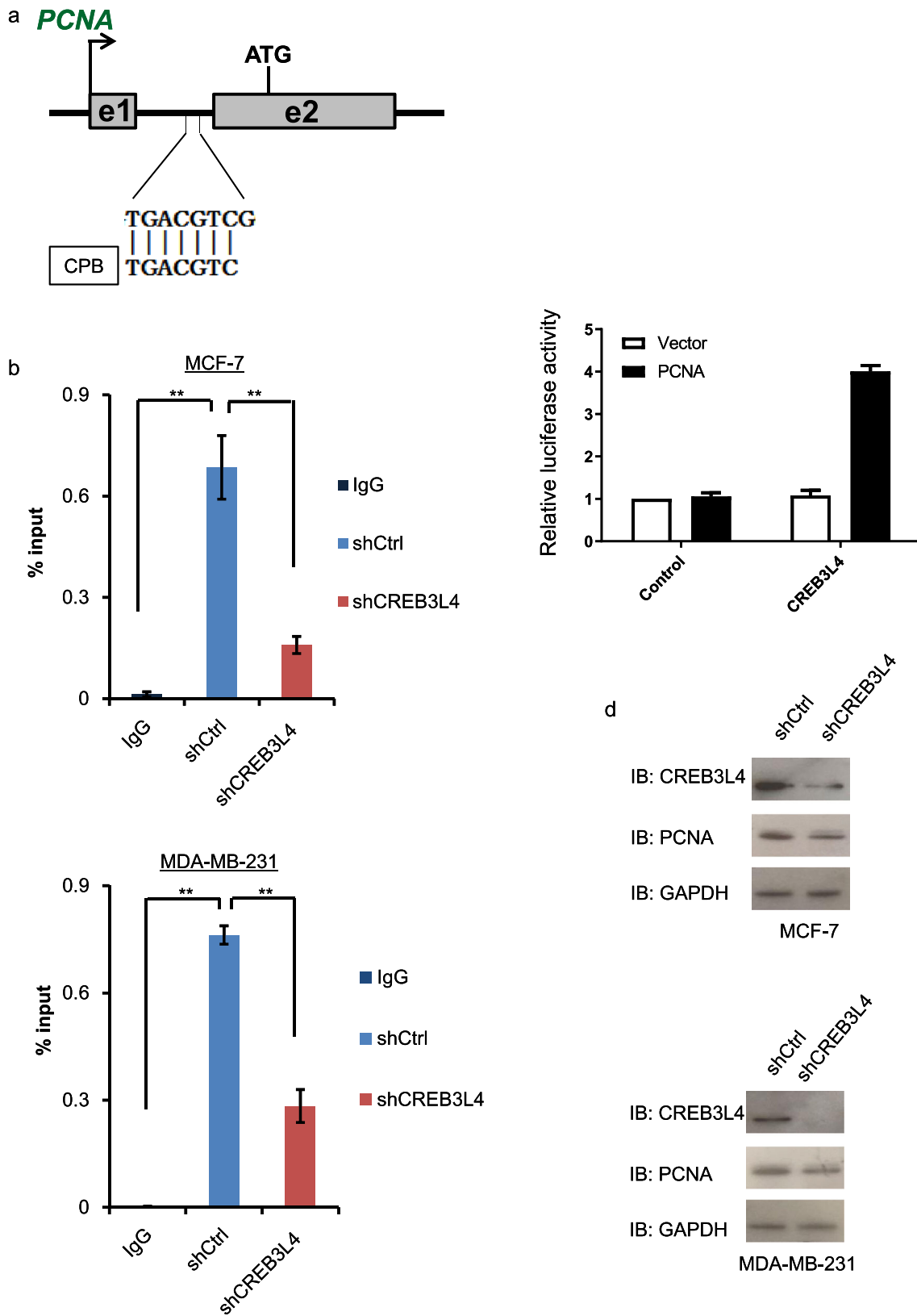
231 and MDA-MB-468, especially in the noninvasive MCF7 and highly aggressive MDA-MB-231 cells. To investigate the function of CREB3L4 in BC cell lines, MDA-MB231 and

MCF7 cells with stable CREB3L4 knockdown were established by the introduction of a shRNA expression vector. As confirmed by real-time PCR and Western blot, the results

Fig. 4 CREB3L4 binds to the promoter region of PCNA. (a) CREB3L4 binding sites present in the PCNA promoter region. (b) ChIP assays were performed to examine CREB3L4-bound DNA using anti-IgG. ChIP was carried out with anti-IgG (negative control) and anti-CREB3L4 antibodies in MCF7 and MDA-MB-231 cells. The qPCR results for the PCNA promoter region are shown as the percentage of input DNA. (c) The promoter-less pGL2 control vector and PCNA promoter vector was transected with CREB3L4 plasmid into MCF7 and MDA-MB-231 cells, and the activity of the region was tested by dual-luciferase reporter assay. The data was shown as mean ± SEM for three independent experiments. (d)The protein expression levels were detected by Western blotting analysis using GAPDH as an internal control. Data are shown as the means ± SDs. ** $p < 0.01$

showed that endogenous CREB3L4 expression was more downregulated in cells transduced with shCREB3L4 than in cells transduced with the control (Fig. 2b, c).

Downregulation of CREB3L4 Suppresses Proliferation and Induces Cell Cycle Arrest and Apoptosis in BC Cells

Next, we analysed the effect of CREB3L4 on breast cancer cells. We applied the MTS assay to evaluate cell proliferation. Three days after transfection, a reduction in cell viability was observed in the shCREB3L4 group, and the inhibitory effect became more significant at 4 and 5 days after transfection ($P < 0.01$). Knockdown of CREB3L4 in MCF7 and MDA-MB-231 cells significantly decreased the extent of cell proliferation compared to that of the respective negative control cells (Fig. 3a). This result suggests that CREB3L4 promotes the proliferation of BC cells. In addition, annexin V/PI double-staining and PI staining tests were used to evaluate apoptosis and cell cycle distribution. The results revealed that cells with CREB3L4 knockdown were significantly more sensitive to cisplatin-induced apoptosis than were the negative control cells ($P < 0.05$) (Fig. 3b). Moreover, we analysed cell cycle distribution using flow cytometry, which showed that CREB3L4 knockdown led to cell cycle arrest at S phase in both MCF-7 and MDA-MB-231 cells at 12 h after release from the thymidine block ($P < 0.01$, Fig. 3c). These results indicate that CREB3L4 downregulation greatly promoted apoptosis and cell cycle arrest in both MCF-7 and MDA-MB-231 cells. In conclusion, we demonstrated that CREB3L4 could modulate the cellular function of BC cells and may play an oncogenic role in breast cancer.

CREB3L4 Binds to the Promoter Region of PCNA

Based on the understanding of the pathologic role of CREB3L4, we continued to explore the underlying regulatory mechanisms. Thus, we investigated the promoter region of PCNA and found that the sequence "GTGACGTC" was located in its promoter region (Fig. 4a). The results of the ChIP assay indicated that CREB3L4 directly binds to the PCNA promoter region, and both MCF7 and MDA-MB-231 cells

with CREB3L4 silencing exhibited reduced CREB3L4 binding with the PCNA promoter (Fig. 4b).This binding can alter gene transcription was also tested by luciferase promoter assay (Fig. 4c). Next, we found that silencing CREB3L4 reduced the protein level of PCNA (Fig. 4d), suggesting a regulatory effect of CREB3L4 on PCNA.

Expression and Correlation of CREB3L4 and PCNA in Breast Cancer Tissues

The relationships between CREB3L4 expression and the clinicopathological features of breast cancers are summarized in Table 2. CREB3L4 is up-regulated in breast cancer tissues and is significantly associated with histological grade and tumour size ($P = 0.001$ and $P < 0.001$, respectively). However, the associations between CREB3L4 expression and patient age, oestrogen receptor (ER), progesterone receptor (PR), HER2, and lymph node status were not statistically significant. The TCGA data set was also used to analyse the correlation between CREB3L4 and ER/PR/HER2, which are typical biomarkers of breast cancer. However, we still found no correlation between CREB3L4 expression and ER/PR/HER2 ($R^2 = 0.1849/0.1024/0.1849$, Fig. 5a). CREB3L4 expression was upregulated in tissue samples derived from patients with a high histologic grade and a large tumour size (Fig. 5b).

Since the molecular mechanism of the interaction between CREB3L4 and PCNA was verified, we further analysed the correlation between CREB3L4 and PCNA expression in tumour tissues using clinical breast cancer samples. The mRNA expression of CREB3L4 and PCNA in 32 pairs of BC tissues and adjacent nontumour tissues was examined using real-time PCR. The levels of CREB3L4 ($P = 0.0108$) and PCNA ($P = 0.001$) mRNA expression were significantly higher in cancerous tissues than in normal tissues (Fig. 5c). To assess the consistency of protein translation with the observed transcription levels, the protein levels of CREB3L4 and PCNA in 80 pairs of BC tissues and adjacent nontumour tissues were examined. The analysis of the relationship between CREB3L4 expression and PCNA expression showed a significantly positive correlation ($R = 0.9139$, 95% CI: 0.8686–0.9441, $p < 0.001$) (Fig. 5d). These results suggest that CREB3L4 is upregulated in breast cancers concomitant with the increase in PCNA expression to promote tumorigenesis. Altogether, our clinical results indicate that CREB3L4 may play a role in the tumorigenesis of BC.

Discussion

CREB typically serves as a proto-oncogenic TF, and its role in human cancers is tissue- and/or cell type-specific [5]. CREB is elevated in breast cancer tissues [26] and positively regulates the proliferation and metastasis of breast cancer as well as

Table 2 Correlation between the CREB4 levels and the clinicopathological features of 80patients with breast cancer

Variables	Cases	CREB4 High(%)	CREB4Low(%)	X2	P
Age (years)				3.810	0.051
≤60	24	8(33.3)	16(66.7)		
>60	56	32(57.1)	24(42.3)		
Histological grade				13.685	0.001
I	27	6(22.2)	21(77.8)		
II	31	18(58.1)	13(41.9)		
III	22	16(72.7)	6(27.3)		
Tumor size (cm)				24.838	<0.001
<2	42	10(23.8)	32(76.2)		
2–5	35	27(77.1)	8(22.9)		
>5	3	3(100)	0(0)		
Lymph node status				6.485	0.090
0	52	22(42.3)	30(57.7)		
1–3	15	9(60.0)	6(40.0)		
4–9	8	7(87.5)	1(12.5)		
>9	5	3(60.0)	2(40.0)		
Molecular subtype				2.671	0.445
LuminalA	26	10(38.5)	16(61.5)		
LuminalB	37	21(56.8)	16(43.2)		
Her-2	8	5(62.5)	3(37.5)		
TNBC	9	4(44.4)	5(55.6)		
ER status				0.050	0.824
negtive	16	8(50.0)	8(50.0)		
positive	64	34(53.1)	30(46.9)		
PR status				0.019	0.890
negtive	22	11(50.0)	11(50.0)		
positive	58	28(48.3)	30(51.7)		
HER2 status				2.222	0.136
negtive	72	34(47.2)	28(38.9)		
positive	8	6(75.0)	2(25.0)		
Ki67 status				0.879	0.348
<14%	28	12(42.9)	16(57.1)		
≥14%	52	28(53.8)	24(46.2)		

bone destruction in breast cancer patients [27]. Among the CREB proteins, CREB3 subfamily members are particularly involved in the UPR. In breast tumours, chronic activation of the UPR is associated with therapy resistance and disease recurrence [28, 29]. However, studies on CREB3s in BC are rare. The development of microarray technology has become an essential component of biology and biomedical research [30]. Therefore, we performed an analysis of the expression of different CREB3 subfamily members in BC patients based on thousands of online analyses on gene expression or copy number variation. These results suggest that CREB3L1 and CREB3L4 mRNA levels are increased in breast cancer tissues. Alison K. Ward demonstrated that decreased CREB3L1 mRNA expression was associated with increased tumour grade and reduced progression-free survival.

CREB3L1 is frequently upregulated in luminal and HER2-amplified breast cancer but not in TNBC [31]. However, recently, Bray Denard1 reported that TNBC tumours with high CREB3L1 expression have a high response rate to doxorubicin-based chemotherapy [32]. Through a comparative analysis, we found that CREB3L4 is a member of the CREB3 subfamily and is more closely related to the development of breast cancer than is CREB3L1. Further analysis of the clinical samples showed that CREB3L4 is highly expressed in BC tissues and positively correlated with tumour size and histological grade. However, the TCGA dataset and our cohort data indicate that CREB3L4 is independent of molecular typing. Our current clinical sample follow-up time is too short to provide meaningful clinical outcome data, and we feel it is too preliminary to make any conclusions at this stage.
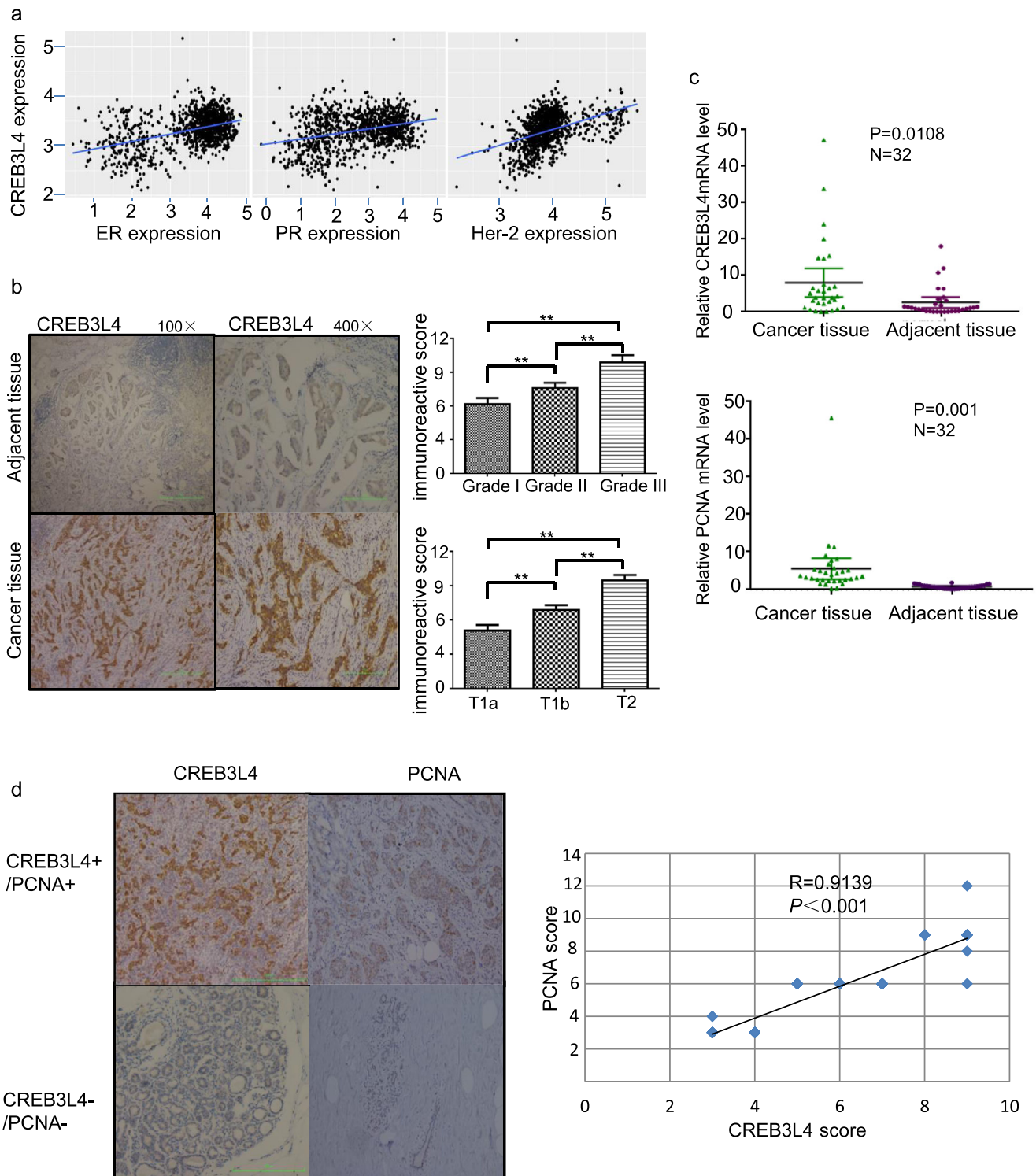
Fig. 5 The expression and correlation of CREB3L4 and PCNA in BC and nontumour tissues. (a) The correlation between CREB3L4 and ER/PR/HER2 was analysed with the TCGA data set. The data is presented as Log fold expression. (b) Representative images from the immunohistochemical staining of CREB3L4 in three serial sections of the same tumour and matched adjacent normal tissues. Scale bars, 50 μm. CREB3L4 protein expression in BC tissues is shown according to histological grade and tumour size. (magnification, ×400; p < 0.05). (c) CREB3L4 and PCNA mRNA expression levels in 32 paired BC and nontumour tissues were examined using real-time PCR. (d) Positive correlation of CREB3L4 with PCNA protein levels. A statistically significant association between CREB3L4 and PCNA expression was observed in breast cancer specimens ($n = 80$). **p < 0.01

We also elucidated its roles in the proliferation, apoptosis and cell cycle arrest of breast cancer cells. First, we generated shRNA to assess the potential role of CREB3L4 in cancer cell proliferation. The results showed that CREB3L4 knockdown significantly reduced cell viability and inhibited breast cancer proliferation. Furthermore, CREB3L4 knockdown also resulted in apoptosis and cell cycle arrest of breast cancer cells. Thus, CREB3L4 is a newly discovered TF associated with the development of breast cancer.

Oestrogen plays an important role in the development and physiology of the normal breast as well as in breast cancer cell proliferation. CREB3L4 has been reported to be highly expressed in sex-related cancers, including prostate, endometrium and uterine cancers [13]. In our experiment, CREB3L4 expression was upregulated in both oestrogen receptor-positive and TNBC cell lines. However, we did not detect a correlation between CREB3L4 protein expression and ER/PR/Her2 expression. In addition, CREB has emerged as a novel effector of p21-activated kinase 4 (PAK4); however, the upstream regulators in the ER/AR and WNT signalling pathways remain to be determined [33]. We wanted to determine whether CREB3L4 plays an important role in oestrogen-induced breast development and oestrogen-dependent breast cancer and whether CREB3L4 is downstream of PAK4. However, to confirm these hypotheses, we will need to quantitate CREB3L4 expression in a larger number of tumour specimens that are collected and processed under standardized conditions. In future studies, it will be of interest to determine whether CREB3L4 expression correlates with other clinically relevant endpoints. These findings will determine whether CREB3L4 may serve as a good immunohistochemical marker for breast cancer cells.

As CREB is a phosphorylation-dependent TF, its phosphorylation is sufficient to induce cellular gene expression in response to mitogen or stress-related signals [34]. Phosphorylation at Ser133 is required for CREBs to bind to CREB-binding proteins (CBPs) [35]; however, PAK4-mediated CREB activation is independent of its phosphorylation at Ser133. Rather, PAK4 activates CREB by phosphorylating CREB-regulated transcriptional cofactor 1 (CRTC1), a coactivator of CREB [36]. The phosphorylation status and phosphorylation site of CREB3L4 need to be further studied in breast cancer. Additionally, whether this mechanism is involved in BC tumorigenesis remains to be ascertained.

CREB mediates the activation of cAMP-responsive genes by binding as a dimer to a conserved cAMP-responsive element (CRE), TGACGTCA [37, 38]. Mayr and Marc Montminy had predicted that PCNA is one of the target genes containing consensus sites for CREB binding [34]. PCNA is a kind of cycle-related protein that is an indicator of tumour proliferation and is related to the occurrence of early cancer and tumour progression [39, 40]. Its main function is to participate in DNA replication, and it is also involved in DNA

excision repair cell cycle control chromatin assembly and RNA transcription [41]. PCNA interacts with the S-phase-specific cyclin-dependent kinase 2 (CDK2)-cyclin A complex to stimulate the phosphorylation of CDK2 substrates, thereby targeting the CDK2-cyclin A complex to bind to DNA replication proteins [42]. ILK overexpression in breast cancer cells promoted proliferation and cell activity by activating the PI3K/Akt pathway and upregulating PCNA [43]. To further investigate the regulatory mechanism by which CREB3L4 plays an oncogenic role, we determined its downstream target gene, PCNA. ChIP assays can realistically reflect the binding of TFs to the promoter in cells, and the results indicated that CREB3L4 directly binds the promoter of PCNA. This binding can alter gene transcription was also tested by luciferase promoter assay. IHC assays on clinical samples demonstrated that CREB3L4 and PCNA protein expression was positively correlated. These results suggest that CREB may promote PCNA expression by directly binding to the PCNA promoter, supporting the notion that a CREB3L4/PCNA axis exists in BC cells. Thus, their regulation and underlying molecular mechanism in BC cells need to be further studied. Whether a novel CREB3L4/PCNA axis participates in the biological behaviours of BC cell is still a key problem to be addressed in our future studies.

Conclusion

Taken together, our results indicate that, among the CREB3 subfamily members, CREB3L4 is most closely linked to the development of breast cancer. Our study demonstrated that CREB3L4 promotes BC oncogenesis via increased proliferation and reductions in apoptosis and cell cycle arrest in breast cancer cells. CREB3L4 binds to the PCNA promoter region. Both are highly expressed in breast cancer tissues, with a positive correlation. Thus, the CREB3L4/PCNA axis may play a crucial role in tumour progression. CREB may upregulate PCNA expression, leading to breast cancer cell proliferation and migration. Therefore, CREB3L4 may be a promising therapeutic target for breast cancer.

Compliance with Ethical Standards

Conflict of Interest The authors declare that there are no conflicts of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the Research Ethics Committee of Qilu Hospital (Qingdao) of Shandong University and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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