Received: 30 January 2019 | Revised: 11 March 2019 | Accepted: 9 May 2019

DOI: 10.1111/1440-1681.13104

ORIGINAL ARTICLE

Cellular and Molecular Biology

CEPP Clinical and Experimental Pharmacology and Physiology WILEY

# Crumbs protein homolog 3 (CRB3) expression is associated with oestrogen and progesterone receptor positivity in breast cancer

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**Funding information**
Natural Science Basic Research Project of Shaanxi Province, Grant/Award Number: 2017JM8015; National Natural Science Foundation of China, Grant/Award Number: 81502413 and 81702631

## Abstract

The crumbs protein homolog 3 (CRB3) regulates the tight junction to help maintain epithelial polarity. Altered CRB3 expression was associated with carcinogenesis of epithelial cells. This study detected CRB3 expression in 192 cases of breast cancer tissues and in the Molecular Taxonomy of Breast Cancer International Consortium (Metabric) and The Cancer Genome Atlas (TCGA) datasets for association with triple negative breast cancer (TNBC) phenotypes. The in vitro experiments confirm the ex vivo data. The data showed that levels of both CRB3 mRNA and protein were associated with TNBC phenotypes, ie, 41.1% (39/95) of ER+ breast cancer was CRB3-positive, whereas 26.9% (25/93) ER– tumour was CRB3-positive ($P$ = 0.046). Moreover, 47.6% (30/63) of PR+ breast cancer was CRB3-positive vs 28.4% (33/116) PR- tumours positive for CRB3 ($P$ = 0.013). In addition, 40.1% (27/66) of ER+/PR+ tumour was CRB3-positive, but only 22.4% (19/85) of TNBC showed CRB3 expression ($P$ = 0.048). Indeed, levels of CRB3 mRNA were higher in non-TNBC than TNBC in both Metabric ($P$ = 3.682e-10) and TCGA datasets ($P$ = 2.501e-07). The in vitro data showed that CRB3 expression was higher in luminal (MCF7 and T47D) than in HER2 (MDA-MB-453 and SK-BR-3) and basal (MDA-MB-231 and BT-549) breast cancer cell lines. More interestingly, ERα regulated expression of CRB3 protein in MCF7 and BT-549 cells and ERα expression was associated with CRB3 expression in breast cancer tissues specimens. This study demonstrated that ERα could be a novel regulator for CRB3 expression in breast cancer.

KEYWORDS

breast cancer, CRB3, Her2/neu, oestrogen receptor, progesterone receptor

Pingping Li and Can Zhou equally contributed to this work.

## 1 | INTRODUCTION

Breast cancer is the most frequently diagnosed malignancy in women worldwide with an estimated 1.7 million new cases and more than a half of million deaths occurring in 2012.[1] In China, breast cancer accounts for 15% of all newly diagnosed cancer cases for Chinese women.[2,3] Molecularly, breast cancer can be grouped according to the expression of oestrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2/neu) as receptor-positive breast cancer or TNBC (triple negative breast cancer). To date, the former can be further divided into defined as Luminal A (oestrogen receptor (ER)+ and/or progesterone receptor (PR)+, HER2−), Luminal B (ER+ and/or PR+, HER2+), and HER2-enriched (ER−, PR− and HER2+) breast cancers,[4,5] most of which have been effectively controlled by using hormone and targeting therapies. The latter remains more difficult to control clinically,[6,7] and can only be treated with surgery plus adjuvant chemotherapy and radiotherapy. Thus, further research on the molecular pathogenesis of the triple negative breast cancer could help us identify and evaluate novel biomarkers for the assessment of treatment responses and prognosis and novel therapeutic strategies.

Breast cancer originates from mammary epithelial cells and the common feature of the epithelium is cell polarity, which includes the shape, structure, and function through the tight junctions, desmosomes, and adherens junctions.[8,9] Thus, establishment and maintenance of the epithelial polarity relies on three cell polarity complexes, ie, the partitioning defective (PAR) complex, the Scribble (SCRIB) complex, and the Crumbs (CRB) complex.[10] Indeed, previous studies demonstrated that apical polarity protein CRB3, a crumbs isoform, maintains epithelial polarity through tight junctions.[11-13] For example, *CRB3* knockout mice revealed that CRB3 was crucial for epithelial morphogenesis and that mice would die shortly after birth with cystic kidneys and proteinaceous debris throughout the lungs,[14] and the *CRB3* knockout mice had improper clearance of the airway, cystic kidney, and villus fusion of the intestinal epithelium.[15] Other recent studies showed that CRB3 could also act as a tumour suppressor in epithelial cells, eg, knockout of CRB3 promoted tumourigenesis potential of cultured epithelial cells and tumour cell metastasis in nude mice.[16,17] Moreover, reduced CRB3 expression induced an increase in the level of cytoplasmic β-catenin in intestinal epithelial cells and β-catenin was accumulated in most cases of colon cancers,[15] while disruption of CRB complex predisposed TGF-β-mediated epithelial-to-mesenchymal transitions (EMT).[18] However, restoration of CRB3 expression re-established cell–cell junctions, cell polarity, and contact growth inhibition, but inhibited tumour cell migration and metastasis.[17] Recently, comparison of transcriptomic and comparative genomic hybridization analysis (CGH) data revealed that *CRB3* is localized at chromosome 19 and CRB3 expression was upregulated in male lactotroph tumours,[19] indicating that aberrant CRB3 expression could associate with tumorigenesis.

In this study, we analyzed expression of CRB3 protein in breast cancer tissues to associate with tumour cell phenotypes and therefore, to identify the role of CRB3 in development and progression of breast cancer.

## 2 | RESULTS

### 2.1 | Association of CRB3 protein expression with TNBC

Our previous study revealed that CRB3 expression was lower in breast cancer compared with that of normal mammary gland tissues.[20] In this study, we detected CRB3 expression in 192 invasive human breast cancers (Table 1) immunohistochemically. The data showed that CRB3 protein was expressed in 65/192 (33.8%) cases of the cytoplasm of tumour cells (Figure 1). We then associated CRB3 expression with clinicopathological data from patients and found that CRB3 expression was associated with ER+/PR+, but inversely with TNBC phenotype, ie, 41.1% (39/95) of ER+ breast cancer was CRB3-positive, whereas 26.9% (25/93) ER− tumour was CRB3-positive ($P = 0.046$; Table 1 and Figure 1A). Moreover, 47.6% (30/63) of PR+ breast cancer was CRB3-positive vs 28.4% (33/116) PR− tumours positive for CRB3 ($P = 0.013$; Table 1 and Figure 1B). In addition, 40.1% (27/66) of ER+/PR+ tumours were CRB3-positive, but only 22.4% (19/85) of TNBC showed CRB3 expression ($P = 0.048$; Table 1 and Figure 1C). However, CRB3 expression was not associated with the age of patients, tumour size, lymph nodal metastasis, pathological grade, or clinical stage (Table 1).

### 2.2 | Association of CRB3 mRNA level with TNBC using Molecular Taxonomy of Breast Cancer International Consortium (Metabric) and TCGA datasets

To validate our data on the association of CRB3 expression with TNBC, we downloaded these two datasets and analyzed accordingly. We found that levels of CRB3 mRNA were higher in ER+ than ER− breast cancer in both Metabric ($P = 2.145932e-09$) and TCGA datasets ($P = 2.919621e-08$; Figure 2A) and was higher in PR+ than PR− breast cancer in Metabric ($P = 4.41747e-08$) and TCGA datasets ($P = 8.92533e-05$; Figure 2B). Overall, levels of CRB3 mRNA were higher in non-TNBC than TNBC in both Metabric ($P = 3.682213e-10$) and TCGA datasets ($P = 2.500548e-07$; Figure 2C). These data further supported our own analysis of CRB3 protein (Table 1).

### 2.3 | Association of CRB3 expression with TNBC phenotype in breast cancer cell lines

To confirm our ex vivo data, we assessed CRB3 expression in different breast cancer cell lines and found that CRB3 expression was higher in luminal (MCF7 and T47D) than in HER2 (MDA-MB-453 and SK-BR-3) and basal (MDA-MB-231 and BT-549) breast cancer cell lines ($P = 0.0001$; Figure 3A,B).

**TABLE 1** Association of CRB3 expression with clinicopathological data from breast cancer patients (n = 192)

| Characteristics | N | CRB3 expression (IHC) Negative (n = 127) | Positive (n = 65) | P |
|---|---|---|---|---|
| Age (y) | 190 | | | 0.61[a] |
| ≥50 | 131 | 85 (64.9%) | 46 (35.1%) | |
| <50 | 59 | 41 (69.5%) | 18 (30.5%) | |
| Tumour size | 183 | | | 0.35 |
| 1 | 68 | 43 (63.2%) | 25 (36.8%) | |
| 2 | 106 | 76 (71.7%) | 30 (28.3%) | |
| 3 | 7 | 6 (85.7%) | 1 (14.3%) | |
| 4 | 2 | 2 (100.0%) | 0 (0%) | |
| Nodal status | 177 | | | 0.16 |
| N0 | 99 | 63 (63.6%) | 36 (36.4%) | |
| N1 | 28 | 22 (78.6%) | 6 (21.4%) | |
| N2 | 41 | 28 (68.3%) | 13 (31.7%) | |
| N3 | 9 | 6 (66.7%) | 3 (33.3%) | |
| Pathological grade | 152 | | | 0.98[a] |
| I | 1 | 1 (100.0%) | 0 (0%) | |
| II | 124 | 83 (66.9%) | 41 (33.1%) | |
| III | 27 | 19 (70.4%) | 8 (29.6%) | |
| Clinical stage | 173 | | | 0.059 |
| 1 | 39 | 20 (51.3%) | 19 (48.7%) | |
| 2 | 85 | 62 (72.9%) | 23 (27.1%) | |
| 3 | 52 | 35 (67.3%) | 17 (32.7%) | |
| ER | 188 | | | 0.046[a] |
| Negative | 93 | 68 (73.1%) | 25 (26.9%) | |
| Positive | 95 | 56 (58.9%) | 39 (41.1%) | |
| PR | 179 | | | 0.013[a] |
| Negative | 116 | 83 (71.6%) | 33 (28.4%) | |
| Positive | 63 | 33 (52.4%) | 30 (47.6%) | |
| HER2 | 170 | | | 0.65[a] |
| Negative | 146 | 96 (65.8%) | 50 (34.2%) | |
| Positive | 24 | 14 (58.3%) | 10 (41.7%) | |
| Molecular phenotype | 181 | | | 0.096 |
| Luminal A | 74 | 46 (62.2%) | 28 (37.8%) | |
| Luminal B | 15 | 8 (53.3%) | 7 (46.7%) | |
| HER2 | 7 | 5 (71.4%) | 2 (28.6%) | |
| TN | 85 | 66 (77.6%) | 19 (22.4%) | |

Abbreviations: ER, oestrogen receptor; PR, progesterone receptor; TNBC, triple negative breast cancer.
[a]Fisher's exact test.

### 2.4 | ERα upregulation of CRB3 protein levels in MCF7 and BT-549 cells

Thus far, our current study demonstrated that CRB3 expression was associated with TNBC phenotypes ex vivo and in vitro. We speculated that ERα could be a novel regulator of CRB3 since ERα is a nuclear transcription factor,[21] and consideration of ER could also regulate PR expression.[22-26] Our data showed that knockdown of ERα expression in luminal cell MCF7 breast cancer cells significantly downregulated levels of CRB3 mRNA and protein (Figure 4A,B). Additionally, CRB3 protein was significantly reduced in MCF7 cells after knockdown of ERα expression in the GEO database (Figure 4C; $P < 0.0001$, GSE27473). CRB3 expression was higher in ER+ breast cancer ZR-75-1 cells than in ER− breast cancer MDA-MB-231 cells isolated from the mouse bone marrow (Figure 4D; $P = 0.0006$, GSE27515). Furthermore, there was an association of ERα and CRB3 expression in the ex vivo GEPIA dataset (gepia.cancer-pku.cn/index.html; $P = 3.6e\text{-}15$, $R = 0.24$; Figure 4E). In addition, we detected CRB3 expression after ERα was overexpressed in TNBC cell line BT-549 and found that levels of CRB3 protein increased after ERα overexpression, while CRB3 mRNA levels were not significantly altered (Figure 4F). Therefore, we speculated that ERα indirectly regulates CRB3 by stabilization of CRB3 protein (Figure 4G). Our findings indicate that ERα could be a novel regulator for CRB3 expression in breast cancer.

## 3 | DISCUSSION

Despite dedicated research efforts, triple negative breast cancer still lacks effective treatment options for the clinical control of TNBC progression and improvement of the survival of patients.[6,7] Thus, a better understanding of TNBC development and molecular pathogenesis could help us discover novel gene alterations that regulate TNBC. In the current study, we focused on CRB3, which regulates epithelial cell tight junctions and maintains epithelial polarity, an important force against epithelial cell carcinogenesis.[11-13,16,17] We found that levels of both CRB3 mRNA and protein were associated with TNBC phenotypes ex vivo and in vitro. Furthermore, knockdown of ERα expression in luminal cell MCF7 breast cancer cells significantly reduced expression of CRB3 mRNA and protein in our experiments and in online GEO data. In addition, ERα expression was associated with CRB3 expression in breast cancer tissues specimens. Our findings indicate that ERα could be a novel regulator for CRB3 expression in breast cancer.

Treatment of TNBC patients generally only includes surgical resection with adjuvant chemotherapy and radiotherapy, whereas there are more options for treatment of receptor-positive invasive breast cancer including hormone, immune, and targeting therapy, all of which depend on the stage of the disease, the age of patients, molecular typing, and other clinical factors.[27-30] However, TNBC may occur more frequently in cases that include younger ages, more aggressive cancers, larger tumour sizes, higher grades, and metastasis,[31,32] recurrence, and poor prognoses.[33] These factors make TNBC cancers more difficult to control.[34,35] In our current study, we sought to identify novel targets for the future control of TNBC and found that CRB3 expression was significantly lost in TNBC tissues and cell lines, but the molecular events responsible for loss of CRB3 expression in human breast tumours remain to be determined. Our previous study revealed that CRB3 expression was lower in breast

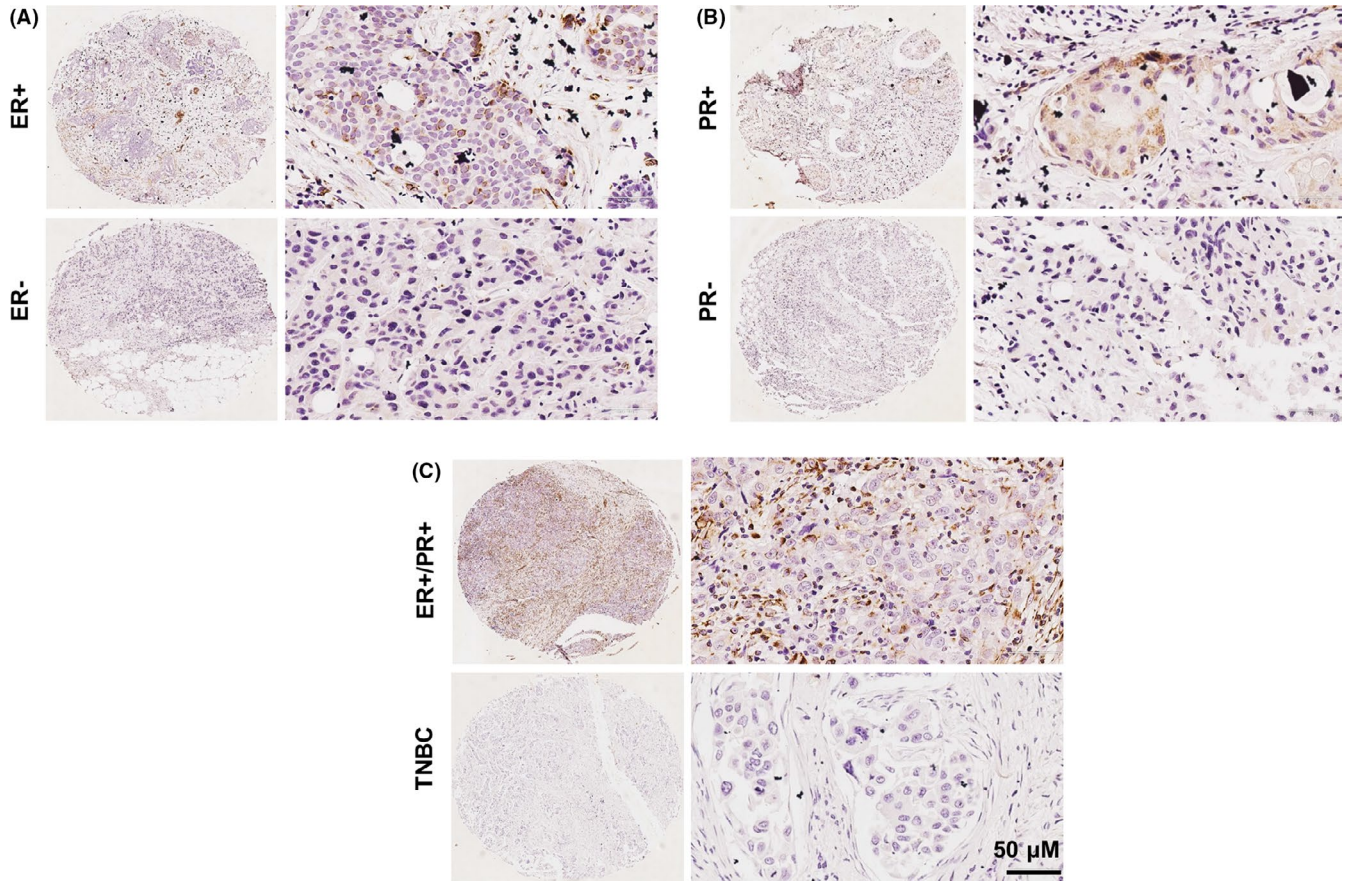


**FIGURE 1** Immunohistochemical detection of CRB3 protein. The TMA sections containing 192 cases of invasive breast cancer were immunostained with CRB3 antibody and quantified. Representative fields of view (FOV) of the TMA cores shows CRB3 expression. A, CRB3 expression in ER+ vs ER− breast cancer. B, CRB3 expressions in PR+ vs PR- breast cancer. C, CRB3 staining in the ER+/PR+ vs TNBC

cancer compared with that of normal mammary gland tissues,[20] while Varelas et al[18] reported that disruption of the CRB complex predisposed TGF-β-mediated mouse mammary epithelial epithelial-mesenchymal transition.

Furthermore, our current study is the first to assess the association between CRB3 and ER and found that ERα knockdown significantly reduced CRB3 expression in breast cancer cells and that ERα expression was associated with CRB3 expression in breast cancer tissue specimens, indicating that ERα is a novel regulator for CRB3 expression and reduced CRB3 expression may contribute to occurrence of ER-negative breast cancer. Our current data also suggest that ERα can post-transcriptionally upregulate CRB3 expression in BT-549 cells. We then analyzed *CRB3* using the tools in the UCSC website (http://genome.ucsc.edu/) and found that there is no ERα binding site in the *CRB3* promoter region. ERα, as a member of the nuclear hormone receptor superfamily,[36] is one of the estradiol (E2)-activated transcription factors to regulate gene expression that are related to cell proliferation, differentiation, and migration.[37,38] Furthermore, ERα can upregulate PR and HER2 expression.[39,40] Our current data on ERα upregulation of CRB3 is novel. However, it remains to be determined how ERα regulates CRB3 expression and what kinds of cell functions are associated with this regulation. What we now know is that ERα expression upregulates cell proliferation and ERα overexpression was associated with the development of breast cancer,[41] whereas CRB3 expression, which functions to suppress cancer development,[11-13,16,17] was lost in invasive breast cancer. It remains to be defined whether the reduction in CRB3 expression is due to a lack of ERα expression in TNBC and whether reduced CRB3 contributes to TNBC pathogenesis. A previous study reported that ERα expression was negatively associated with the progressive grade of invasive breast cancer.[42] Moreover, ERα expression was inversely associated with tumour cell EMT and cancer stem-like cell phenotypes.[43,44]

In the current study, we found that CRB3 expression was inversely associated with tumour size (Table 1), which is consistent with previous studies showing that downregulation of CRB3 expression induced breast cancer cell proliferation, whereas CRB3 overexpression inhibited proliferation of human breast cell lines.[20,45,46] Our previous study also showed that the downregulation of CRB3 expression increased migration and invasion of breast cancer cells, whereas CRB3 overexpression inhibited migration and invasion of human breast and kidney cancer cell lines.[20,47] Our previous study also showed that CRB3 expression was an independent favourable predictor for renal clear cell carcinoma.[47]

In summary, our current study is a proof-of-principle. The data from our current study demonstrated that expression of CRB3

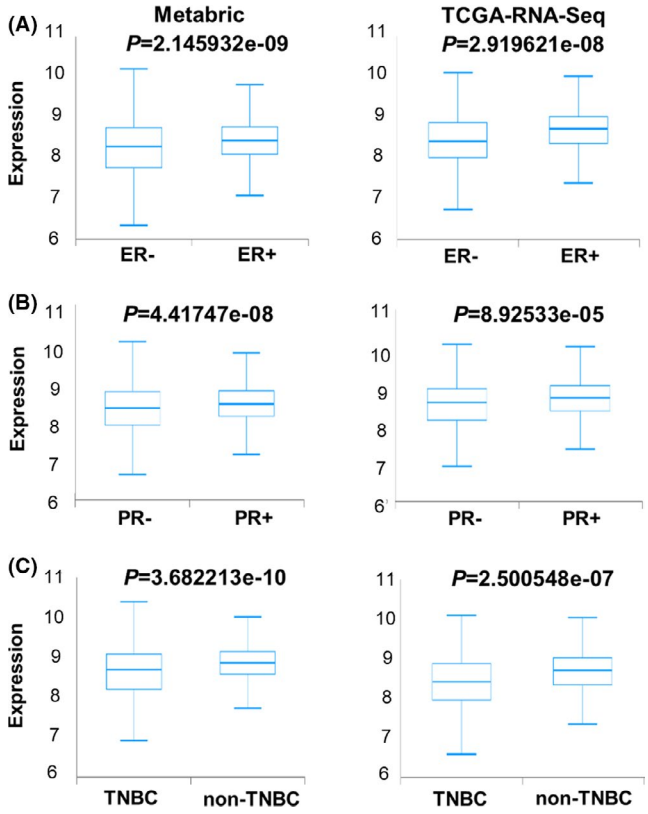


**FIGURE 2** Expression of CRB3 mRNA in breast cancer. The box plots and *P* values were generated by using BCIP (http://www.omicsnet.org/bcancer/)

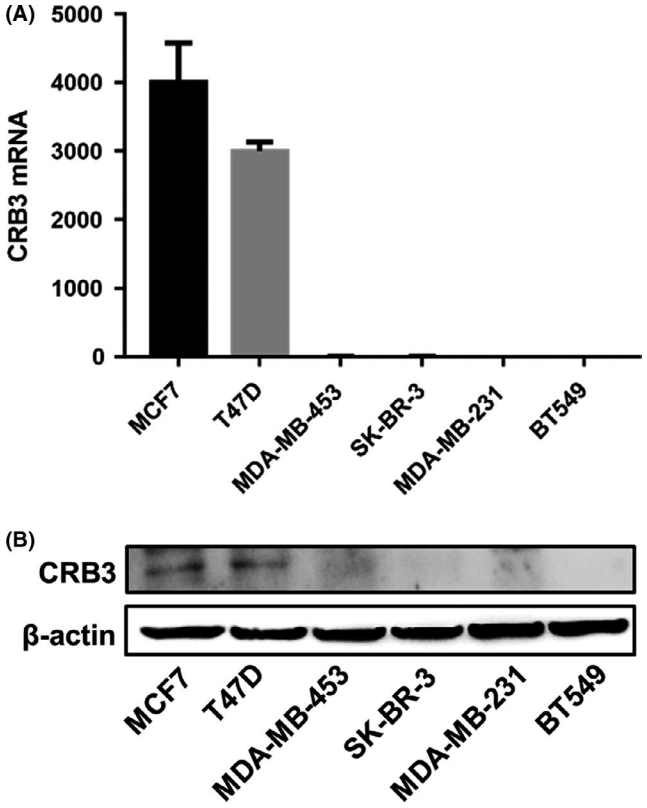


**FIGURE 3** CRB3 expression in breast cancer cell lines. A, Expression analysis of CRB3 mRNA in six breast cancer cells detected by using qRT-PCR. B, Western blot. Expressions of CRB3 mRNA in six breast cancer cells detected by using western blot

mRNA and protein was reduced in breast cancer, which was associated with TNBC phenotypes ex vivo and in vitro. Knockdown of ERα expression significantly reduced CRB3 expression in breast cancer cells and ERα expression was associated with CRB3 expression in breast cancer tissues specimens.

## 4 | MATERIALS AND METHODS

### 4.1 | Immunohistochemical analysis (IHC)

This study was approved by the ethics committee of human subject research of The First Affiliated Hospital, Xi'an Jiaotong University (Xi'an, China). The TMAs were obtained from Shanghai Outdo Biotech Co., Ltd. (#HBreD140Su03 and HBre-Duc052Bch-01). The HBre-Duc052Bch-01 TMAs contained 52 TNBC tissues, while the HBreD140Su03 TMAs contained 140 breast cancer tissues. All patients were histologically diagnosed with invasive breast cancer and their clinicopathological data were retrospectively retrieved from their medical records (Table 1). Tissue specimens were obtained from surgical resection of tumour lesions before any chemoradiation therapy.

TMA sections were deparaffinized and rehydrated into water and immunostained with an anti-CRB3 antibody using an immunostaining kit (#SP-9001; Beijing Zhongshan Golden Bridge Biotechnology Co. Beijing, China) according to the manufacturer's instructions. The anti-ER (Cat. #187260); PR (#32085) and HER2 antibodies (#245702) were purchased from Abcam. In brief, the TMA sections were microwaved in 0.01 mol/L sodium citrate (pH 6.0) in a microwave oven (100 W for 6 minutes and 50 W for 13 minutes) to repair antigens and subsequently in 3% $H_2O_2$ solution for 10 minutes to block the potential endogenous peroxidase activity. After that, the sections were incubated with the blocking solution (20% normal goat serum) at room temperature for 50 minutes and then with a primary antibody anti-CRB3 (#HPA013835; 1:200; Sigma-Aldrich) at 4°C overnight. On the next day, the sections were washed with phosphate buffered saline (PBS) and then incubated with the immunostaining kit (#SP-9001; Beijing Zhongshan Golden Bridge Biotechnology Co.) at room temperature for 1 hour in the dark and subsequently developed the colour reaction using the 3,3′-diaminobenzidine (DAB) solution and counterstained with hematoxylin. Immunostained TMA sections were reviewed and scored under a Leica microscope (SCN 400; Leica, Wetzlar, Germany). Each tissue core of the TMA sections was scored by a pathologist twice on three different microscopic fields each time in a blinded fashion, ie, the staining intensity was scored as 0 (negative), 1 (weakly positive), 2 (moderately positive), or 3 (strongly positive), while percentage (%) of staining was scored as 0 (<10%), 1 (10%–40%), 2 (40%–70%), or 3 (>70%). The staining index was reached by combination of these two scores and the staining index between 0 and 2 was considered a negative case, whereas the staining index between 3 and 6 was a positive case.

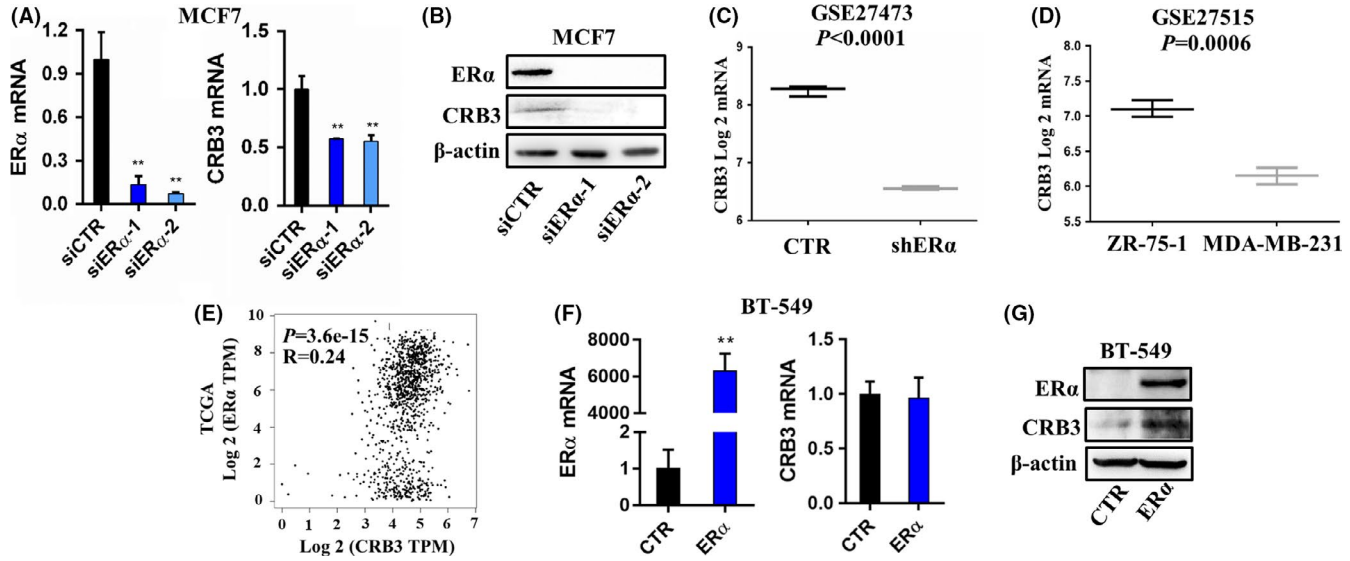


**FIGURE 4** Regulation of CRB3 expression after knockdown of ERα expression in MCF7 cells. A, Levels of ERα and CRB3 mRNA in MCF7 cells after knockdown of ERα expression were assessed using qRT-PCR. B, Levels of ERα and CRB3 proteins in MCF7 cells after knockdown of ERα expression were assessed using western blot. C, Association of ERα and CRB3 expression in MCF7 cells. The box plot of CRB3 mRNA levels in pre- and post-ERα-silenced MCF7 cells. D, CRB3 expression in ER positive breast cancer cell ZR-75-1 and ER negative breast cancer cell MDA-MB-231 isolated from the mouse bone marrow using qRT-PCR. E, Association of ERα and CRB3 expression in breast cancer tissues. Expression of ERα and CRB3 was positively correlated in breast cancer tissues utilizing GEPIA (gepia.cancer-pku.cn/index.html) dataset. F, Levels of ERα and CRB3 mRNA in BT-549 cells after overexpression of ERα expression were assessed using qRT-PCR. G, Levels of ERα and CRB3 proteins in BT-549 cells after overexpression of ERα expression were assessed using western blot. $^{**}P < 0.01$. CTR, negative control

## 4.2 | Breast cancer integrative platform (BCIP)

In this study, we utilized BCIP from the website (http://www.omicsnet.org/bcancer/) to analyze and visualize gene expression in breast cancer tissue specimens from patients. BCIP data are derived from the European Bioinformatics Institute of European Molecular Biology Laboratory (EMBL-EBI), The Cancer Genome Atlas (TCGA), and Gene Expression Omnibus (GEO) datasets. It has been characterized by multi-omic integrated analysis types (transcriptome, copy number variation, microRNA, pathway and gene functional network analysis), and dividing the breast cancer samples into several subgroups according to histopathological features and clinical information.[48] We retrieved data from BCIP and analyzed CRB3 expression data and its association with clinicopathological features.

## 4.3 | Cell culture, RNA interference and transfection

Human breast cancer cell lines MCF7, T47D, MDA-MB-453, SK-BR-3, MDA-MB-231, and BT-549 were obtained from the National Infrastructure of Cell Line Resource. MCF7, MDA-MB-453, SK-BR-3 and MDA-MB-231 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, USA) supplemented with 10% fetal bovine serum (FBS; HyClone). T47D cells were cultured in Roswell Park Memorial Institute medium (RPMI)-1640 (HyClone) supplemented with 10% FBS (HyClone) and 0.2 units/mL of bovine insulin (#I-1882, Sigma-Aldrich) and BT549 cells were cultured in RPMI-1640 (HyClone, Logan, USA) supplemented with 10% FBS (HyClone) and 0.023 IU/mL bovine insulin (Sigma-Aldrich, St Louis, MO, USA) in a humidified atmosphere containing 5% $CO_2$ at 37°C.

ERα siRNA to knockdown ERα expression was purchased from GenePharma Company and the ERα siRNA sequences were: ERα-1, 5′-GCA UUC UAC AGG CCA AAU UTT-3′ and ERα-2, 5′-GGA UUU GAC CCU CCA UGA UTT-3′. These siRNAs and negative control siRNAs were transiently transfected into breast cancer cells in 6-cm plates for 48 hours using 166 pmol siRNA and 17 μL of Lipofectamine 2000 (Invitrogen) in 250 μL of Opti-MEM medium (Invitrogen).

Lentivirus carrying ERα cDNA was were purchased from GeneChem Company, which then infected BT-549 cells in the presence of 5 μg/mL of polybrene for 72 hours and maintained in the growth medium containing 2 μg/mL of puromycin to generate stable sublines.

## 4.4 | Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated from cells using the RNA Fast 200 (#220010, Fastagen Biotech, Shanghai, China) and reversely transcribed into cDNA using the PrimeScript™ RT Master Mix (#RR036A, TaKaRa Biotechnology [Dalian] Co., Dalian, China) according to the manufacturers' protocols. qPCR was amplified using the TB Green™ Premix Ex Taq™ II (#RR820A, TaKaRa) using primers in Bio-Rad CFX96 system according to the manufacturer's instructions. The primers used were: ERα, 5′-TCC GGC ACA TGA GTA ACA AA-3′ and 5′-TGA AGA CGA TGA GCA TCC AG 3′; CRB3, 5′-CTT CTG CAA ATG AGA ATA

GCA CTG-3′ and 5′-GAA GAC CAC GAT GAT AGC AGT GA-3′; β-actin, 5′-CAT GTA CGT TGC TAT CCA GGC-3′ and 5′-CTC CTT AAT GTC ACG CAC GAT-3′. The experiment was performed in triplicate and level of CRB mRNA was normalized to β-actin using the $2^{-(Ct-Ct)}$ method.

## 4.5 | Western blot

Whole-cell lysates were prepared using a modified RIPA buffer and protein samples were separated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE) gels and transferred onto PVDF membranes (Millipore). We then followed a standard Western blotting protocol to blot these membranes using an anti-CRB3 antibody (#292449; 1:500; Santa Cruz Biotechnology, Dallas, USA), anti-ERα antibody (#75635; 1:1000; Abcam, Cambridge, USA) or anti-β-actin antibody (AC026; 1:10 000; ABclonal, Boston, USA) and the horseradish peroxidase (HRP)-conjugated secondary antibody (7074, Cell Signaling Technology, Beverly, USA). The chemiluminescent signals were visualized by using the ECL Plus (Millipore, Temecula, USA) and detected by ChemiDoc™ XRS+ (Bio-Rad, Hercules, USA.

## 4.6 | Statistical analysis

Statistical analyses were performed using GraphPad Prism Version 7.0 (GraphPad Software). The correlation between CRB3 expression and clinicopathological characteristics was assessed by using Fisher's exact test or the chi-square test, while in vitro data between two groups were assessed by using unpaired *t* tests and Pearson correlation coefficient tests, while the data on three groups were compared using one-way analysis of variance (ANOVA) followed by using Dunnett's multiple comparisons test. All statistical tests were two-sided and all results were expressed as the mean ± SEM for $^{*}P < 0.05$ or $^{**}P < 0.01$ All in vitro data were obtained from at least three experimental replicates with similar results.

## ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Natural Science Foundation of China (#81702631 and #81502413) and Natural Science Basic Research Project of Shaanxi Province (#2017JM8015). This manuscript has been edited and proofread by Medjaden Bioscience Limited (Hong Kong, China).

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**How to cite this article:** Li P, Zhou C, Yan Y, et al. Crumbs protein homolog 3 (CRB3) expression is associated with oestrogen and progesterone receptor positivity in breast cancer. *Clin Exp Pharmacol Physiol*. 2019;46:837–844. https://doi.org/10.1111/1440-1681.13104