CHFR-mediated epithelial-to-mesenchymal transition promotes metastasis in human breast cancer cells

GUOBIN JIANG, HONGYAN FANG, XI SHANG, XIAOPIN CHEN and FEILIN CAO

Department of Thyroid and Breast Surgery, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, Luqiao, Zhejiang 318050, P.R. China

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Abstract. Checkpoint with FHA and RING finger domains (CHFR) is a G_2 phase/mitosis checkpoint. Several studies have reported that CHFR is downregulated in multiple cancer types and serves a tumor suppressor role. However, the biological function of CHFR in breast cancer (BRCA), particularly regarding metastasis, are yet to be elucidated. In the present study, it was revealed that CHFR is upregulated in BRCA compared with normal tissues, according to The Cancer Genome Atlas database. In addition, subgroup analysis of BRCA revealed that CHFR was upregulated in both human epidermal growth factor receptor 2-positive and triple-negative BRCA. Meanwhile, patients with high expression levels of CHFR exhibited poorer overall survival rates. Furthermore, the present data revealed that the overexpression of CHFR in SKBR3 cells resulted in enhanced cell migration and invasiveness, and also significantly upregulated mesenchymal markers, such as N-cadherin, vimentin, transcription factor Slug and tight junction protein claudin-1. Furthermore, knockdown of CHFR in MDA-MB-231 cells significantly inhibited cell migration and invasiveness, and also downregulated mesenchymal markers, such as N-cadherin, vimentin and tight junction protein claudin-1. In conclusion, the current results indicated that CHFR expression was associated with cell metastasis in BRCA by mediating epithelial-to-mesenchymal transition.

Introduction

Worldwide, breast cancer (BRCA) is the second most common cause of cancer-associated mortality in women, and it has a high incidence rate in China (1,2). Triple negative breast cancer (TNBC) refers to a type of BRCA where patients lack human epidermal growth factor receptor 2 (HER2), ER estrogen receptors (ER) and progesterone receptors (PR), and this subtype is characterized by large visceral metastatic spread and increased rate of nodal invasion (3). Due to the metastasis of BRCA, particularly in TNBCs, the prognosis remains poor. Therefore, it is necessary to further identify the molecular mechanism underlying metastasis in BRCA, particularly in TNBCs.

Checkpoint with FHA and RING finger domains (CHFR) serves a key role in regulating the cell cycle by regulating the transition to metaphase in reaction to microtubule stress (4). In a previous study, CHFR was revealed to be significantly downregulated by promoter methylation or mutation in gastric cancer (5), human non-small cell lung carcinoma (NSCLC) (6), and esophageal (7) and colorectal cancer (8). However, aberrant hypermethylation of the CHFR promoter is uncommon in primary BRCA (9). However, the role of CHFR in metastasis in BRCA is yet to be characterized.

Cancer cell metastasis is a multistep process involving proliferation, epithelial-to-mesenchymal transition (EMT), migration and invasion (10,11). EMT was originally considered to be a growth-like process, during which epithelial cells exhibit a migratory and invasive mesenchymal phenotype (12). A hallmark of EMT is the functional loss of the epithelial maker E-cadherin and the upregulation of the mesenchymal markers N-cadherin, vimentin and fibronectin (13).

In the present study, according to The Cancer Genome Atlas (TCGA) database, CHFR is upregulated in BRCA tissues compared with normal tissues. In addition, subclass analysis of BRCA revealed that CHFR is upregulated in $HER2^+$ and TNBC. Notably, patients with higher levels of CHFR exhibited poorer overall survival rates. However, the biological function of CHFR on the metastasis of BRCA is yet to be elucidated. The current data revealed that overexpression of CHFR in SKBR3 cells resulted in enhanced migratory and invasive abilities, and also significant upregulation of mesenchymal markers, such as N-cadherin, vimentin, transcription factor Slug and tight junction protein claudin-1. Furthermore, knockdown of CHFR in MDA-MB-231 cells significantly inhibited migratory and invasive abilities, and also downregulated mesenchymal markers, such as N-cadherin, vimentin and tight junction protein claudin-1. In conclusion, the current results indicated that CHFR enhanced cell metastasis in BRCA

Correspondence to: Professor Feilin Cao, Department of Thyroid and Breast Surgery, Taizhou Hospital of Zhejiang Province Affiliated to Wenzhou Medical University, 1 Tongyang Road, Luqiao, Zhejiang 318050, P.R. China
E-mail: caofl@enzemed.com

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by mediating EMT. Moreover, the present study indicated that CHFR may provide a potential therapeutic target for metastatic BRCA treatment.

Materials and methods

Cell culture. All BRCA cell lines cells (SKBR3, MDA-MB-231 and MCF-7) were purchased from the American Type Culture Collection. All cells were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Hyclone; Cytiva), 2 mM L-glutamine (Gibco; Thermo Fisher Scientific, Inc.), 1% penicillin (100 U/ml) and streptomycin (100 μg/ml) (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37˚C, 5% CO_2 in a humidified incubator and passaged at ≥80% confluence using trypsin (Gibco; Thermo Fisher Scientific, Inc.).

Western blotting. Cells were lysed in RIPA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The supernatants of lysates were collected and concentrations of protein were quantified with the Protein Quantitative Kit (TransGen Biotech Co., Ltd.) using a microplate reader (Molecular Devices, LLC). Then, ~50 μg protein was loaded onto a 10% gel, and separated via SDS-PAGE, then separated proteins were transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat milk at room temperature for 2 h, and then incubated at 4˚C overnight with primary antibodies against CHFR (cat. no. 904S; 1:1,000), N-cadherin (cat. no. 13116; 1:1,000), β-catenin (cat. no. 8480; 1:1,000), vimentin (cat. no. 5741; 1:1,000), Snail (cat. no. 3879; 1:1,000), Slug (cat. no. 9585; 1:1,000), claudin-1 (cat. no. 4933; 1:1,000) and E-cadherin (cat. no. 3195; 1:500), all from Cell Signaling Technology, Inc., as well as β-actin (cat. no. 2228; 1:5,000), which was purchased from Sigma-Aldrich (Merck KGaA). To determine transfection efficiency following CHFR knockdown, a different antibody against CHFR was used (cat. no. 12169-1-AP; 1:500), which was purchased from ProteinTech Group, Inc. The corresponding anti-rabbit IgG (cat. no. HS101-01; 1:2,000) and anti-mouse IgG (cat. no. HS201-01; 1:2,000) horseradish peroxidase (HRP)-conjugated secondary antibodies (TransGen Biotech Co., Ltd.) was added and incubated at room temperature for 1 h. Signals were visualized after an electrochemiluminescence reaction with HRP substrate (cat. no. P0018S; Beyotime Institute of Biotechnology) and semi-quantified using ImageJ (version 1.52v; National Institutes of Health).

Transfection and RNA interference of CHFR. Small interfering (si)RNAs targeting CHFR (5'-CACCACGCCAUG AAAUUCATT-3') and non-targeting siRNA negative controls (5'-UUCUCCGAACGUGUCACGU-3') were obtained from Santa Cruz Biotechnology, Inc. MDA-MB-231 cells were seeded into a 6-well plate at $1x10^5$ and transfected with 4.0 μg siRNA using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Prior to any treatment, cells were incubated for 24 h and the transfection efficiency of the siRNA was determined via western blotting.

Plasmid construction and transfection. The coding sequences of human CHFR mRNA were synthesized and subcloned into the pcDNA3.1 vector (cat. no. 128034; Addgene, Inc.) to construct the CHFR overexpression plasmid. The integrity of the respective plasmid constructs was confirmed via DNA sequencing. When SKBR3 cells reached 75% confluency in the 6-well plate, cells were used to overexpress CHFR. A complex was formed between the 4.0 μg plasmid and Lipofectamine for 20 min at room temperature, and transfection was carried out at 37˚C for 24 h. Then, 800 μg/ml G418 was used to select cells transfected with pcDNA3.1 and CHFR overexpression plasmid for 48 h. Subsequently, the cells were cultured with 400 μg/ml G418 for maintenance. The cells transfected with pcDNA3.1 vector and CHFR plasmid were defined as the control group and CHFR group, respectively.

In vitro migration and invasion assays. For the migration assay, Transwell inserts (24 wells; 8-μm pore size; poly-carbonate membrane; Corning, Inc.) were used according to the manufacturer's protocol. Cells were transfected with plasmids (pcDNA3.1 and CHFR plasmids) and siRNA (siR-control and siR-CHFR), and the cells were seeded into the upper chambers at $1x10^5$/chamber and cultured in serum-free DMEM. The lower compartment was filled with DMEM, with 10% FBS used as a chemoattractant. After incubation for 24 h, cells remaining in the upper chamber were removed, and cells at the bottom of the insert were fixed with 4% paraformaldehyde for 30 min at room temperature, stained in 0.5% crystal violet for 20 min at room temperature and counted under a light microscope (magnification, x400; Olympus Corporation). The results were averaged over three independent experiments. For invasion assays, the inserts were coated with Matrigel (BD Biosciences) at 37˚C for 4 h before the cells were added. The proceeding steps were the same as migration assay.

Cell proliferation assay. After CHFR overexpression or silencing, cells at a density of 1,000/well were seeded in a 96-well plate and incubated for the indicated times (24, 48, 72 and 96 h). The medium was discarded and cells were incubated with 50 ml of 1 mg/ml MTT (Sigma-Aldrich; Merck KGaA) in PBS for up to 4 h at 37˚C. The purple formazan was then solubilized by DMSO and absorbance at 570 nm was read by a microplate reader (Molecular Devices, LLC).

Morphological analysis. Cells were transfected with pcDNA3.1 and pcDNA3.1-CHFR plasmids. Then, 48 h after transfection, the morphology of the cells was observed with an inverted microscope (CKX53; Olympus Corporation).

Survival analysis. The samples were divided into two groups based on the expression of CHFR. The expression of CHFR was listed in ascending order, the patients in whom expression of CHFR was <the median were defined as low expression groups; otherwise, the patients were defined as high expression groups. The clinical relevance of CHFR in patients with BRCA was analyzed using the UALCAN database (14) and Kaplan-Meier plotter (www.KMplot.com). The gene symbol chosen was CHFR (Affymetrix ID no.223931_s_at). Patients were split by auto select best cutoff, and to restrict the analysis

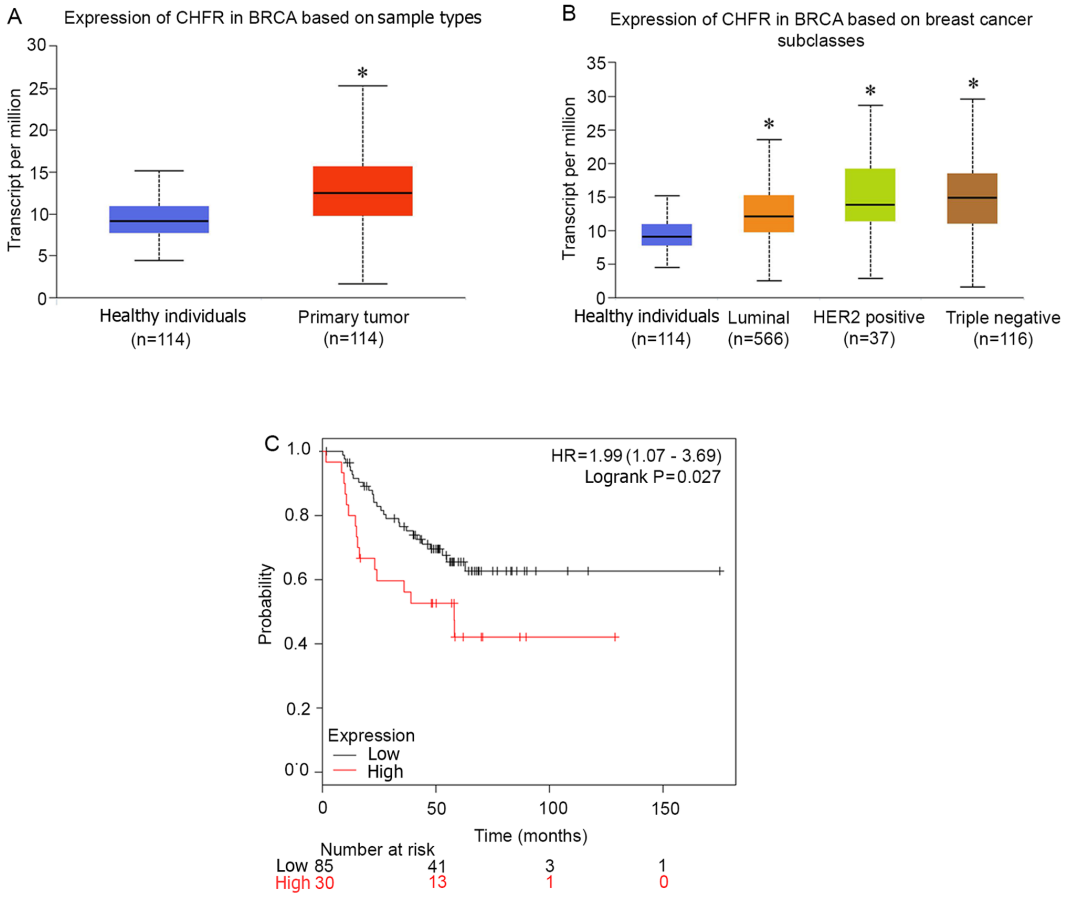

Figure 1. CHFR expression profiles and clinical relevance in BRCA. (A) Graph displaying the expression of CHFR in BRCA according to sample types. Data were obtained from TCGA database. (B) Graph describing the expression of CHFR in subclasses of BRCA. Data were obtained from TCGA database. (C) Overall survival curves according to the expression levels of CHFR in patients with TNBC, generated using data from the UALCAN database and a web application named Kaplan-Meier plotter. *P<0.05 vs. healthy individuals. BRCA, breast cancer; TNBC, triple negative breast cancer; TCGA, The Cancer Genome Atlas; CHFR, checkpoint with FHA and RING finger domains; HER2, human epidermal growth factor receptor 2.

into subtypes, patients negative for PR, HER2 and lymph node status were chosen. Then, the Kaplan-Meier plot was constructed, and the overall survival of patients with TNBC was obtained using a log-rank test.

Statistical analysis. All data are expressed as the mean ± SD from at least three independent experiments. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc.) and SPSS 13.0 (SPSS, Inc.) software packages. Statistical significance between two groups was determined using the two-sided Student's t-test, and for multiple group comparisons an ANOVA followed by Bonferroni's post hoc test was performed. P<0.05 was considered to indicate a statistically significant difference.

Results

CHFR expression analysis in BRCA dataset. Data from TCGA was used to determine the clinical relevance of CHFR expression in human BRCA, and the results revealed that CHFR mRNA was upregulated in BRCA tissues compared with normal tissues (Fig. 1A).

In addition, subgroup analysis of BRCA revealed that CHFR expression was upregulated in HER2$^+$ and TNBC types compared with the normal subclass (Fig. 1B). Notably, patients with higher levels of CHFR exhibited poorer overall survival rates in patients with TNBC (Fig. 1C). Taken together, these data indicated that CHFR is significantly upregulated in BRCA, and exerts a significant pro-tumor effect.

CHFR overexpression enhances migratory and invasive abilities of BRCA cells, and inhibits cell proliferation. To further investigate the role of CHFR in BRCA, three human BRCA cell lines were selected, and their basal expression of CHFR was detected via a western blot assay. As depicted in Fig. 2A, CHFR expression was higher in MAD-MB-231 cells, compared with MCF-7 and SKBR3 cells. Thus, SKBR3 cells were transfected with an expression plasmid of CHFR to study the biological role of CHFR.

Firstly, the transfection efficiency was investigated, and the results demonstrated that CHFR levels were significantly upregulated in SKBR3 cells that were transfected with a CHFR expression plasmid (Fig. 2B), and overexpression of CHFR significantly increased cell migration compared with the control group at 24 h (Fig. 2C and E). In addition, as displayed in Fig. 2D and E, overexpression of CHFR significantly increased the number of invaded cells compared with the control group at 24 h. Therefore, the current data demonstrated that CHFR positively regulates BRCA cell

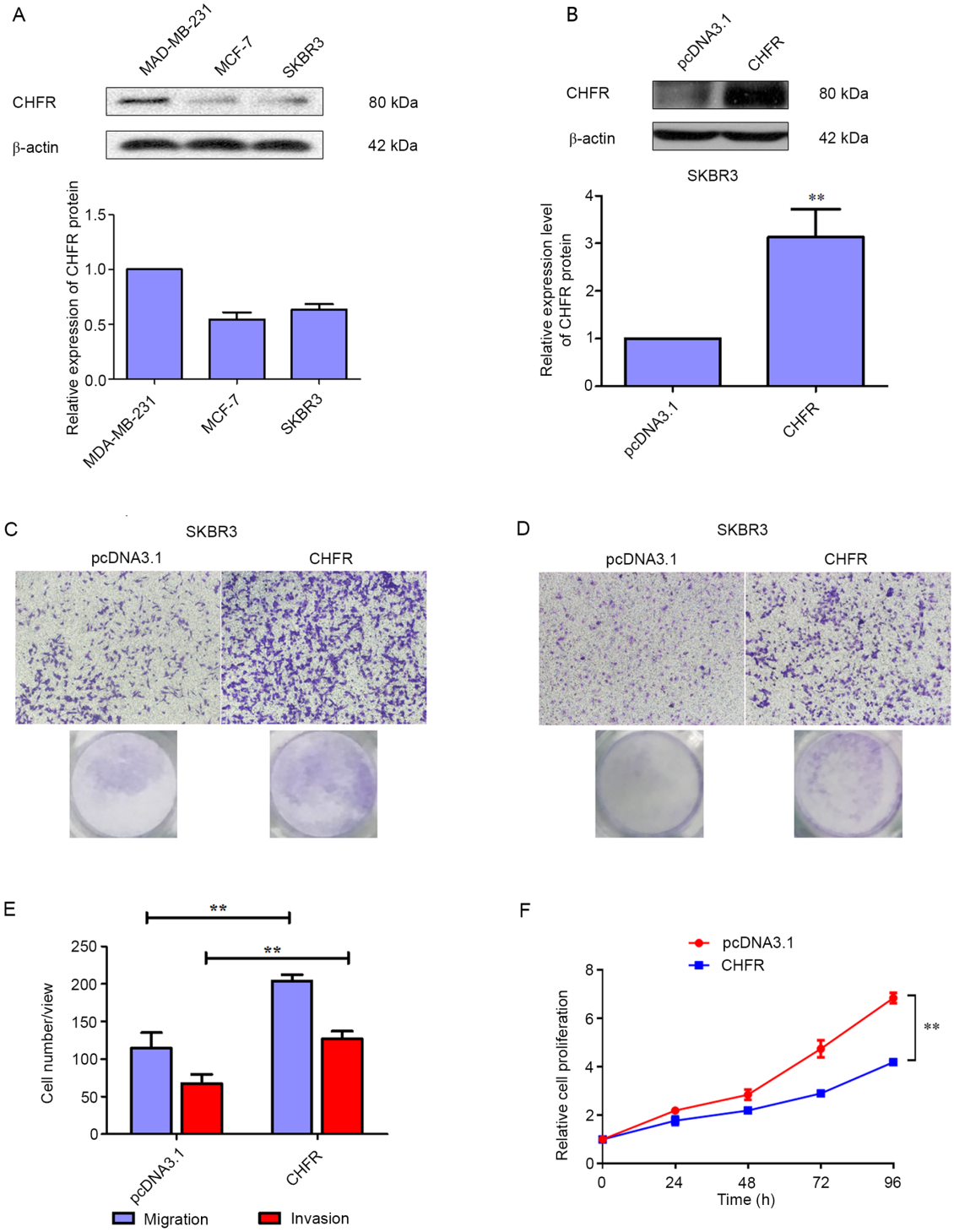

Figure 2. CHFR overexpression promotes migratory and invasive abilities of BRCA cells. (A) CHFR expression in human BRCA cell lines was detected via western blotting with anti-CHFR antibody. β-actin was used as a loading control. (B) Confirmation of transient CHFR overexpression in SKBR3 cells at the protein level. Representative images of (C) Transwell migration and (D) invasion assays. (E) Quantification of Transwell assays demonstrated that CHFR overexpression enhanced SKBR3 cell migration and invasion. (F) CHFR overexpression significantly suppressed cell proliferation. The data are derived from three independent experiments. **P<0.01 vs. pcDNA3.1 group. BRCA, breast cancer; CHFR, checkpoint with FHA and RING finger domains.

migration and invasion. However, CHFR overexpression significantly suppressed the proliferative activity of SKBR3 cells (Fig. 2F).

CHFR knockdown inhibits the migratory and invasive abilities of BRCA cells, and promotes cell proliferation. To further verify the effects of CHFR on migration and invasion in BRCA, MAD-MB-231 cells were transfected with siRNA to knockdown the expression of CHFR. Firstly, the transfection efficiency was evaluated and the results revealed that CHFR levels were significantly decreased in MAD-MB-231 cells that were transfected with CHFR siRNA (Fig. 3A), and knockdown of CHFR significantly reduced cell migration compared with the control group at 24 h (Fig. 3B and D). In addition, as

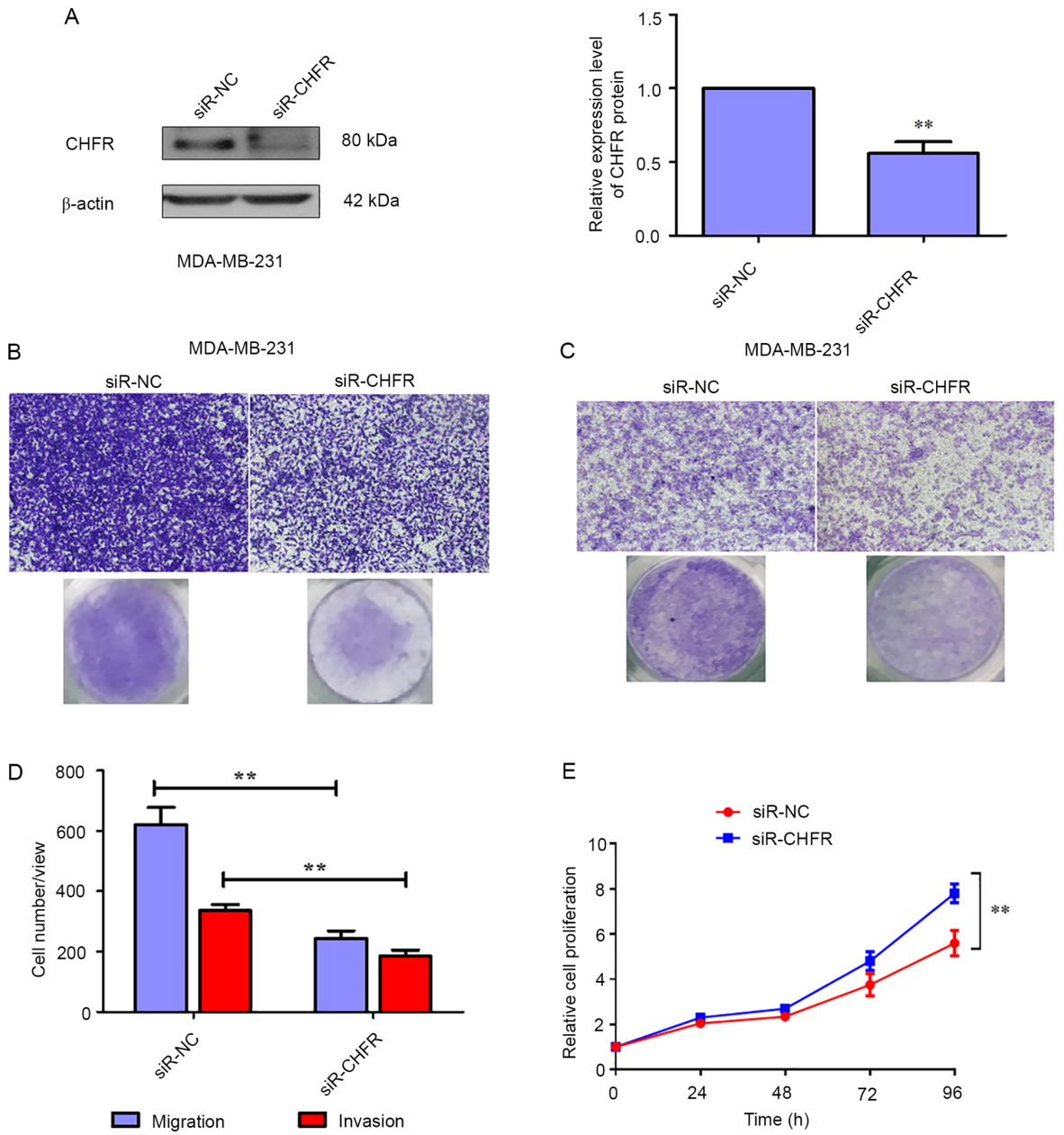

Figure 3. CHFR knockdown suppresses migratory and invasive abilities of BRCA cells. (A) Verification of transient CHFR knockdown in MDA-MB-231 cells via western blotting with anti-CHFR antibody. Representative images of (B) Transwell migration and (C) invasion assays. (D) Transwell assays demonstrated that CHFR knockdown suppressed MDA-MB-231 cell migration and invasion. (E) CHFR silencing significantly promoted cell proliferation. The data are derived from three independent experiments. **P<0.01 vs. siR-NC group. BRCA, breast cancer; CHFR, checkpoint with FHA and RING finger domains; siR, small interfering RNA; NC, negative control.

indicated in Fig. 3C and D, knockdown of CHFR significantly decreased cell invasion compared with the control group at 24 h. Therefore, the current data also demonstrated that knockdown of CHFR negatively regulated BRCA cell migration and invasion. On the other hand, CHFR knockdown promoted the proliferation of MDA-MB-231 cells (Fig. 3E).

CHFR may promote cell metastasis via EMT in BRCA cells. To investigate the underlying mechanisms behind the role of CHFR in the regulation of cell metastasis in BRCA cells, mesenchymal markers were examined, such as N-cadherin, vimentin, transcription factors Slug and Snail, and tight junction proteins E-cadherin, claudin-1 and β-catenin. Initially, as displayed in Fig. 4A ectopic expression of CHFR was evaluated, and the results revealed that overexpression of CHFR significantly upregulated the mesenchymal markers N-cadherin, vimentin and its transcription factor Slug, and tight junction protein claudin-1. But, CHFR overexpression significantly suppressed the expression of E-cadherin, an epithelial cell marker. Furthermore, using RNA interference technology, the expression of CHFR was knocked down, which resulted in the reduction of N-cadherin, vimentin and claudin-1 expression, and upregulation of the expression of epithelial marker E-cadherin (Fig. 4B). Finally, the morphological change of SKBR3 cells following CHFR overexpression was also examined. As shown in Fig. 4C, after CHFR overexpression,

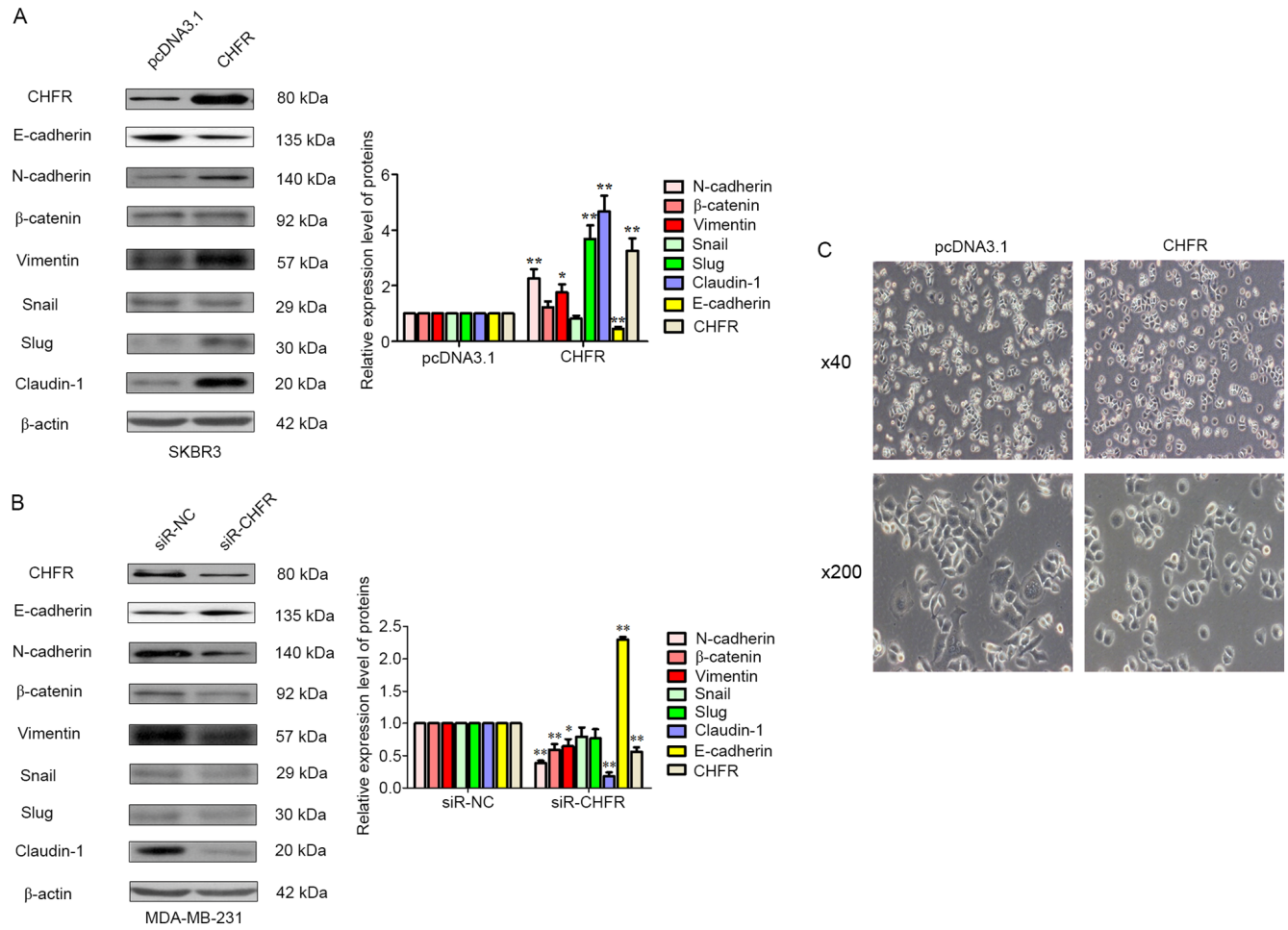

Figure 4. CHFR mediates the EMT of BRCA cells. (A) CHFR overexpression promoted the EMT in SKBR3 cells. (B) CHFR knockdown inhibited the EMT in MDA-MB-231 cells. The data are derived from three independent experiments. (C) The morphological changes in SKBR3 cells following CHFR overexpression. *P<0.05 and **P<0.01 vs. the corresponding control group (pcDNA3.1 and siR-NC). EMT, epithelial-to-mesenchymal transition; BRCA, breast cancer; CHFR, checkpoint with FHA and RING finger domains; siR, small interfering RNA; NC, negative control.

mesenchymal cells that were rounded became more polygon, which is more favorable for EMT. In other words, more mesenchymal characteristics could be observed in SKBR3 cells when CHFR was overexpressed compared with the control. These results combined indicated that CHFR-mediated EMT promoted human BRCA cell metastasis.

Discussion

CHFR is a G_2 phase/mitosis checkpoint protein that works by promoting the degradation of target proteins, such as PARP-1, to delay entry into metaphase depending on its E3-ubiquitin ligase activity (4,15). Inactivation of CHFR in numerous tumors was revealed to result from methylated CpG islands on its promotor region (16). Although CHFR is a frequent target of novel promoter hypermethylation in other cancer types, such as colorectal and esophageal cancer, it is significantly less frequent in NSCLC, and independently associated with a poor outcome in acute myeloid leukemia (17-20). However, aberrant hypermethylation of the CHFR promoter is uncommon in primary BRCA (9).

In the current study, the role of CHFR in the metastasis of BRCA cells was investigated. According to data retrieved from TCGA, CHFR was upregulated in BRCA tissues compared with normal tissues. In addition, CHFR was upregulated in $HER2^{+}$ and TNBC subtypes. Notably, patients with TNBC with higher levels of CHFR exhibited poorer overall survival rates compared with patients in the low CHFR expression group. Therefore, the aforementioned summarized data indicated that CHFR expression, and not its promoter hypermethylation, may represent a biomarker able to predict a poorer therapeutic response in patients with the $HER2^{+}$ or TNBC subtypes of BRCA. However, the effect and mechanism underlying the role of CHFR expression in the regulation of TNBC metastasis is yet to be elucidated.

TNBC is a highly aggressive subclass, accounting for ~10-20% of all BRCA diagnoses (21). Due to poor overall survival, early relapse and distant metastasis, TNBC clinical treatment of BRCA represents a notable challenge (21,22). A hallmark of cancer is abnormal activation of EMT, and this is associated with the metastasis of TNBC (23). EMT was originally speculated to be a growth process, during which epithelial cells display a migratory and invasive mesenchymal phenotype (12). From a molecular perspective, EMT is characterized by downregulation of the epithelial cell marker E-cadherin, and the upregulation of mesenchymal

cell markers vimentin and N-cadherin (24). The majority of these regulate various transcription factors implicated in EMT, such as Snail, Slug and zinc finger E-box-binding homeobox 1 (25). Previous studies have reported that there are four major epigenetic factors that regulate EMT in TNBC and are responsible for distant metastases, comprising long non-coding and microRNAs, and acetylation or methylation of histones or DNA (22). In the current study, there were two bands of CHFR in the MDA-MB-231 cells with siR-CHFR. The CHFR antibody used in the siR-CHFR transfection was different from the other CHFR antibody batches. It is possible that the specificity of the antibody was inferior for the siR-CHFR experiment, which could explain the presence of the two CHFR bands on the western blots in the siR-CHFR MDA-MB-231 cells. Overexpression of CHFR in SKBR3 cells significantly upregulated the expression of mesenchymal markers N-cadherin, vimentin and its transcription factor Slug, and tight junction protein claudin-1, while downregulated the expression of epithelial cell marker E-cadherin. As expected, silencing of CHFR in MDA-MB-231 decreased the expression of mesenchymal markers N-cadherin, vimentin and transcription factor Slug, while upregulated the expression of epithelial cell marker E-cadherin. Although there is little publication concerning how CHFR influences the EMT of cancer cells, especially in human BRCA, we speculate that the E3-ubiquitin ligase activity might contribute this function. Therefore, affinity purification of CHFR combined with mass spectrometry will be perform in the future to determine the underlying mechanism for its regulation in EMT of BRCA cells. Overall, the current data demonstrated that CHFR modulated the metastasis of BRCA cells via mediating EMT.

As cell migration and invasion are important components of cell metastasis, the observed effects of CHFR on BRCA cell migration and invasion revealed that it may also affect cell metastasis. One characteristic of malignancy is increased cell motility. Using a Transwell assay, with or without Matrigel, it was revealed that overexpression of CHFR significantly promoted BRCA cell SKBR3 migration and invasion, while knockdown of CHFR notably inhibited the rate of BRCA cell MDA-MB-231 motility. More importantly, ectopic expression of CHFR effectively impaired the cell proliferation of SKBR3 cells, while silencing of CHFR significantly enhanced the proliferation of MDA-MB-231 cells. These findings indicated that exogenous CHFR successfully acted as a cell cycle checkpoint. Taken together, the present data is consistent with a previous study, which focused on the role of CHFR in human gastric cancer cells (26). In a previous study, reduced CHFR expression was found to lead to a notable increase in population growth and a higher percentage of mitotic cells when observed *in vitro*. Importantly, reduced CHFR expression resulted in an increase in the number of mitotic (metaphase and anaphase) cells in the population. Reduced CHFR expression resulted in the acquisition of a number of phenotypes associated with malignant progression, including increased growth rate, increased mitotic index, increased invasion, increased motility, increased aneuploidy and increased colony formation in soft agar, further supporting the role of CHFR in cancer (27).

In conclusion, the current findings indicated that CHFR was upregulated in $HER2^+$ and TNBC subclasses of BRCA. In addition, patients with higher levels of CHFR exhibited poorer overall survival rates. Notably, CHFR was found to function as a novel oncogene to regulate the metastasis of BRCA cells via mediating EMT. Therefore, CHFR may represent a novel molecular therapeutic target for the treatment of BRCA, via regulation of metastatic mechanisms.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The results published here are in part based upon data generated by the TCGA Research Network: https://www.cancer.gov/tcga.

Authors' contributions

GJ and FC conceived and designed the experiments. GJ, XC and FC confirmed the authenticity of all the raw data. GJ, XS and HF performed the experiments. GJ and XC analyzed the data. GJ and FC wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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