Knockdown of NCOA5 suppresses viability, migration and epithelial-mesenchymal transition, and induces adhesion of breast cancer cells

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Abstract. Nuclear receptor coactivator 5 (NCOA5) has been reported to be involved in the regulation of several malignancies; however, to the best of our knowledge, its role in breast cancer is still unknown. The present study aimed to reveal the biological function of NCOA5 in breast cancer cells. NCOA5 expression in breast cancer tissues and cell lines was examined using reverse transcription-quantitative PCR and western blotting. Small interfering RNA (siRNA) against NCOA5 (siNCOA5) was transfected into MDA-MB-453 and MCF-7 cells to knock down NCOA5. MTT, transwell migration and cell adhesion assays were performed to determine cell viability, migration and adhesion abilities of breast cancer cells, respectively. In addition, the expression levels of N-cadherin, Vimentin and E-cadherin were examined by western blotting. It was revealed that NCOA5 expression was significantly increased in breast cancer tissues and cell lines. Knockdown of NCOA5 suppressed breast cancer cell viability and migration, and induced cell adhesion. Compared with those in cells transfected with non-targeting negative control siRNA, the protein expression levels of N-cadherin and Vimentin were significantly decreased, whereas the protein expression levels of E-cadherin were significantly increased in cells transfected with siNCOA5. The present study demonstrated that knockdown of NCOA5 suppressed cell viability and migration, induced cell adhesion, and inhibited epithelial-mesenchymal transition of breast cancer cells, indicating that NCOA5 may serve a tumor-promoting role in breast cancer.

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Introduction

Breast cancer is the most common malignancy in women and the second leading cause of cancer-associated mortality in women worldwide (1). Patients with early-stage breast cancer can be cured (2). Early diagnosis and early treatment of breast cancer are the key to improving its prognosis (3). Despite marked advances in its treatment, breast cancer remains a major health problem (4).

Nuclear receptor coactivator 5 (NCOA5) is a nuclear receptor coregulator, which interacts with α and β estrogen receptors, as well as an orphan nuclear receptor (nuclear receptor subfamily 1 group D member 2), independently of the AF2 domain (5). NCOA5 is considered to have both coactivator and corepressor functions, and could modulate estrogen receptor α-mediated transcription (6). In addition, NCOA5 interacts with tumor suppressor Tat-interacting protein (30 kDa) and regulates c-myc transcription (7). NCOA5 has been reported to be involved in the regulation of several malignancies, such as hepatocellular carcinoma, colorectal cancer and papillary thyroid cancer (8-14). Ye *et al* (15) reported that NCOA5 expression was increased in luminal breast cancer tissues, and high NCOA5 expression was associated with the progression and prognosis of patients with luminal breast cancer. However, to the best of our knowledge, the mechanisms underlying the role of NCOA5 in breast cancer are still unknown.

In the present study, the expression levels of NCOA5 in breast cancer tissues and a series of breast cancer cell lines were detected. The effects of NCOA5 on the viability, migration, adhesion and epithelial-mesenchymal transition (EMT) of breast cancer cells were evaluated using loss-of-function experiments. The present study aimed to reveal the biological function of NCOA5 in breast cancer, and provided a potential therapeutic target for patients with breast cancer.

Materials and methods

Tissue collection. Breast cancer tissues and adjacent normal tissues (distance from tumor margin, 2 cm) were collected from 25 female patients with breast cancer who underwent surgical resection at the People's Hospital of Deyang City

(Deyang, China). These patients were recruited between January 2019 and November 2020. The patients were aged 35-67 years with a mean age of 51.3 years, and all patients had not been treated before surgical resection. The inclusion criteria were: i) Female subjects aged 18-70 years; ii) histology or cytology confirmed breast cancer; iii) ECOG score was 0-1; iv) the expected survival time of the case was >3 months; v) left ventricular ejection fraction ≥55%; vi) laboratory examination data met the following criteria: Hemoglobin ≥90 g/l, absolute neutrophil count $\geq 1.5 \times 10^9$/l, platelets $\geq 100 \times 10^9$/l, total bilirubin ≤1.5 times upper limit of normal (x ULN), aspartate transaminase/alanine transaminase ≤2.5x ULN or ≤5x ULN in case of liver metastasis, creatinine ≤1.5x ULN and international normalized ratio ≤1.5x ULN, partial thromboplastin time ≤1.5x ULN; and vii) volunteered to participate in the research and signed the informed consent form. The exclusion criteria were: i) Patients with heart, liver, kidney and hematopoietic system diseases; ii) brain metastasis; iii) with double or multiple cancers; iv) suffering from clinically significant active, acute, chronic infection or bleeding; v) hypertension is not under control; vi) pregnant or lactating women, mental disorders; vii) participate in any other clinical trials within 1 month before enrollment; viii) received therapy before surgery; and ix) the researcher judged that the subjects had any other conditions that were not suitable for the trial. The study was approved by the Ethics Committee of the People's Hospital of Deyang City (Deyang, China). All patients signed an informed consent form prior to enrollment in the present study.

Cell culture and transfection. The human MCF-10A normal breast epithelial cell line and human breast cancer cell lines (MDA-MB-231, MDA-MB-453 and SK-BR-3 breast adenocarcinoma cell lines, and MCF-7 invasive ductal carcinoma cell line) were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. The cells were cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FCS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Gibco; Thermo Fisher Scientific, Inc.) and 100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37˚C. The small interfering RNA (siRNA/si) directly against human NCOA5 (siNCOA5) and the non-targeting negative control siRNA (siNC) were purchased from Shanghai GenePharma Co., Ltd. The sequences of siRNAs were as follows: siNCOA5 sense, 5'-AGGGAUCUUAGAGACUUUCGUTT-3' and antisense, 5'-ACGAAAGUCUCUAAGAUCCCUTT-3'; siNC sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'. Cells (5×10^5 cells/ml) were plated into the wells and transfected with 50 nM siRNA using Lipofectamine® 3000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 6 h, according to the manufacturer's protocols. At 48 h after transfection, cells were collected for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from tissues or cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.). The isolated RNA was then reverse transcribed into cDNA using a Prime-Script® RT reagent kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. The primers used in the present study were as follows: NCOA5 forward, 5'-CAAGTGCTCCCCTCTGCTAC-3' and reverse, 5'-CTGTTTGCTGCTGTGGAAAA-3'; GAPDH forward, 5'-CGACCACTTTGTCAAGCTCA-3' and reverse, 5'-AGGGGTCTACATGGCAACTG-3'. The specific reaction procedure was: 42˚C for 15 min, 85˚C for 5 sec, and 4˚C until further use. The cDNA was used as a template for qPCR. The PCR reaction was performed with the SYBR Green PCR Master Mix (Quantabio) on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Amplification was performed using the following conditions: Initial denaturation at 95˚C for 1 min, followed by 40 cycles of 95˚C for 5 sec and 60˚C for 20 sec. GAPDH was used as a reference gene. Relative gene expression of NCOA5 was calculated using the $2^{-\Delta\Delta Cq}$ method (16).

Western blotting. Proteins were extracted from the tissues or cells using RIPA Lysis Buffer (EMD Millipore). A BCA Protein Assay Kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration of lysates. A total of 50 μg protein per lane was separated via 10% SDS-PAGE, and the protein was then transferred to a polyvinylidene difluoride membrane (EMD Millipore). Next, the membrane was blocked with 10% skim milk at 4˚C overnight, followed by incubation at 37˚C for 2 h with the following antibodies: NCOA5 (cat. no. ab70831; dilution, 1:500), N-cadherin (cat. no. ab18203; dilution, 1:1,000), Vimentin (cat. no. ab45939; dilution, 1:800), E-cadherin (cat. no. ab212059; dilution, 1:800) and GAPDH (cat. no. ab9485; dilution, 1:2,000). All these antibodies were purchased from Abcam. After washing three times with PBS with 0.05% Tween-20 at room temperature for 5 min each, the membrane was incubated with the Goat Anti-Rabbit IgG H&L (HRP) antibody (cat. no. ab6721; dilution, 1:2,000; Abcam) at 37˚C for 1 h. Finally, the membrane was treated with ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and the band density was determined by densitometric analysis (Image Lab v4.0; Bio-Rad Laboratories, Inc.).

MTT. To determine cell viability, a total of 1.5×10^4 cells were seeded in each well of 96-well plates. The cells were cultured in DMEM supplemented with 10% FCS at 37˚C in a humidified atmosphere of 5% CO_2 for 24, 48, 72 and 96 h. Cell viability was determined using a MTT Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Cells were incubated with MTT solution at 37˚C for 4 h, and then dimethyl sulfoxide (Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan crystals. Absorbance was detected at a wavelength of 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Transwell migration assay. Cell migration was analyzed using 6-well transwell insert chambers with a pore size of 8 μm (Corning, Inc.). The cells were suspended in DMEM without FCS and then 5×10^5 cells were cultured in the upper chamber. DMEM supplemented with 10% FCS was added to the bottom chamber as a chemoattractant. Following incubation at 37˚C for 24 h, the migrated cells were fixed with 95% ethanol for 15 min at 37˚C. Subsequently, the cells were stained with 0.3% hematoxylin (Leagene Biotechnology) for 15 min at 37˚C. Cells were observed under a light microscope (Nikon Corporation), and the number of migrated cells was calculated for five random fields.

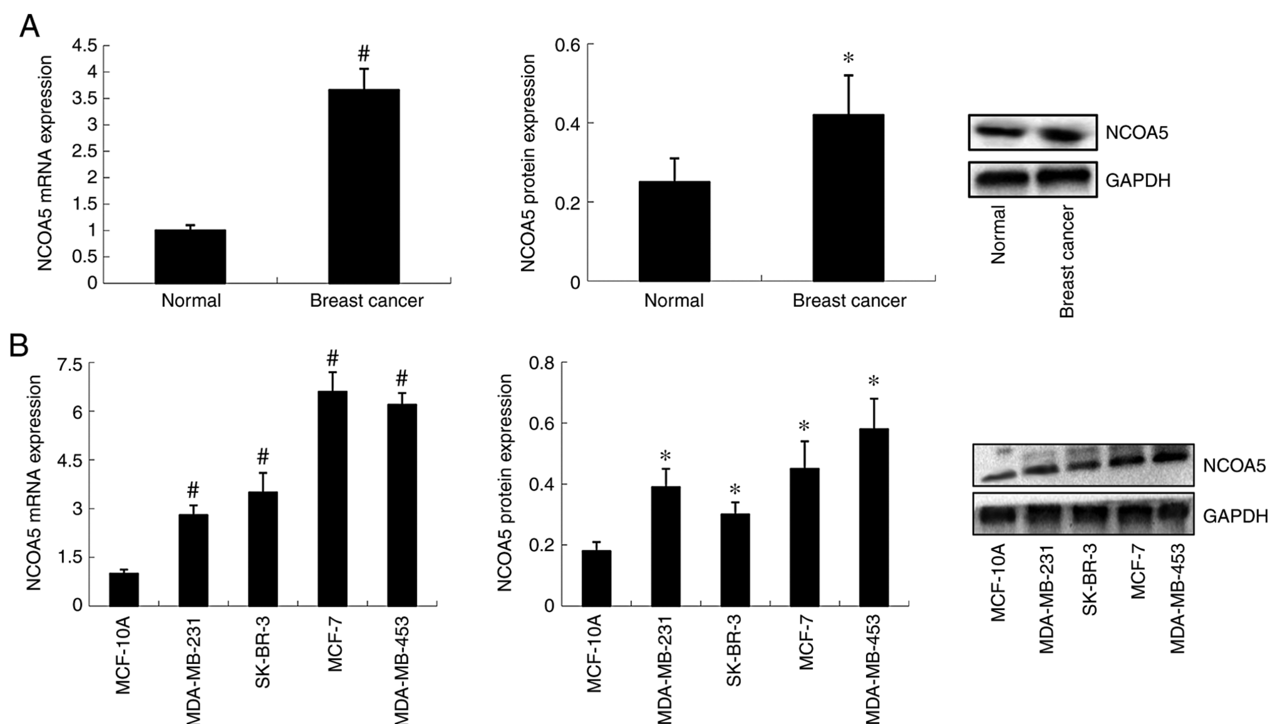

Figure 1. NCOA5 expression in human breast cancer tissues and breast cancer cell lines. (A) NCOA5 expression in human breast cancer tissues and adjacent normal tissues. *P<0.05 and #P<0.01 vs. normal. (B) NCOA5 expression in the MCF-10A normal breast epithelial cell line and a series of breast cancer cell lines (MDA-MB-231, MDA-MB-453, MCF-7 and SK-BR-3). *P<0.05 and #P<0.01 vs. MCF-10A. NCOA5, nuclear receptor coactivator 5.

Cell adhesion assay. The 96-well plates were pre-coated with fibronectin (Sigma-Aldrich; Merck KGaA) and blocked with 1% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at 37˚C for 2 h. Cells were suspended in serum-free medium and then seeded into the 96-well plates at a density of $4x10^3$ cells/well. Following incubation at 37˚C for 1 h, the cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 15 min. The adhesive cells were stained with 0.5% crystal violet (Leagene Biotechnology) at room temperature for 2 h, and the crystals were dissolved with sodium dodecyl sulphate (Amresco, LLC) at room temperature for 30 min. The absorbance at 570 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc.). Cell adhesion activity was normalized to the optical density values of the siNC group (which was set as 100%).

Statistical analysis. All data were obtained from experiments performed in triplicate and the experiments were repeated at least three times. The data are presented as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc.). A paired t-test was used for the analysis of tumor and adjacent non-tumor samples. An unpaired t-test was used for comparisons between two groups for cell experiments. One-way ANOVA followed by Tukey's post hoc test was used for analysis among three or more groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NCOA5 expression in human breast cancer tissues. Expression levels of NCOA5 in human breast cancer tissues and adjacent normal tissues were detected by RT-qPCR and western blotting. As shown in Fig. 1A, compared with that in adjacent normal tissues, NCOA5 expression was increased in breast cancer tissues at both the mRNA and protein levels.

NCOA5 expression in breast cancer cell lines. NCOA5 expression in the MCF-10A normal breast epithelial cell line and a series of breast cancer cell lines (MDA-MB-231, MDA-MB-453, MCF-7 and SK-BR-3) was detected by RT-qPCR and western blotting. The results demonstrated that the mRNA and protein expression levels of NCOA5 were significantly increased in breast cancer cell lines compared with the normal cell line (Fig. 1B). Among the breast cancer cell lines, MDA-MB-453 and MCF-7 cells exhibited higher NCOA5 mRNA and protein expression than MDA-MB-231 and SK-BR-3 cells. Subsequently, loss-of-function experiments were performed using the MDA-MB-453 and MCF-7 cell lines, which exhibited high expression levels of NCOA5.

Effect of NCOA5 on the viability of breast cancer cells. To investigate the biological function of NCOA5 in breast cancer cells, siNCOA5 was transfected into MDA-MB-453 and MCF-7 cells to knock down NCOA5 expression. The results of western blot analysis demonstrated that compared with those in the cells transfected with siNC, the protein expression levels of NCOA5 were significantly decreased in MDA-MB-453 and MCF-7 cells transfected with siNCOA5 (Fig. 2A). Subsequently, an MTT assay was performed using MDA-MB-453 and MCF-7 cells to examine viability. As shown in Fig. 2B, compared with the cells transfected with siNC, cell viability was significantly decreased in both MDA-MB-453 and MCF-7 cell lines transfected with siNCOA5 at 48, 72 and 96 h.

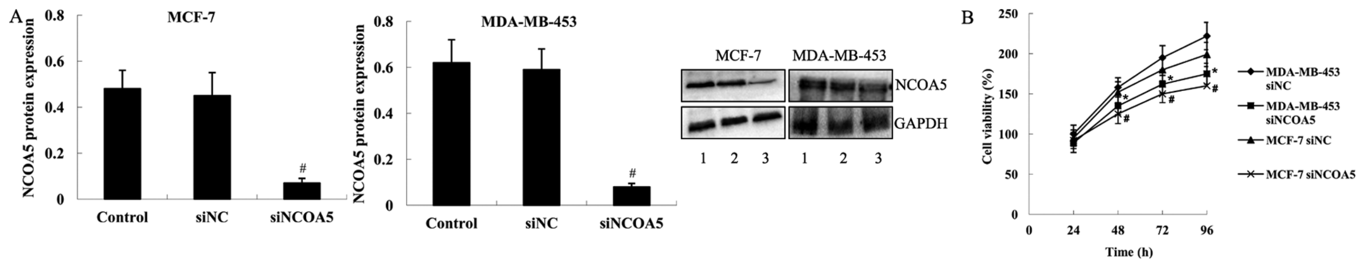

Figure 2. Effect of NCOA5 on the viability of breast cancer cells. (A) NCOA5 expression in MCF-7 and MDA-MB-453 cells following transfection with siNCOA5. Lane 1, control; lane 2, siNC; lane 3, siNCOA5. #P<0.01 vs. siNC. (B) Effect of siNCOA5 on the viability of MCF-7 and MDA-MB-453 cells. *P<0.05 vs. MDA-MB-453 siNC; #P<0.05 vs. MCF-7 siNC. NCOA5, nuclear receptor coactivator 5; si, small interfering RNA; NC, negative control.

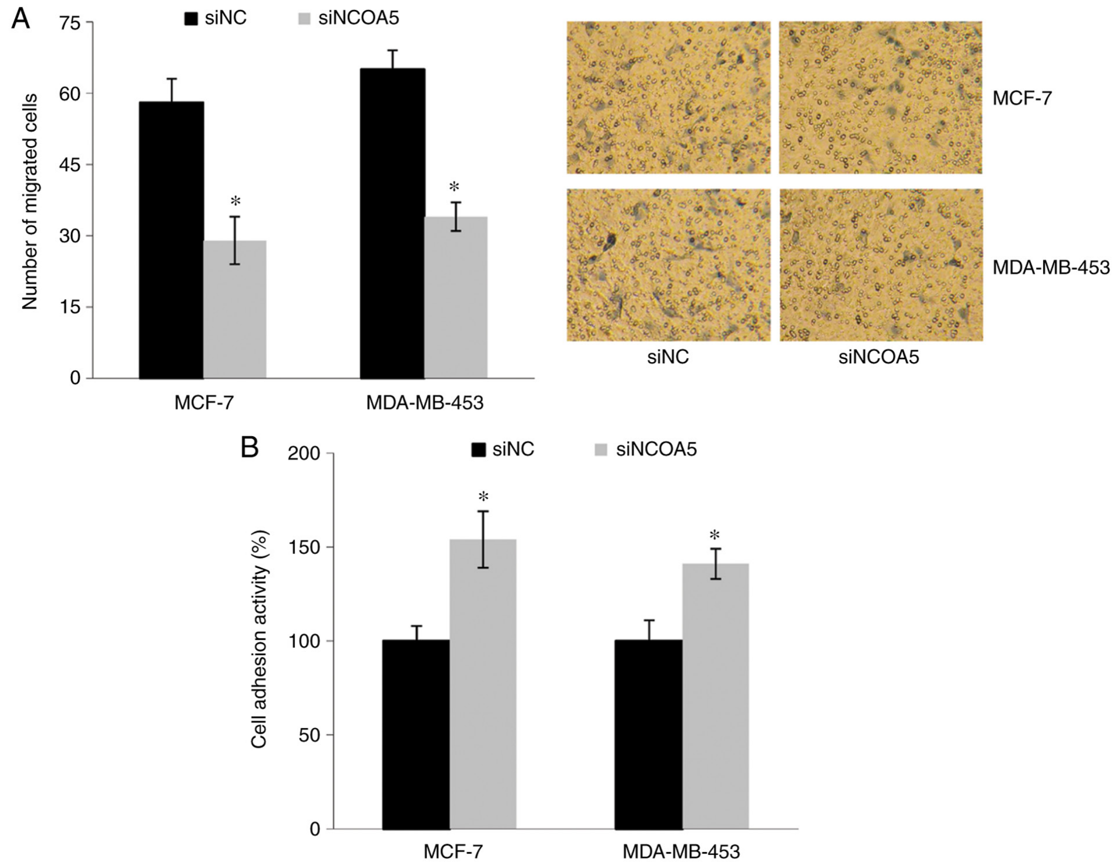

Figure 3. Effect of NCOA5 on the migration and adhesion of breast cancer cells. (A) Effect of NCOA5 on cell migration. Magnification, x200. (B) Effect of NCOA5 on cell adhesion. *P<0.05 vs. siNC. NCOA5, nuclear receptor coactivator 5; si, small interfering RNA; NC, negative control.

Effect of NCOA5 on the migration and adhesion abilities of breast cancer cells. Transwell migration assays were performed to investigate the effect of NCOA5 on cell migration. As shown in Fig. 3A, the number of migrated cells was significantly decreased in the siNCOA5 group compared with the siNC group. Furthermore, a cell adhesion assay revealed that the adhesion abilities of MDA-MB-453 and MCF-7 cells were significantly increased following transfection with siNCOA5 (Fig. 3B).

Effect of NCOA5 on EMT of breast cancer cells. The protein expression levels of N-cadherin, Vimentin and E-cadherin were examined by western blot analysis to investigate the effect of NCOA5 on EMT of breast cancer cells. Compared with those in the cells transfected with siNC, the protein expression levels of N-cadherin and Vimentin were significantly decreased, whereas the protein expression levels of E-cadherin were significantly increased in MDA-MB-453 cells transfected with siNCOA5. In MCF-7 cells, the expression levels of N-cadherin and Vimentin were significantly decreased, and E-cadherin expression was significantly increased in siNCOA5-tranfected cells compared with siNC-transfected cells (Fig. 4).

Discussion

NCOA5 is a unique nuclear receptor coactivator with both co-activation and co-repression functions (6). The abnormal expression of estrogen receptor is associated with various types of cancer (17), and previous studies have demonstrated

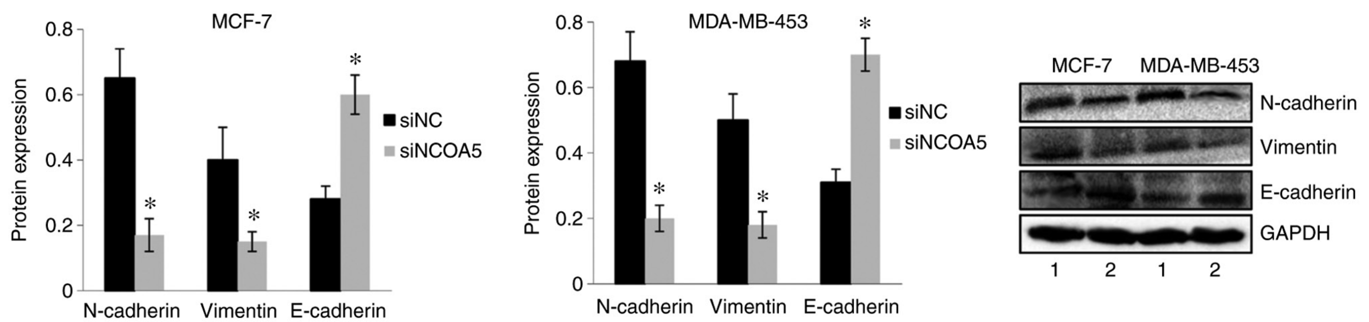

Figure 4. Effect of NCOA5 on the expression levels of epithelial-mesenchymal transition-related proteins in breast cancer cells. Lane 1, siNC; lane 2, siNCOA5. *P<0.05 vs. siNC. NCOA5, nuclear receptor coactivator 5; si, small interfering RNA; NC, negative control.

that NCOA5 can regulate estrogen receptor-mediated transcription in human cells (5-7). The role of NCOA5 in human cancer has attracted increasing attention. Abnormal expression of NCOA5 has been reported in a variety of tumors, including esophageal squamous cell carcinoma, hepatocellular carcinoma and cervical cancer (15,18-21). The present study demonstrated that NCOA5 expression was significantly increased in human breast cancer tissues compared with adjacent normal tissues. Furthermore, NCOA5 expression was significantly increased in breast cancer cell lines compared with a normal breast epithelial cell line. The present results were consistent with a previous report showing that NCOA5 was upregulated in luminal breast cancer tissues compared with adjacent normal tissues both in a validated cohort and The Cancer Genome Atlas cohort (15). Additionally, upregulation of NCOA5 has been identified in colorectal cancer (13). Conversely, NCOA5 has been reported to be downregulated in hepatocellular carcinoma, cervical cancer, esophageal squamous cell carcinoma, papillary thyroid carcinoma and osteosarcoma (18-21). These reports suggest that alterations of NCOA5 are involved in the carcinogenesis and progression of human cancer.

Accumulating evidence has demonstrated that NCOA5 serves as a tumor suppressor or an oncogene in different tumor types (12-14,19). For example, NCOA5 expression is associated with the clinicopathological features of patients with colorectal cancer (13). Knockdown of NCOA5 markedly suppresses the proliferation, migration and invasion of colorectal cancer cells, induces cell cycle G1 phase arrest, and inhibits *in vivo* xenograft growth of colorectal cancer cells (13). Similarly, knockout of NCOA5 inhibits proliferation and migration in hepatocellular carcinoma cells (12). By contrast, Zheng *et al* (14) reported that NCOA5 is a tumor suppressor gene in papillary thyroid carcinoma, and reduced NCOA5 expression is associated with the aggressive clinicopathological features of patients with papillary thyroid carcinoma. Additionally, a study revealed that low NCOA5 expression predicts poor prognosis in human cervical cancer, and downregulation of NCOA5 results in an increase in proliferation, migration and invasion of HeLa cells (19). Ye *et al* (15) demonstrated that high NCOA5 expression was an independent high risk factor in luminal breast cancer, and patients with high NCOA5 expression had a lower overall survival rate. Yuan *et al* (22) reported that NCOA5 expression could be stimulated by methionine and leucine in bovine mammary epithelial cells; however, phosphatidylinositol 3-kinase inhibition could abolish the stimulatory effect of methionine and leucine on NCOA5. NCOA5 can bind to the mTOR promoter, and induce mTOR phosphorylation and β-casein synthesis (22). However, the precise role and the cellular mechanism for NCOA5 in breast cancer are still largely unknown. The present study explored the biological function of NCOA5 *in vitro*. NCOA5 expression was significantly increased in breast cancer cell lines. Therefore, loss-of-function experiments were performed in MDA-MB-453 and MCF-7 cell lines, which exhibited higher expression levels of NCOA5 than MDA-MB-231 and SK-BR-3 cell lines. It was demonstrated that knockdown of NCOA5 suppressed the viability and migration, and induced adhesion of breast cancer cells, indicating that NCOA5 may act as a novel oncogene to promote the progression of breast cancer. The present finding was consistent with previous studies demonstrating that NCOA5 acts as an oncogene in colorectal cancer and hepatocellular carcinoma (12,13). However, in papillary thyroid carcinoma and cervical cancer, NCOA5 acts as a tumor suppressor (14,19). It was hypothesized that the role of NCOA5 in human cancer may be dependent on the tissue type. The mechanisms underlying the biological effects of NCOA5 in different types of human cancer should be further explored.

The present study further demonstrated that NCOA5 knockdown decreased the expression levels of N-cadherin and Vimentin, and increased the expression levels of E-cadherin. It has been demonstrated that N-cadherin, Vimentin and E-cadherin are key mediators of EMT (23-25). EMT is an important process for epithelial cancer metastasis (26-28). The loss of E-cadherin expression or gain of N-cadherin and Vimentin expression contributes to EMT (25,29,30). The present results supported the hypothesis that NCOA5 induced EMT in breast cancer cells, thus promoting tumor cell migration and invasion. Additional functional experiments are required to demonstrate the effects of the EMT phenotype of the cells.

Interestingly, the present findings suggested that NCOA5 could promote proliferation and aggressiveness of breast cancer cells. However, among all the breast cancer cell lines examined, the expression levels of NCOA5 were high in the least aggressive MCF-7 cell line (31). At present, there are few studies regarding the role of NCOA5 in breast cancer (15,22), and the association between NCOA5 expression and the aggressiveness of breast cancer cell lines is still unclear.

Whether the expression levels of NCOA5 could be used to predict the aggressiveness of breast cancer cell lines requires further investigation.

A limitation of the present study was that loss-of-function experiments were performed only in breast cancer cells. More studies on MCF-10A normal breast epithelial cell line are required to provide a negative control for the experiments performed using the breast cancer cell lines. Another limitation of the present study was that it only used an MTT assay to determine cell viability. Other assays are required to further examine the effect of NCOA5 on cell proliferation.

In conclusion, the present study demonstrated that NCOA5 expression was upregulated in human breast cancer tissues and breast cancer cell lines. Furthermore, the present study revealed the cellular mechanisms for NCOA5 in breast cancer, and demonstrated that knockdown of NCOA5 suppressed cell viability and migration, induced cell adhesion, and inhibited EMT of breast cancer cells, indicating that NCOA5 serves a tumor-promoting role in the progression of breast cancer. The present study suggested NCOA5 as a novel target for the treatment of breast cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YT and FL designed the study, prepared the manuscript, and confirmed the authenticity of all the raw data. YT, FL and PX conducted the experiments. All authors were substantially involved in the research, acquisition of data, analysis and manuscript preparation. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the People's Hospital of Deyang City (Deyang, China). All patients signed an informed consent form prior to enrollment in the present study.

Patient consent for publication

All patients signed an informed consent for enrollment and publication.

Competing interests

The authors declare that they have no competing interests.

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