Knockdown of NCAPG promotes the apoptosis and inhibits the invasion and migration of triple-negative breast cancer MDA-MB-231 cells via regulation of EGFR/JAK/STAT3 signaling

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Abstract. Triple-negative breast cancer (TNBC) is the most aggressive subtype of breast cancer and the treatment options are extremely limited. Non-SMC condensing I complex subunit G (NCAPG) expression is upregulated in TNBC, but its specific regulatory mechanism in TNBC has not been previously reported. The expression levels of NCAPG in TNBC were analyzed using data obtained from the UALCAN database. RT-qPCR and western blotting were used to detect the expression of NCAPG in various breast cancer cell lines. The expression of NCAPG was knocked down, and cell viability was then detected using a CCK-8 assay, apoptosis was detected using a TUNEL assay, and the expression of the apoptosis-related proteins Bcl-2, Bax and Bad were detected by western blotting. Wound healing and Transwell assays were used to assess migration and invasion. Western blotting was also used to determine the expression levels of migration and invasion-related proteins MMP2 and MMP9, as well as EGFR/JAK/STAT3 pathway-related proteins. Following exogenous treatment with EGF and the JAK/STAT3 signaling pathway agonist colivelin, cell viability, apoptosis, invasion and migration were assessed. The expression of NCAPG in TNBC MDA-MB-231 cells was significantly increased. Inhibition of NCAPG inhibited the activity, promoted apoptosis, and inhibited the invasion and migration of TNBC MDA-MB-231 cells, potentially via regulation of the EGFR/JAK/STAT3 signaling pathway. In conclusion, downregulation of NCAPG can promote apoptosis and inhibit invasion and migration of TNBC MDA-MB-231 cells via EGFR/JAK/STAT3 signaling.

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Key words: NCAPG, EGFR/JAK/STAT3, triple-negative breast cancer, apoptosis, invasion, migration

Introduction

Triple-negative breast cancer (TNBC) is a type of breast cancer in which estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor-2 (HER-2) expression is absent (1). TNBC accounts for ~20% of all breast cancer cases, is characterized by a high recurrence rate and high rate of metastasis, and is difficult to treat (2). TNBC is the most common malignant tumor in women, and its incidence and death rates are increasing annually, and worryingly, the age of onset is decreasing meaning a larger number of younger individuals are being diagnosed with TNBC (3).

Non-SMC condensin I complex subunit G (NCAPG) is a protein related to cell proliferation and division, and it participates in the occurrence and development of several types of cancer (4). A previous study found that NCAPG was associated with a poorer prognosis in breast cancer patients. Compared with non-TNBC, the expression levels of NCAPG in TNBC is higher, and knockdown of its expression was found to cause cell cycle arrest in MCF-7 cells (5). Meanwhile, studies also found that NCAPG expression is upregulated in TNBC through microarray analysis where it was found to be associated with a poorer prognosis, suggesting that NCAPG may serve as a potential biomarker of TNBC (6,7). Moreover, NCAPG expression has been shown to be upregulated in trastuzumab-resistant HER2-positive breast cancer cells, and it enhanced the drug resistance of HER2-positive breast cancer cells via activation of the SRC/STAT3 signaling pathway (8). However, there are no studies on the related mechanism of NCAPG action in TNBC cells.

It was found that NCAPG silencing reduced epidermal growth factor receptor (EGFR) expression in hepatocellular carcinoma (9). This suggests that NCAPG can affect the development of cancer by regulating the expression of EGFR. EGFR is a glycoprotein receptor on the cell membrane, which can promote the growth and proliferation of tumor cells. In TNBC, EGFR was found to promote the development of TNBC through JAK/STAT3 signaling (10).

Therefore, it was hypothesized that downregulation of NCAPG affects the development of TNBC cells through

EGFR/JAK/STAT3 signaling. Our study provides a theoretical basis for the targeted treatment of TNBC.

Materials and methods

Cell culture. Normal mammary epithelial MCF-10A cells, luminal A human breast cancer MCF-7 cells, luminal B human breast cancer BT-474 cells, $HER2^+$ breast cancer HCC1954 cells and TNBC MDA-MB-231 cells were obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin solution at 37˚C in a humidified incubator supplied with 5% CO_2.

RT-qPCR. Total RNA from the cells was extracted using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA from each sample was reverse transcribed to single-stranded cDNA, which was next used for qPCR. The mRNA expression levels of NCAPG were detected using a SYBR Premix Ex Taq kit (Takara Bio, Inc.). The thermocycling conditions for qPCR were as follows: 95˚C in a 20-μl reaction volume for 10 min, followed by 40 cycles at 95˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. The fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (11). The sequences were as follows: NCAPG forward: 5'-TCCACATAGAGAAGAATGATGCTGA-3' and reverse: 5'-GCAAACACGGGGAAGAACAC-3'; GAPDH forward: 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse: 5'-GCG CCCAATACGACCAAATC-3'.

Western blot assay. RIPA lysis buffer was used to extract the total proteins from the cells, and the protein concentration was quantified using a BCA kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. A total of 30 μg protein/lane was loaded on a 10% SDS-gel, resolved using SDS-PAGE and transferred to a PVDF (Roche Diagnostics GmbH). The membranes were blocked with 5% nonfat milk for 1 h at room temperature, and then incubated with the corresponding primary antibodies anti-NCAPG (1:800, ab70350, Abcam), anti-Bcl-2 (1:1,000, ab32124, Abcam), anti-Bax (1:1,000, ab182733, Abcam), anti-Bad (1:1,000, ab32445, Abcam), anti-MMP2 (1:1,000, ab92536, Abcam), anti-MMP9 (1:1,000, ab76003, Abcam), anti-p-EGFG (1:1,000, ab40815, Abcam), anti-EGFR (1:1,000, ab52894 Abcam), anti-p-JAK1 (1:1,000, ab138005, Abcam), anti-JAK1 (1:800, ab133666, Abcam), anti-STAT3 (1:1,000, ab68153, Abcam), anti-p-STAT3 (1:1,000, ab76315, Abcam), anti-GAPDH (1:1,000, ab9485, Abcam) overnight at 4˚C. The following day, the membranes were incubated with goat anti-rabbit IgG H&L (HRP)-conjugated secondary antibodies (1:5,000, ab7090, Abcam) for 1 h at room temperature. The expression levels of the different proteins were detected using enhanced chemiluminescence reagent (Bio-Rad Laboratories, Inc.). The data were analyzed using ImageJ version 1.46 (National Institutes of Health).

Cell transfection. Short hairpin RNA NCAPG#1 (sh-NCAPG#1), sh-NCAPG#2 and the control adenovirus vector (sh-NC) were obtained from GeneCopoeia, Inc. For transfections, the cells were seeded into 6-well plates for 24 h, and then transfected according to the manufacturer's instructions. Two human shRNA-NCAPG sequences were as follows: sh-NCAPG#1: CCGGGCTATGCAGAAGCATCTTCTTCT CGAGAAGAAGATGCTTCTGCATAGCTTTTTTG and sh-NCAPG#2: CCGGCGGGCAGTGTTATCATGTATTCT CGAGAATACATGATAACACTGCCCGTTTTTTG. sh-NC is as follows: CAACAAGATGAAGAGCACCAA. After determining the optimal transfection efficiency, cells were divided into a control group, sh-NC group and sh-NCAPG group. Then, 50 ng/ml EGF (exogenous EGFR agonist) and 2 μM colivelin (JAK/STAT3 signaling pathway agonist) were added, and the transfected cells were divided into a control, sh-NC and sh-NCAPG, sh-NCAPG+EGF and sh-NCAPG+ colivelin group.

Cell viability assay. The cell viability was measured using a CCK-8 assay (Dojindo Molecular Technologies, Inc.). Briefly, after treatment of the cells as above, 10 μl CCK-8 solution was added and cells were incubated for 2 h. Cell viability was measured at an absorbance wavelength of 450 nm (optical density) using a microplate reader (Bio-Rad Laboratories, Inc.).

TUNEL assay. A TUNEL staining kit (Beyotime Institute of Biotechnology) was used to detect cell apoptosis according to the manufacturer's instructions. TUNEL solution was then added to stain the nucleus. Different areas of the sample were randomly selected and captured under a fluorescence microscope (Olympus Corporation, magnification, x200) to count the number of TUNEL-positive cells.

Wound healing assay. Cells were seeded into 6-well plates in serum-free DMEM. When the cells reached 80-90% confluence, a 10-μl pipette tip was used to produce a wound in the monolayer at the bottom of the plate. The wound width was then measured at 0 and 24 h on a light microscope (magnification, x200). The cell migration rate was calculated as follows: (Initial width-final width)/Initial width.

Transwell invasion assay. Cell suspensions (100 μl) were added to the upper chambers of the Transwell inserts coated with Matrigel™ (50 mg/l; 1:8 diluted solution; BD Biosciences) and 600 μl supplemented medium was added to the lower chambers. After incubation for 24 h, the membrane was fixed with 4% paraformaldehyde for 15 min and sequentially stained with 0.1% crystal violet solution for 30 min (all at room temperature). The inside of the membrane was gently wiped with a cotton swab to remove any cells that had not migrated. Finally, the number of cells that had migrated were counted under a light microscope (magnification 200).

Database. The UALCAN database (ualcan.path.uab.edu/) was used to analyze the expression levels of NCAPG.

Statistical analysis. All data are presented as the mean ± standard deviation and were analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc.). A Student's t-test was used for comparisons between two groups, and a one-way ANOVA followed by Tukey's post hoc test was used for

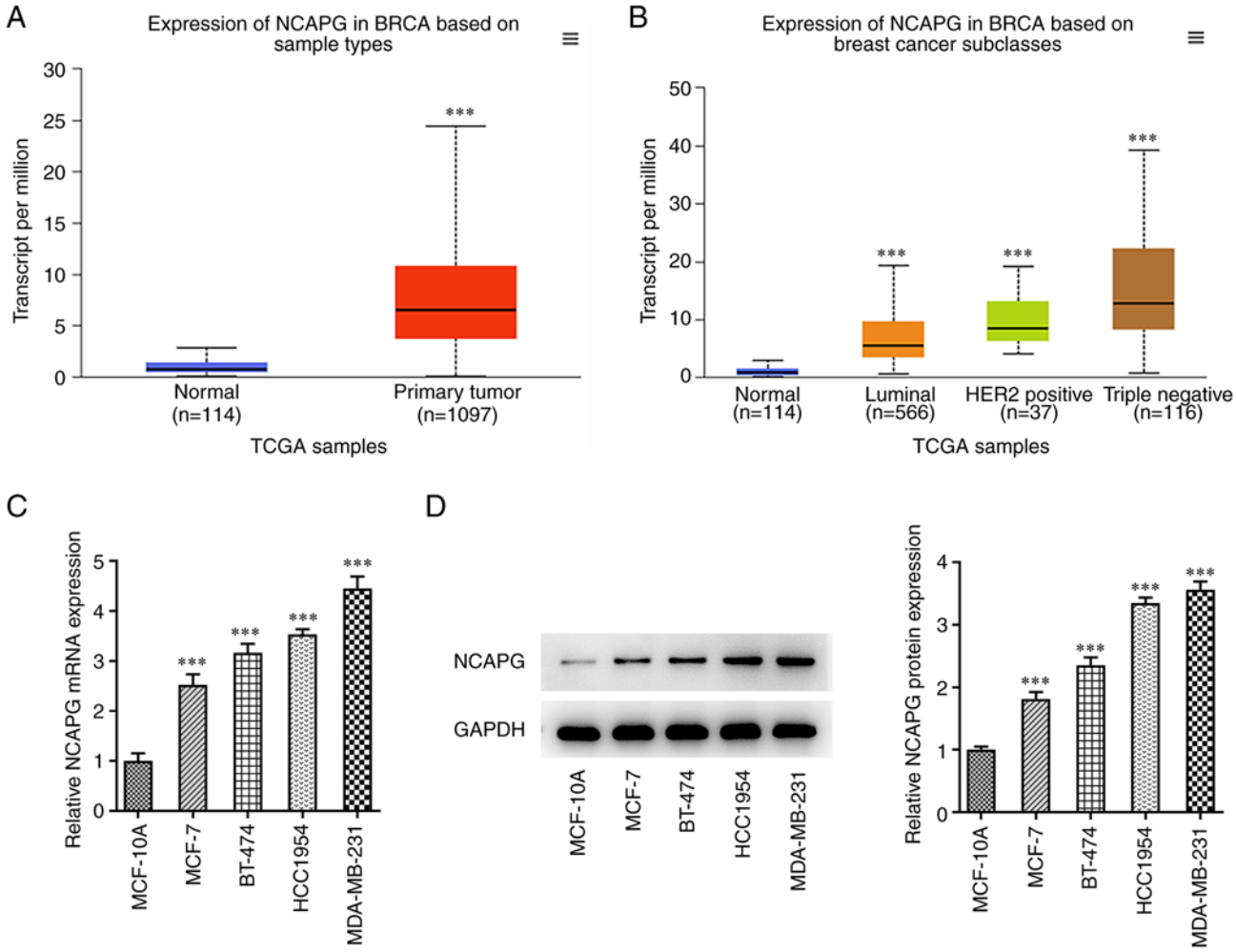

Figure 1. The expression of NCAPG is upregulated in TNBC MDA-MB-231 cells. (A and B) The expression level of NCAPG was analyzed by UALCAN database. ***P<0.001 vs. the normal tissues. The expression level of NCAPG in cells was analyzed by (C) RT-qPCR and (D) western blot analysis. ***P<0.001 vs. the MCF-10A cell line. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer.

comparisons between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

Results

NCAPG expression is upregulated in TNBC MDA-MB-231 cells. UALCAN database analysis showed that the expression of NCAPG was significantly increased in TNBC patients compared with healthy patients (Fig. 1A and B). RT-qPCR and western blot analysis were used to detect the expression of NCAPG in various breast cancer cell lines. The results showed that the expression of NCAPG was significantly increased in MCF-7, BT-474, HCC1954, and MDA-MB-231 cells compared with the MCF-10A cells (Fig. 1C and D). The above results indicated that NCAPG had higher expression in the TNBC cell line. Thus, MDA-MB-231 cells were selected for the following experiments.

Knockdown of NCAPG promotes apoptosis of TNBC MDA-MB-231 cells. The interference plasmid of NCAPG was constructed, and the transfection efficiency was detected by RT-qPCR and western blotting. Compared with the sh-NC group, the expression of NCAPG in the sh-NCAPG#1 and sh-NCAPG#2 groups was significantly decreased, and the transfection efficiency of the sh-NCAPG#2 group was the better of the two, thus it was selected for the subsequent experiments (Fig. 2A and B). CCK-8 analysis showed that cell viability in the sh-NCAPG group was significantly decreased compared with that in the sh-NC group (Fig. 2C). TUNEL staining and western blotting analysis showed that compared with the sh-NC group, apoptosis was significantly increased in the sh-NCAPG group, and this was accompanied by decreased expression of Bcl-2 and increased expression of Bax and Bad (Fig. 2D-F).

Knockdown of NCAPG inhibits migration and invasion of TNBC MDA-MB-231 cells. Wound healing and Transwell assays were used to assess the migration and invasion of TNBC cells, and the results showed that compared with the sh-NC group, the migration and invasion of the sh-NCAPG group was significantly decreased (Fig. 3A and B). Western blotting was used to detect the expression of matrix metalloproteinase (MMP)2 and MMP9, and the results showed that the expression of MMP2 and MMP9 in the sh-NCAPG group were significantly decreased compared with the sh-NC group (Fig. 3C).

Activation of EGFR/JAK/STAT3 signaling reduces the effect of the knockdown of NCAPG on the apoptosis of

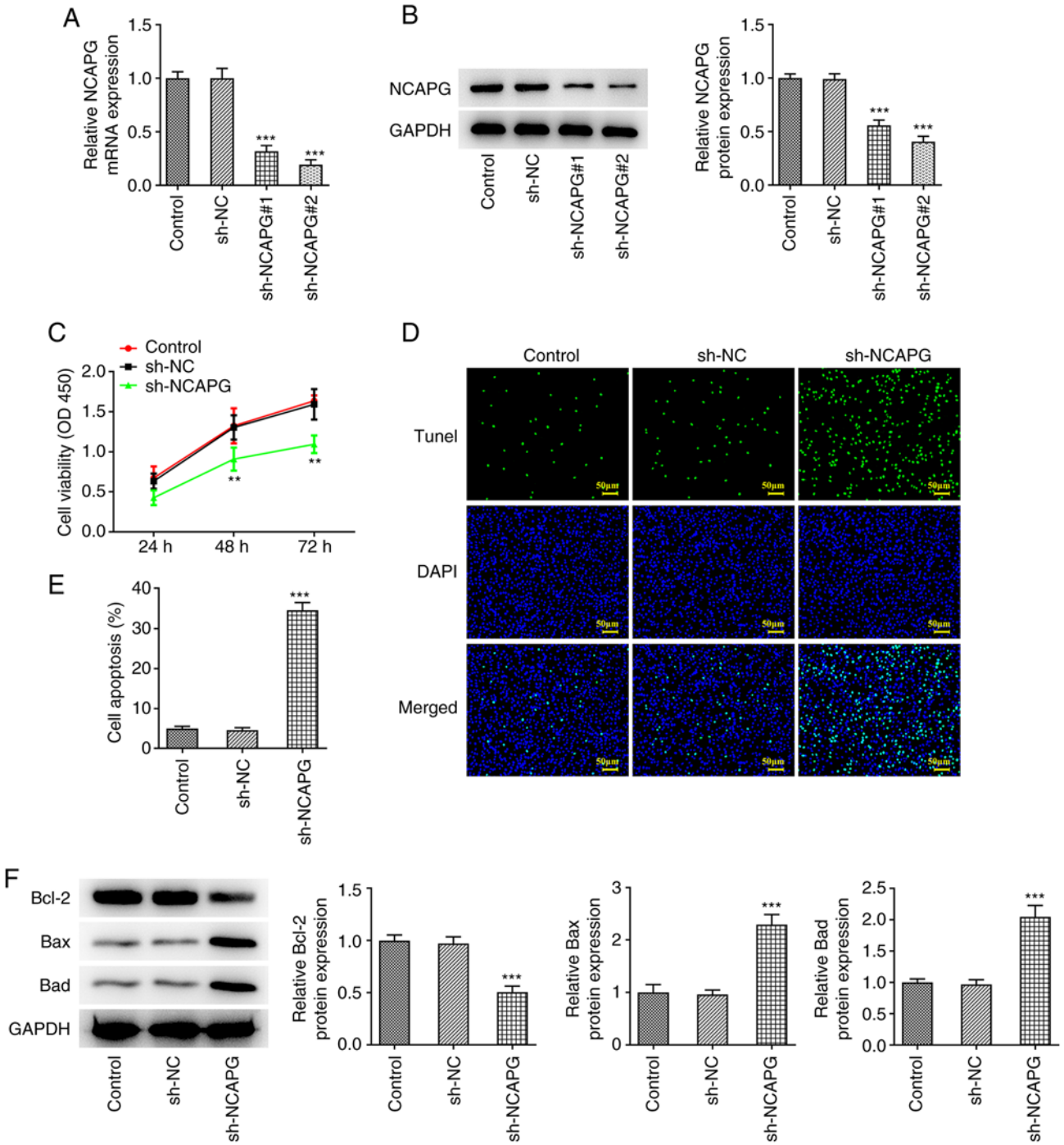

Figure 2. Downregulation of NCAPG promotes the apoptosis of TNBC MDA-MB-231 cells. The expression level of NCAPG in cells was analyzed by (A) RT-qPCR and (B) western blot analysis after cell transfection. (C) CCK-8 kit was used to detect the cell viability. (D) TUNEL assay was used to detect the cell apoptosis. (E) Statistical analysis of apoptosis. (F) Apoptosis-related proteins were detected by western blot analysis. **P<0.01, ***P<0.001 vs. sh-NC. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer.

TNBC cells. The expression levels of the EGFR/JAK/STAT3 signaling pathway-related proteins were detected by western blotting, and the results showed that the expression of phosphorylated (p-)EGFR, phosphorylated Janus kinase 1 (p-JAK1), and phosphorylated signal transducer and activator of transcription 3 (p-STAT3) were significantly decreased in the sh-NCAPG group compared with the sh-NC group (Fig. 4). Subsequently, after the addition of EGF and colivelin, the expression of p-EGFR was increased as detected by western blot analysis compared with the sh-NCAPG group (Fig. 5A). CCK-8 results showed that relative to the sh-NC group, cell viability in the sh-NCAPG group was significantly reduced. Moreover, cell activity was significantly increased in the sh-NCAPG+ EGF and sh-NCAPG+colivelin groups compared with the sh-NCAPG group (Fig. 5B). TUNEL and western blotting results showed that compared with the sh-NCAPG group, apoptosis was significantly decreased in the sh-NCAPG+ EGF and sh-NCAPG+colivelin groups, and this was accompanied by increased Bcl-2 expression, and decreased Bax and Bad expression (Fig. 5C and D).

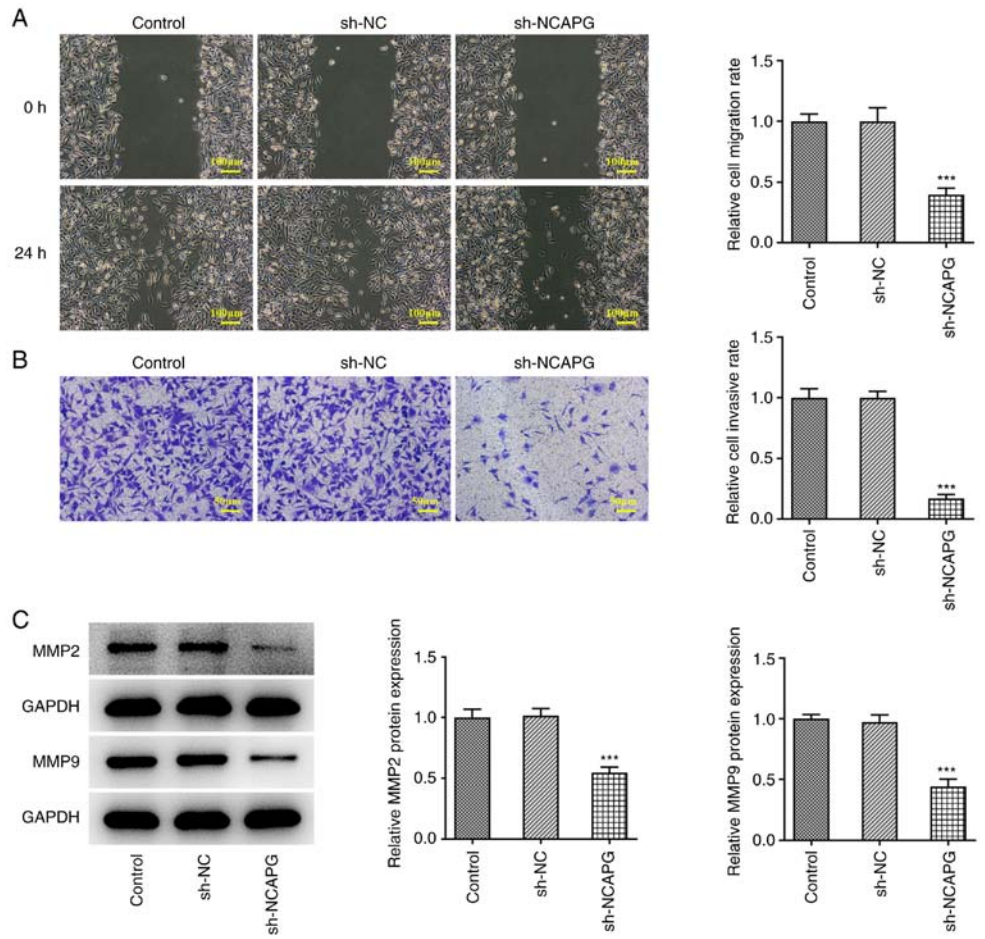

Figure 3. Downregulation of NCAPG inhibits the migration and invasion of TNBC MDA-MB-231 cells. (A) Wound healing and (B) Transwell assay were used to assess cell invasion and migration. (C) MMP2 and MMP9 proteins were detected by western blot analysis. ***P<0.001 vs. sh-NC. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer; MMP, matrix metalloproteinase.

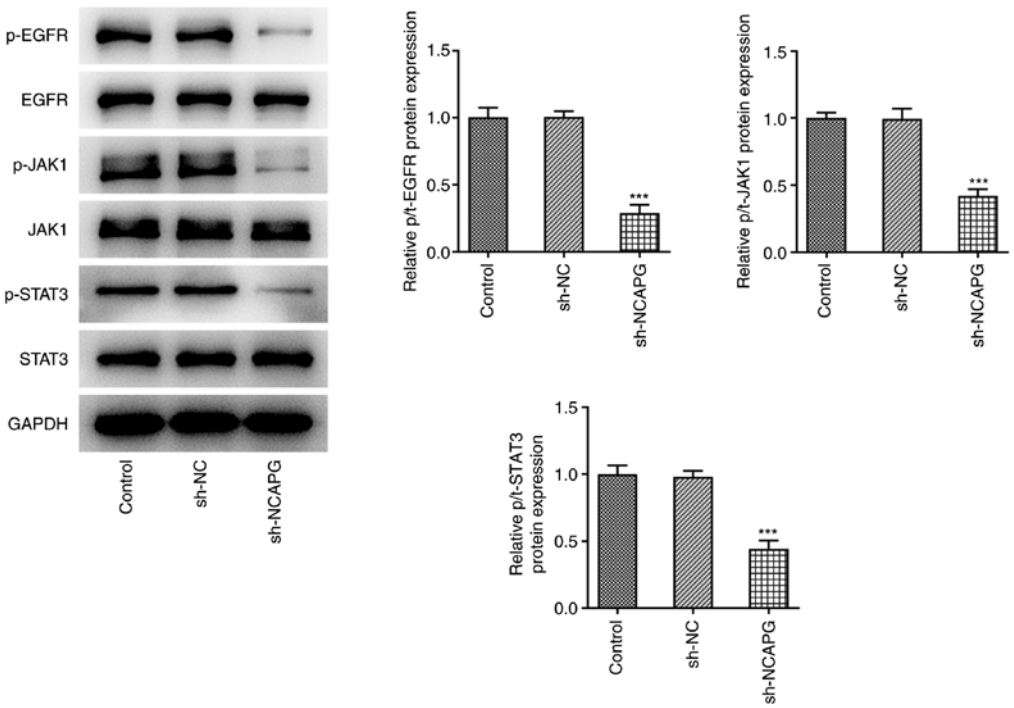

Figure 4. Downregulation of NCAPG inhibits EGFR/JAK1/STAT3 signaling in TNBC MDA-MB-231 cells. EGFR/JAK1/STAT3 signaling-related proteins were detected by western blot analysis. ***P<0.001 vs. sh-NC. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer; EGFR, epidermal growth factor receptor; JAK1, Janus kinase 1; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated.

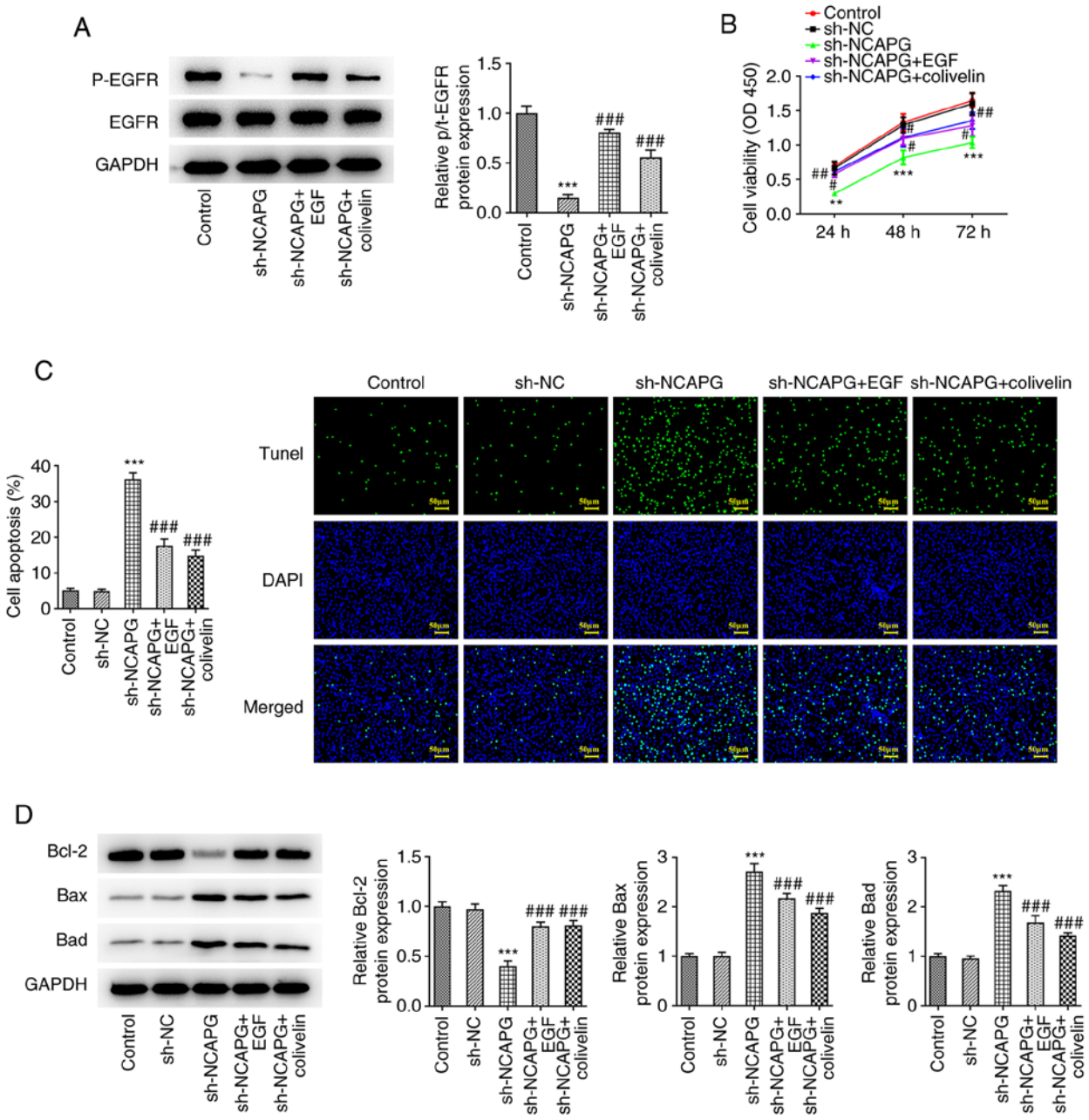

Figure 5. Activation of EGFR/JAK1/STAT3 signaling reduces the promoting effect of the downregulation of NCAPG on apoptosis of TNBC MDA-MB-231 cells. (A) Western blot assay was used to detect the expression of p-EGFR and EGFR. ***P<0.001 vs. control; $^{###}$P<0.001 vs. sh-NCAPG. (B) CCK-8 kit was used to detect cell viability. (C) TUNEL assay was used to detect cell apoptosis. (D) Apoptosis-related proteins were detected by western blot analysis. **P<0.01, ***P<0.001 vs. sh-NC; $^{#}$P<0.05, $^{##}$P<0.01, $^{###}$P<0.001 vs. sh-NCAPG. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer; EGFR, epidermal growth factor receptor; JAK1, Janus kinase 1; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated.

Activation of EGFR/JAK/STAT3 signaling reduces the inhibitory effect of knockdown of NCAPG on invasion and migration of TNBC MDA-MB-231 cells. Wound healing and Transwell assays showed that EGFR/JAK/STAT3 signal activation significantly increased the invasion and migration of cells (Fig. 6A), and the expression of MMP2 and MMP9 in cells was significantly increased (Fig. 6B) compared to the sh-NCAPG group.

Discussion

At present, with the continuous in-depth research on the pathogenic mechanisms underlying the development and progression of triple-negative breast cancer (TNBC), several targeted drugs have been discovered (12). However, due to the heterogeneity of TNBC and acquisition of drug resistance, the clinical effects of targeted drugs for TNBC is not always significant (13). Therefore, there is an urgent need to identify novel effective targets for early screening, prognosis assessment and treatment of TNBC.

Through UALCAN database analysis, we found that non-SMC condensin I complex subunit G (NCAPG) expression was significantly elevated in TNBC. This is consistent with the results screened out by Zeng *et al* (14) through microarray analysis. A previous study showed that the protein expression levels of NCAPG were significantly increased in 16 different types of tumors, and upregulated expression of

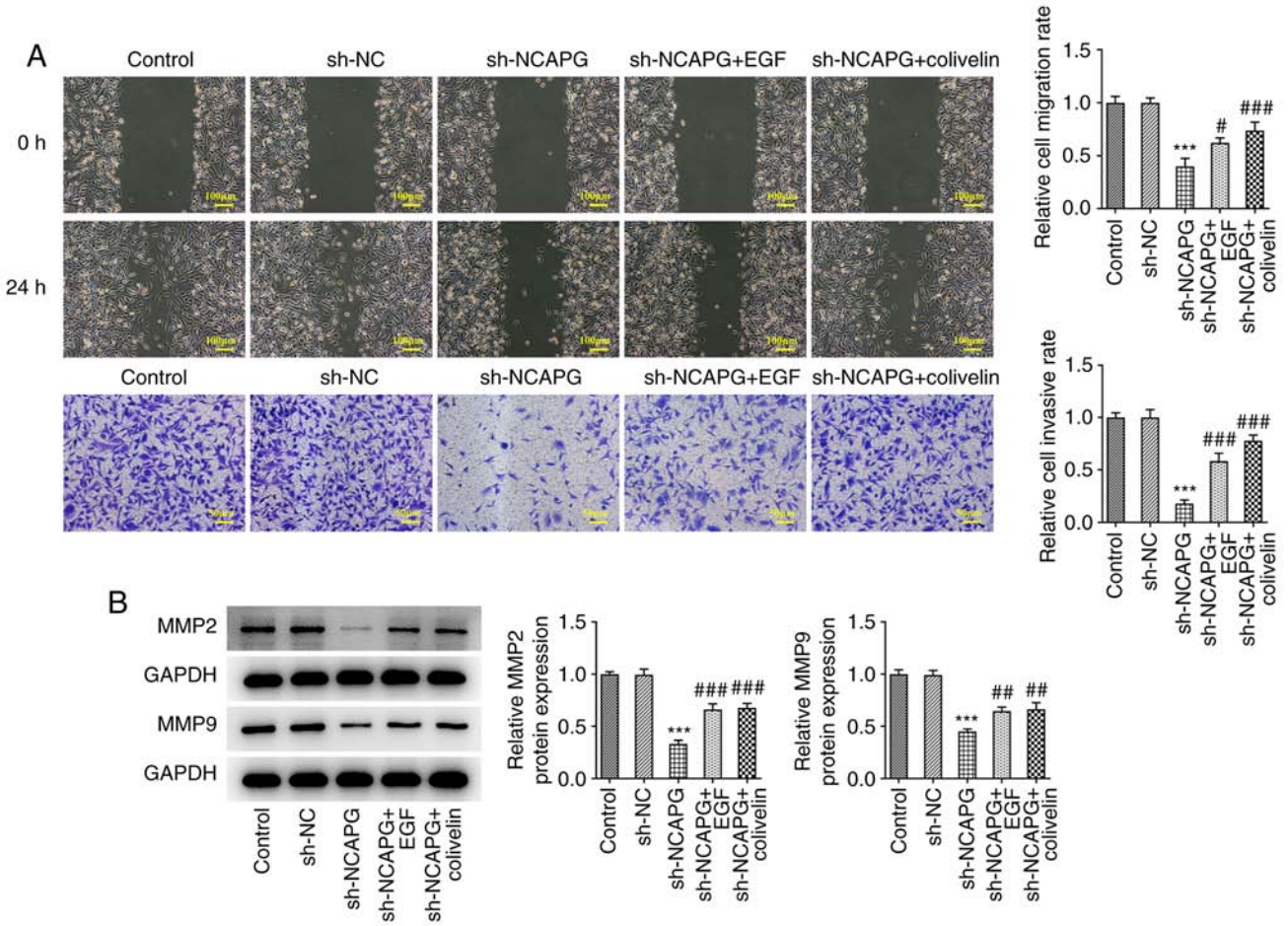

Figure 6. The activation of EGFR/JAK1/STAT3 signaling reduces the inhibitory effect of the downregulation of NCAPG on the invasion and migration of TNBC MDA-MB-231 cell. (A) Wound healing and Transwell assays were used to assess cell invasion and migration. (B) MMP2 and MMP9 proteins were detected by western blot analysis. ***P<0.001 vs. sh-NC; $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. sh-NCAPG. NCAPG, non-SMC condensin I complex subunit G; TNBC, triple-negative breast cancer; EGFR, epidermal growth factor receptor; JAK1, Janus kinase 1; STAT3, signal transducer and activator of transcription 3; MMP, matrix metalloproteinase.

NCAPG was significantly correlated with a poor survival rate in liver cancer, breast cancer, lung cancer and ovarian cancer, amongst others (4). NCAPG has also been shown to be significantly associated with a poor prognosis in TNBC (7). Additionally, it has been shown that NCAPG promotes hepatocellular carcinoma proliferation and reduces apoptosis through the PI3K/AKT signaling pathway in hepatocellular carcinoma cells (15). In lung cancer, NCAPG promotes lung cancer cell proliferation and migration through the TGF-β signaling pathway (16). However, the mechanism by which NCAPG is upregulated in TNBC has not been determined, nor have the downstream effects of its upregulation. In the present study, it was shown that the expression of NCAPG in breast cancer cells, including MCF-7, BT-474, HCC1954 and MDA-MB-231 cells, was significantly increased. After NCAPG expression was inhibited, the activity of MDA-MB-231 cells was decreased, apoptosis was increased, and invasion and migration were significantly decreased. These results showed that downregulation of NCAPG expression inhibited the malignant phenotype of TNBC MDA-MB-231 cells.

Subsequently, the downstream regulatory mechanisms of NCAPG in TNBC MDA-MB-231 cells were explored. It was previously shown that NCAPG silencing in hepatoma cells inhibited cell proliferation and promoted cell apoptosis by reducing the expression of epidermal growth factor receptor (EGFR) (9). In human lung adenocarcinoma, mutations in the EGFR kinase domain mediate signal transducer and activator of transcription 3 (STAT3) activation through interleukin (IL)-6 production (17). EGFR silencing *in vivo* can significantly inhibit the growth of breast cancer cells by regulating the Janus kinase (JAK)/STAT3 signaling pathway (10). Therefore, whether NCAPG could regulate EGFR and its downstream JAK/STAT3 signaling pathway was assessed. It was shown that EGFR/JAK/STAT3 signaling pathway activity was inhibited after NCAPG inhibition. EGFR/JAK/STAT3 signaling pathway activators can reverse the effects of NCAPG on proliferation, invasion, migration and apoptosis of TNBC MDA-MB-231 cells. These results suggest that downregulation/knockdown of NCAPG promotes apoptosis and inhibits invasion and migration of TNBC MDA-MB-231 cells through EGFR/JAK/STAT3 signaling.

There are also certain limitations to the present study. We only assessed the expression of NCAPG in TNBC MDA-MB-231 cells, which will be verified in more breast cancer cell lines in future experiments. In addition, we only examined our findings in cell experiments and did not verify

our results in animal experiments. We will further verify our experimental results in animal experiments in the future.

In conclusion, the results of the present study showed that knockdown of NCAPG promoted apoptosis and inhibited invasion and migration of TNBC MDA-MB-231 cells, and this was achieved through NCAPG-mediated regulation of EGFR/JAK/STAT3 signaling.

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Availability of data and materials

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

Authors' contributions

JL, JZ and BL conceived and designed the study. JL, HS, SL and DW performed experiments. BL and HH wrote the paper. JL, JZ, HS and HH reviewed and edited the manuscript. Data acquisition and analysis were performed by JL and HH. JL and SL confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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