

Long noncoding RNA LINC00885 upregulates NCK1 to promote cell viability and migration of triple-negative breast cancer cells through sponging miR-654-3p

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Abstract.

BACKGROUND: LINC00885 is a novel oncogenic long noncoding RNA (LncRNA) which is upregulated in various types of cancer, but its function in triple-negative breast cancer (TNBC) remains unknown.

OBJECTIVE: This study aimed to determine the role of LINC00885 on TNBC development.

METHODS: Clinical interrelation and survival analysis were determined using online database. The CCK-8 and Transwell assays were used to detect the proliferation and migration behaviors in TNBC cell lines. The interaction among genes was detected by RNA pull down assay.

RESULTS: LncRNA LINC00885 was highly expressed in TNBC compared to normal breast like. Low levels of LINC00885 was related to good prognosis in TNBC patients compared to TNBC patients with high LINC00885. LINC00885-downregulation inhibited, whereas LINC00885-overexpression promoted the proliferation and migration capability of TNBC cell lines. In TNBC cell lines, noncatalytic region of tyrosine kinase 1 (NCK1) expression was positively associated with LINC00885 expression, and shRNA-mediated the depletion of NCK1 significantly abolished LINC00885 upregulation-mediated pro-tumor effects. Combined with online databases, miR-654-3p was screened as the direct target gene of LINC00885, which could directly bind to 3'-untranslated regions (3'-UTR) of NCK1, resulting in the decreased expression of NCK1 in TNBC cell lines. LINC00885 overexpression-mediated the upregulation of NCK1 was abrogated by miR-654-3p mimics. MiR-654-3p mimics significantly rescued the tumor promotive role caused by LINC00885-overexpression. However, exogenous NCK1 notably eliminated the anti-tumor effects caused by miR-654-3p mimics in LINC00885-overexpressed cells.

CONCLUSIONS: LINC00885 is expressed at a high level in TNBC. LINC00885 promoted proliferation and migration by regulating the miR-654-3p/NCK1 axis in TNBC cell lines. Possibly, LINC00885 can be served as a potential therapeutic target for TNBC.

Keywords: Long noncoding RNA LINC00885, triple-negative breast cancer, noncatalytic region of tyrosine kinase 1, miR-654-3p

1. Introduction

Breast cancer (BC) is the most widespread malignancy and the second leading cause of cancer-related

death among the female population worldwide, accounting for ~1/4 of all cancers in females [1,2]. BC can be divided into four subtypes, including luminal A, luminal B, HER2 enrichment and triple-negative breast cancer (TNBC), according to the expression levels of the estrogen receptor (ER), progesterone receptor (PR), HER2 and the proliferation marker protein Ki-67 [3].

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The first-line treatments for BC are surgery, radiotherapy and adjuvant chemotherapy and/or endocrine therapy [4,5]. However, due to the lack of ER/PR/HER2 receptors, TNBC is not responsive to endocrine and target therapy; therefore, the clinical management of TNBC remains a challenging problem [6,7].

TNBC is more aggressive than other BC subtypes because it is associated with increased angiogenesis and epithelial-mesenchymal transition (EMT) [8–10]. At the molecular level, EMT alters the adhesion, polarity and differentiation of epithelial cells and renders them more prone to migration and invasion [10–12]. The noncatalytic region of tyrosine kinase 1 (NCK1) is an adaptor protein acting as a linker of the proximal and downstream signaling events that are related to the occurrence and development of a variety of tumors [13,14]. NCK1 upregulation promotes the development of ovarian carcinoma (OC) [15,16], colorectal cancer (CoC) [17] and cervical squamous cell carcinoma [18]. NCK1 specifically localizes in invadopodia but not in podosomes and can be used to recognize cell surface degradative structures of metastatic mammary carcinoma cells [19]. NCK1 is implicated in podosome formation and ECM degradation [20,21]. Our previous study also indicates that NCK1 elevation promotes the migration of TNBC cell lines and NCK1 may serve as a diagnostic and prognostic marker of metastatic TNBC [22]. Therefore, the ECM degradation and the subsequent EMT process may be involved in NCK1-mediated cell migration.

Long non-coding RNAs (lncRNAs), a cluster of transcripts > 200 bp in length, are implicated in the molecular diagnostics and therapy of several tumors [23,24]. Previous evidence suggests that lncRNAs are frequently abnormally expressed in cancer cells and participate in various cellular processes [25,26]. LncRNAs are frequently dysregulated in a variety of cancer types, including BC, ovarian cancer, hepatocellular carcinoma and others [25,27–30]. Several studies have identified several misregulated lncRNAs in TNBC that act as competitive endogenous RNA (ceRNA) for miRNAs, which are important in tumorigenesis [31,32]. LncRNA NCK1-AS1 can promote NCK1 expression to facilitate tumorigenesis in OC [16]. Thus, lncRNAs may affect the migration of cancer cells by affecting the expression of NCK1. LINC00885 is one of the upregulated lncRNAs in aggressive ductal carcinoma *in situ* lesions [33]. However, if LINC00885-mediated tumor progression involves NCK1 in TNBC remains unclear.

In the current study, the expression of LINC00885 in patients with TNBC and TNBC cell lines was analyzed.

Combining database analysis and RNA pull-down assay, the downstream target genes of LINC00885 were screened and verified. The effects of LINC00885 and its downstream signaling axis on cell growth and migration were also investigated. LINC00885 may serve as a prognostic and predictive marker and as a novel therapeutic target.

2. Materials and methods

2.1. Data collection and analysis

Breast Cancer Gene-Expression Miner v4.4 (<http://bcgenex.centregauducheau.fr/BC-GEM/GEM-Accueil.php>) was used to analyse the mRNA expression levels of LINC00885 in different molecular subtypes of human breast carcinoma (BRCA) as well as in normal tissues. The probability of overall survival of patients with BC was calculated using the Kaplan-Meier Plotter online tool (<https://kmplot.com/analysis/>). The association between genes was evaluated using SPSS 22.0 software (IBM Corp.). The miRNA-lncRNA interactions were investigated using the Encyclopedia of RNA Interactomes (ENCORI; <http://starbase.sysu.edu.cn/>) and LncRNASNP (<http://bioinfo.life.hust.edu.cn/lncRNASNP/#/>) online databases. The binding capability of miR-654-3p to NCK1 was investigated using the TargetScan (<https://www.targetscan.org/>), miRDB (<https://mirdb.org/>), ENCORI and miRWalk (<http://mirwalk.umm.uni-heidelberg.de/>) online databases.

2.2. Subjects

A total of 40 pairs of TNBC and normal tissue samples were acquired from The First People's Hospital of Pingdingshan, China. The present study was approved by the Ethics Committee of Pingdingshan University. Matched pericarcinomatous tissue was defined as tissue > 2 cm distal to the tumor margin. Total RNA was extracted from the tissue for gene expression analysis.

2.3. Cell culture and transfection

A total of four TNBC cell lines (MDA-MB-468, BT549, Hs578t and MDA-MB-231) and one normal breast epithelial cell line (MCF-10A), were pur-

Table 1
Primer sequences of RT-PCR

| Gene | Forward | Reverse |
|------------|---------------------------------|----------------------------------|
| LINC00885 | 5'-GCAGAATGGATCTAATCTGATTCCT-3' | 5'-ATCATCTTTACGGAGGTAGAGAAGAG-3' |
| GAPDH | 5'-AGTCAACGGATTTGGTCGTATTG-3' | 5'-AAACCATGTAGTTGAGGTCAATGAA-3' |
| U6 | 5'-CTCGCTTCGGCAGCACA-3' | 5'-AACGCTTACGAATTTGCGT-3' |
| NCK1 | 5'-GGAAATGCAGGAAGATCAATGG-3' | 5'-CAAACCTTCCAGTGAGTGAAGG-3' |
| MiR-654-3p | 5'-GGGATGTCTGCTGACCA-3' | 5'-CAGTCCGTGTCGTGGA-3' |

chased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences. MDA-MB-468, Hs578t, MDA-MB-231 and MCF-10A cells were cultured in complete Dulbecco's modified Eagle's medium (cat. no. 11965092; Lot. no. 8121295; Gibco; Thermo Fisher Scientific, Inc.) and BT549 cells were cultured in RPMI 1640 medium (cat. no. 11875119; Lot. no. 8117293; Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C with 5% CO₂. All media were supplemented with 10% fetal bovine serum (cat. no. 16140071; Lot. no. 1428478; Gibco; Thermo Fisher Scientific, Inc.), 100 mg/ml streptomycin and 100 U/ml penicillin (Cat. no. 15070063, Lot. no. 2289239; Gibco; Thermo Fisher Scientific, Inc.). The small-interfering (si)RNA targeting LINC00885 (siRNA-LINC00885) and the negative control (NC)-siRNA were from Shanghai GenePharma Co., Ltd. LINC00885 or NCK1 gene fragments were cloned into the pcDNA3.1 vector plasmid to synthesize pcDNA3.1-LINC00885 and pcDNA3.1-NCK1 while the empty pcDNA3.1 vector was used as the NC (pcDNA3.1). The sequences of the short hairpin (sh)RNA targeting NCK1 (shRNA-NCK1) and its NC-shRNA were as follows: 5'-GATCCGGGGTTCTCTGTCTCAGAGAAATTCAAGAGATTTCTCTGACAGAGAACCCTTTTTTACGCGTG-3' and 5'-AATTCACGCGTAAAAAAGGGTTCTCTGTCAGAGAAATCTCTTGAATTTCTCTGACAGAGAACCCCG-3', respectively. In addition, synthesis of miR-654-3p mimic/inhibitor (anti-miR) and NC-miR/anti-miR-NC was performed by Guangzhou RiboBio Co., Ltd. Cells seeded in 24-well plates were transfected using LipofectamineTM 3000 (Cat. no. L3000008; Lot. no. 1991277; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 48 h transfection, cells were harvested for further experiments.

2.4. Reverse transcription-quantitative (RT-q) PCR

Total RNA was extracted from cultured cells and tumour tissue using TRIzolTM reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The miScript reverse transcription

kit (Qiagen GmbH) was used for the reverse transcription of miRNAs. qPCR was performed using the SYBR Premix Ex TaqTM II kit (Cat. no. RR820A; Lot. no. AL13568A; Takara Bio.) on the ABI Prism 7700[®] sequence detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers used are listed in Table 1. The RNA expression levels of LINC00885 and NCK1 were normalized to the internal reference gene GAPDH, whereas U6 was used to normalize the RNA expression levels of miR-654-3p. RNA levels were quantitated using the 2^{-ΔΔC_q} method [34].

2.5. Cell Counting Kit-8 (CCK-8) assay

Cell viability was assessed using the Cell Counting Kit-8 assay (CCK-8; cat. no. CA1210; Lot. no. 20220715; Solarbio), according to the manufacturer's instructions. After transfection for 48 h, cells were plated into 96-well plates at the density of 5 × 10³ cells/well, followed by the addition of 10 μl of the CCK-8 solution for 3 h at 37°C with 5% CO₂. Thereafter, the absorbance at 450 nm of each well was measured using a microplate reader (EnSpire 2300; PerkinElmer, Inc.) at 0, 24, 48 and 72 h.

2.6. Western blot analysis

Total protein from tumor cells was quantified using a bicinchoninic acid assay kit and 20 μg protein/lane was separated by SDS-PAGE on a 15% gel and transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk for 1 h at 25°C, followed by incubation with primary antibodies against NCK1 (cat. no. 15247-1-AP; Proteintech Group, Inc.) and β-actin (cat. no. BL005B; Lot. no. 152848; Biosharp; CN) overnight at 4°C. The membrane was washed three times with phosphate-buffered saline with 0.1% Tween 20 and then incubated with secondary antibody (1:5,000; cat. no. ZB-2305; Lot. no. 216790201; Zhongshan, CN) for 1 h at 25°C. The protein bands were visualized with electrochemiluminescence reagents. Blots were performed in triplicate and protein expression was quantified using ImageJ software (V 1.8.0, National Institutes of Health) with β-actin as the loading control.

2.7. Dual-luciferase reporter gene experiment

The NCK1 and LINC00885 wild type (WT) and mutant (MUT) sequences of the 3'-untranslated regions binding to miR-654-3p were subcloned in the luciferase reporter plasmid pGL3.1 to synthesize NCK1-WT/-MUT and LINC00885-WT/-MUT plasmids, respectively. Plasmids were co-transfected with miR-654-3p mimic or negative mimic for 48 h. Subsequently, a dual-luciferase reporter assay system (cat. no. E1910; Lot. no. 0000449129; Promega Corporation, US) was used to assess the relative luciferase activity, according to the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

2.8. RNA pulldown

Briefly, 1×10^7 MDA-MB-231 cells were harvested, lysed and processed by ultrasonication. To generate probe-coated beads, a miR-654-3p probe labeled with biotin or the negative control probe were incubated with magnetic beads (cat. no. 88817; Lot. no. KC1206538; Thermo Fisher Scientific, Inc.) at 25°C for 2 h. Cell lysates were then incubated with either miR-654-3p or oligo probes at 4°C overnight. After washing with washing buffer, the bead-bound RNA complexes were eluted and extracted using an RNeasy Mini Kit (cat. no. 74104; Lot. no. 154014194; Qiagen GmbH) for qPCR using the LINC00885 and NCK1 primers. The total cell lysate was used as input and set to one.

2.9. Lactate dehydrogenase (LDH) activities measurement

Cells were inoculated in 6-well plates. After transfection and incubation, cells were digested with 0.25% trypsin and washed with phosphate buffer (PBS). After homogenized on ice, protein concentration was quantified by the BCA Protein Assay Kit (cat. no. 23227, lot. no. OB184645, Thermo Fisher Scientific, Inc.). LDH activity was measured by colorimetric assay using specific test kit (cat. no. BC0685, lot. no. 20220926, Solarbio, CN). The absorbance of LDH was measured at the wavelength of 450 nm. The activity of LDH was calculated according to the manufacturer's introduction. The activity of LDH in control group was normalized to 1.0.

2.10. Statistical analysis

All assays were performed ≥ 3 times. Comparisons between groups were performed using GraphPad Prism

(version 6.0, GraphPad Software; Dotmatics), using a paired Student's *t*-test or a one-way ANOVA. Association analysis was performed using Pearson's correlation. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. LINC00885 is upregulated in TNBC tumor tissues and cells

Firstly, the expression pattern of LINC00885 in different subtypes of BC was analyzed according to online databases. Increased LINC00885 expression was observed in patients with basal-like ($n = 864$) and patients with HER2-enrichment (HER2-E; $n = 347$) subtype cancer, while it was downregulated in patients with luminal A subtype ($n = 1,022$), compared with that in the normal breast-like population ($n = 796$) (Fig. 1A). In addition, using BC Gene-Expression Miner v4.4, the LINC00885 expression was also confirmed to be increased in basal-like subtype ($n = 712$) compared with that in normal breast-like cancer ($n = 3,273$), according to basal-like (PAM50) status (Fig. 1B). In the collected fresh human TNBC tissues, LINC00885 mRNA expression was increased in TNBC compared with that in paracarcinoma (Fig. 1C). Additionally, LINC00885 expression was also significantly increased in human TNBC cell lines including MDA-MB-468, T549, BH460, Hs578t, MDA-MB-231, compared with that in the normal mammary epithelial cell line MCF-10A (Fig. 1D). Hence, the present study subsequently tried to investigate the role of LINC00885 in TNBC. Importantly, patients with TNBC and low LINC00885 presented a good prognosis compared with that in TNBC patients with high LINC00885 (Fig. 1E). Therefore, LINC00885 may also be a prognostic predictor for TNBC.

3.2. LINC00885 promotes tumor biological behaviors of TNBC cell lines

Given the upregulation of LINC00885 in TNBC, the effects of LINC00885 on the malignant behaviors of TNBC cell lines were investigated. Firstly, LINC00885-overexpressed and LINC00885-knocked down MDA-MB-231 and BT549 cells were constructed (Fig. S1). A significant increase in cell growth was observed at 48 and 72 h in LINC00885-overexpressed MDA-MB-231 and BT-549 cells, whereas cell growth de-

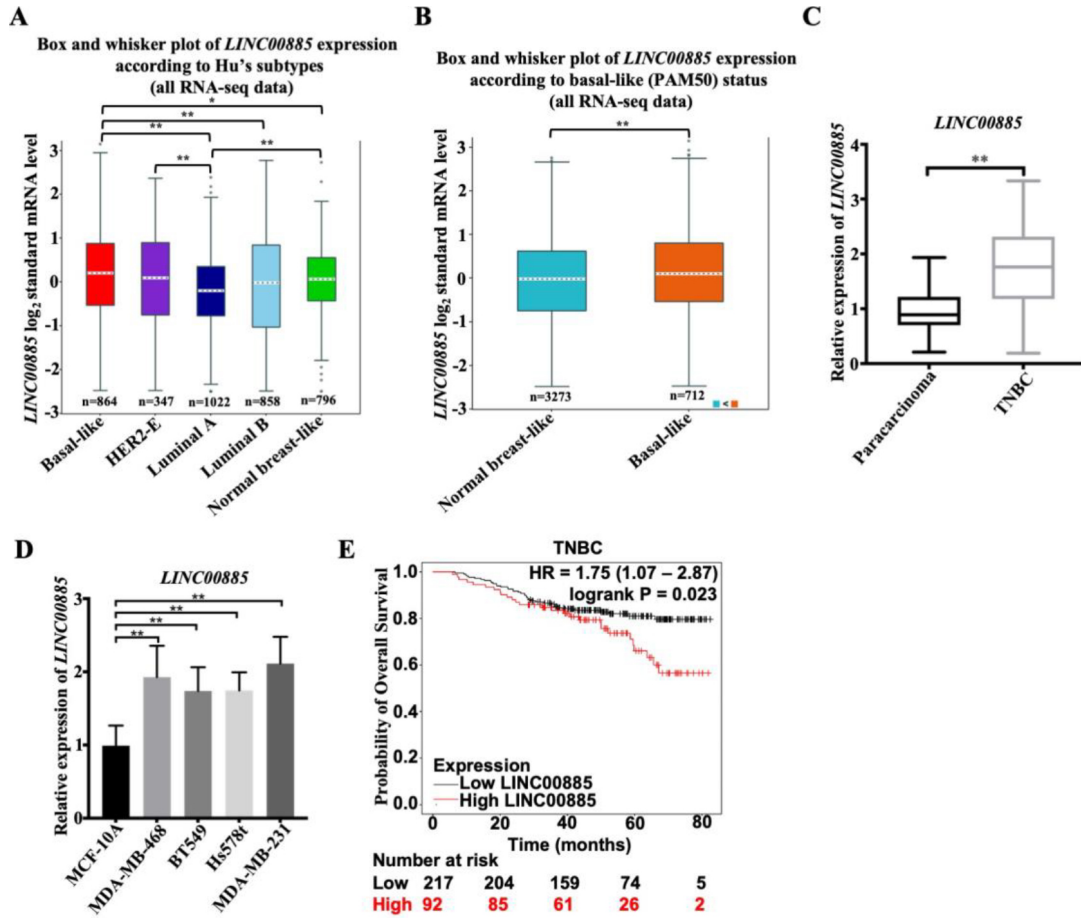


Fig. 1. Relative LINC00885 expression in TNBC and its clinical significance. (A) The differential expression of LINC00885 in different BC Subtypes including basal-like ($n = 864$), HER2-E ($n = 347$), luminal A ($n = 1,022$), luminal B ($n = 858$), normal breast-like ($n = 796$) was determined using the Hu's subtypes. $**P < 0.01$, basal-like vs. normal breast-like. (B) The differential expression of LINC00885 was determined by base-like (PAM50) status in basal-like ($n = 712$) and normal breast-like ($n = 3,273$) of BC. $**P < 0.01$, basal-like vs. normal breast-like. (C) LINC00885 expression in TNBC specimens and para-carcinoma ($n = 40$) was determined using reverse transcription-quantitative PCR. $**P < 0.01$, basal-like vs. normal breast-like. (D) LINC00885 expression in normal mammary epithelial cell MCF-10A, MDA-MB-468, BT549, Hs578t and MDA-MB-231 cells were detected by reverse transcription-quantitative PCR. $**P < 0.01$, basal-like vs. MCF-10A cells. (E) Patients with TNBC were classified into two groups according to the expression state of LINC00885 (low and high LINC00885). Overall Survival (OS) was calculated using the online Kaplan-Meier Plotter (<https://kmpplot.com/analysis/>). Data are presented as mean \pm standard deviation ($n = 3$). $*P < 0.05$ and $**P < 0.01$. BC, breast cancer; TNBC, triple-negative BC; HER2-E, HER2 enrichment.

creases in LINC00885-knocked down MDA-MB-231 and BT-549 cells, compared with that in the control group (Fig. 2A and B). These results demonstrated that the overexpression of LINC00885 could promote cell viability of TNBC cell lines, whereas LINC00885 knockdown could negatively impact the growth rate of TNBC cell lines (Fig. 2A and B). Consistently, Transwell migration assays also indicated that LINC00885 knockdown suppressed the migration of MDA-MB-231 and BT549 cells, whereas this was increased following the LINC00885 overexpression (Fig. 2C and D). Additionally, the levels of lactate dehydrogenase (LDH) that were detected using colorimetric analy-

sis increased in LINC00885-overexpressed MDA-MB-231 cells and moderately decreased in LINC00885-downregulated MDA-MB-231 cells (Fig. S2A). These results indicated that LINC00885 facilitated the viability and migration of TNBC cell lines *in vitro* and might also be related to glycolysis.

3.3. LINC00885 and NCK1 in TNBC are positively associated

NCK1 is a protein that has previously been reported to be closely associated with cancer progression [17,18]. In the present study, NCK1 expression

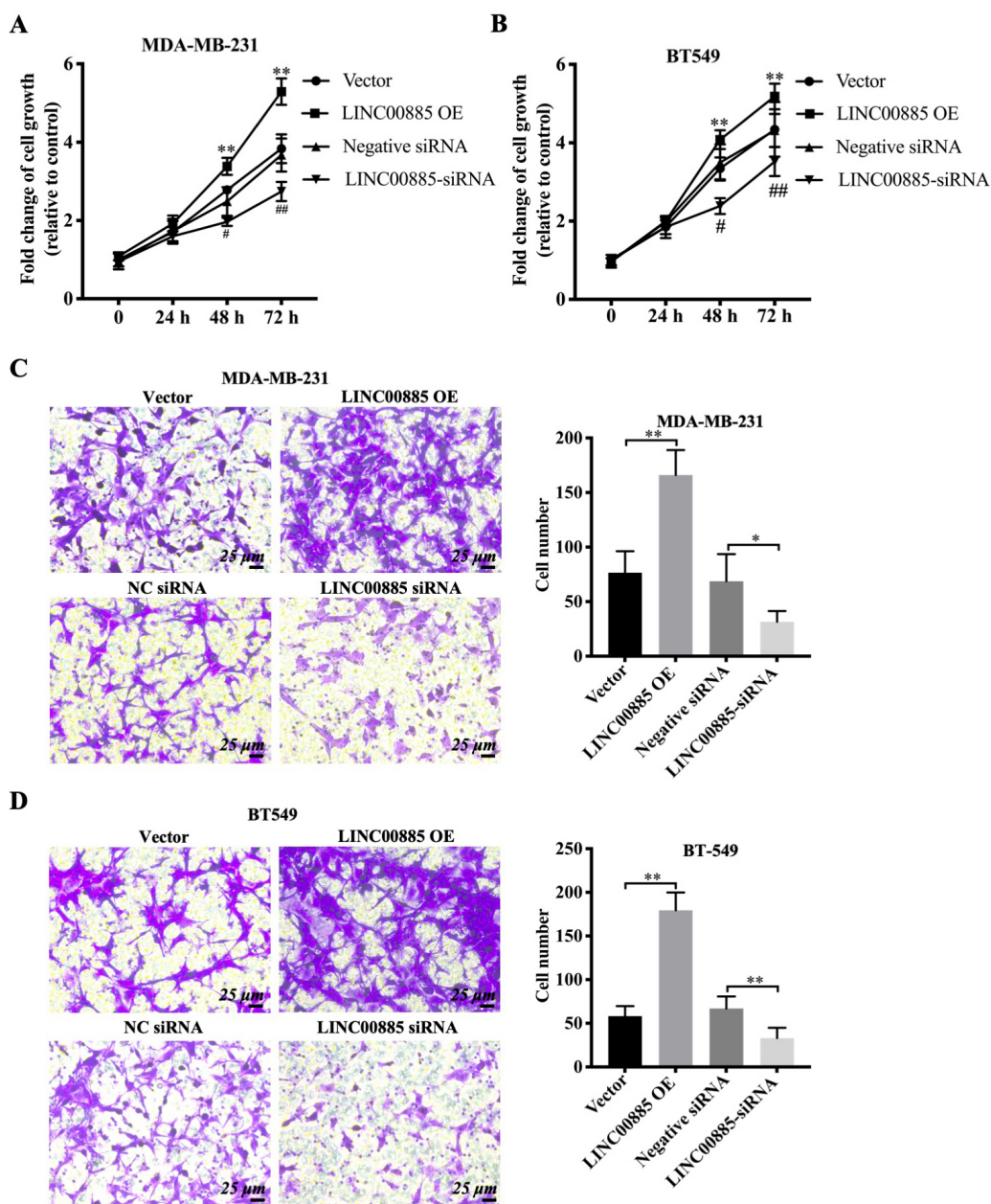


Fig. 2. Effect of LINC00885 on the migration of triple-negative breast cancer cells. The plasmid overexpressing LINC00885, LINC00885 siRNA and the corresponding control vector/NC-siRNA were injected into BT549 and MDA-MB-231 cells. MDA-MB-231 (A) and BT549 cell (B) viabilities are determined using the Cell Counting Kit-8 assay. Transwell assay for pcDNA3.1-LINC00885 and siRNA-LINC00885 (C) MDA-MB-231 and (D) BT549 cells. Relative quantification of migrated cells is shown in the right panel (scale bar, 25 μ m). ** $P < 0.01$, pcDNA3.1-LINC00885 or siRNA-LINC00885 vs. pcDNA3.1 or NC-siRNA. NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885.

was positively correlated with LINC00885 expression in 40 patients with TNBC (Fig. 3A). In addition, mRNA expression of NCK1 was increased by 2.5-fold along with the increased LINC00885 expression, while NCK1 expression was effectively knocked down

by 75% in MDA-MB-231 and BT549 cells (Fig. 3B). Moreover, NCK1 protein level increased following LINC00885 overexpression, while it decreased after LINC00885 knockdown in MDA-MB-231 and BT549 cells (Fig. 3C). These results indicated that NCK1 ex-

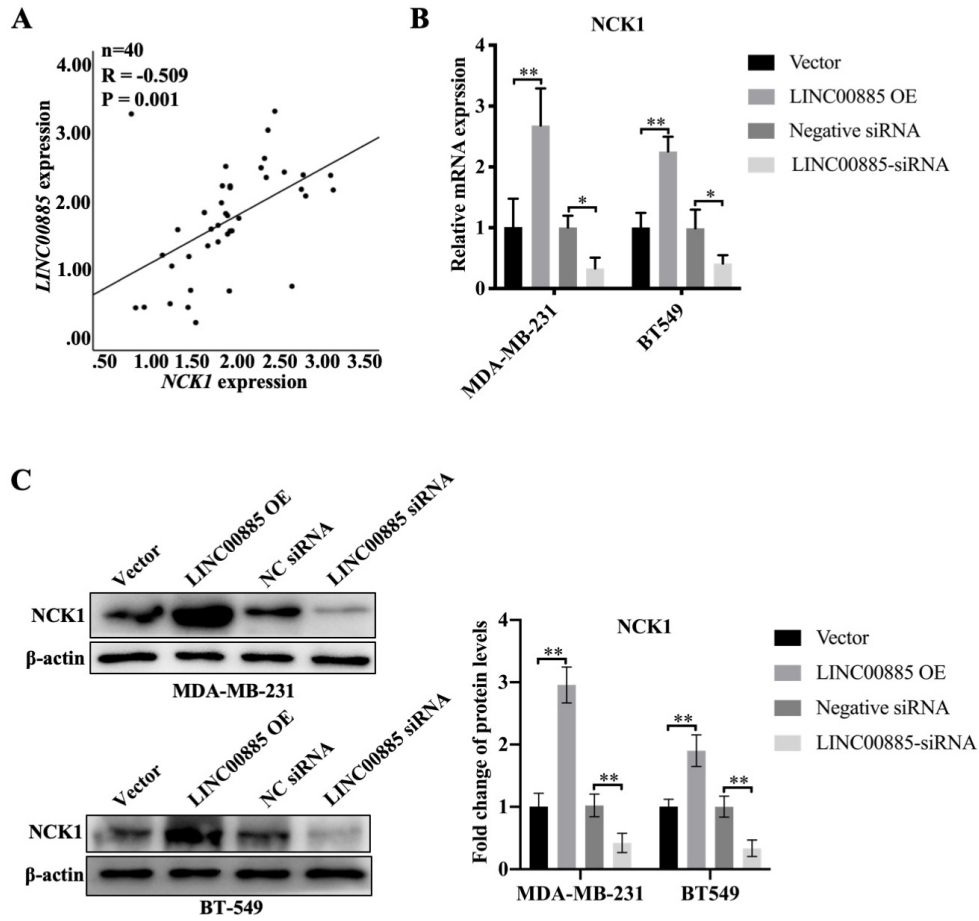


Fig. 3. Association between LINC00885 and NCK1 in TNBC. (A) The expression of LINC00885 and NCK1 are determined by RT-qPCR. The association between LINC00885 and NCK1 expression in fresh TNBC specimens as analyzed using SPSS 22.0 software (IBM Corp.). (B) MDA-MB-231 and BT549 cells were transfected with pcDNA3.1-LINC00885, siRNA-LINC00885 and the corresponding negative controls pcDNA3.1 or NC-siRNA. NCK1 mRNA was detected by RT-qPCR in MDA-MB-231 and BT549 cells. $**P < 0.01$, pcDNA3.1-LINC00885 or siRNA-LINC00885 vs. pcDNA3.1 or NC-siRNA, respectively. (C) The NCK1 protein levels of MDA-MB-231 and BT549 cells were determined by WB. Data are presented as mean \pm standard deviation ($n = 3$). $*P < 0.05$ and $**P < 0.01$. NCK1, noncatalytic region of tyrosine kinase 1; RT-qPCR, reverse transcription-quantitative PCR; TNBC, triple-negative breast cancer; R, Pearson's coefficient; NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885.

pression was positively associated with LINC00885 expression.

3.4. NCK1 downregulation abolished the promotive role of LINC00885 in cell viability and migration of TNBC cell lines

Subsequently, the functional role of NCK1 in LINC00885-mediated alteration of cell proliferation and migration was investigated. Overexpression of LINC00885 significantly increased the viability of MDA-MB-231 and BT549 cells from 48 to 72 h (Fig. 4A). However, shRNA-mediated NCK1 knock-

down decreased the cell viability of LINC00885 overexpression plasmid-transfected MDA-MB-231 and BT549 cell lines, compared with that of MDA-MB-231 and BT549 cells transfected with negative control shRNA and pcDNA3.1-LINC00885 plasmid (Fig. 4A). Consistently, LINC00885 upregulation promoted the migration of TNBC cell lines, which were not affected by the co-transfection of pcDNA3.1-LINC00885 and negative control shRNA (Fig. 4B and C). Once NCK1 was downregulated, cell migration and migrated cell number were restrained in LINC00885-overexpressing TNBC cell lines (Fig. 4B and C). In addition to that, LINC00885-overexpression-mediated the increase of

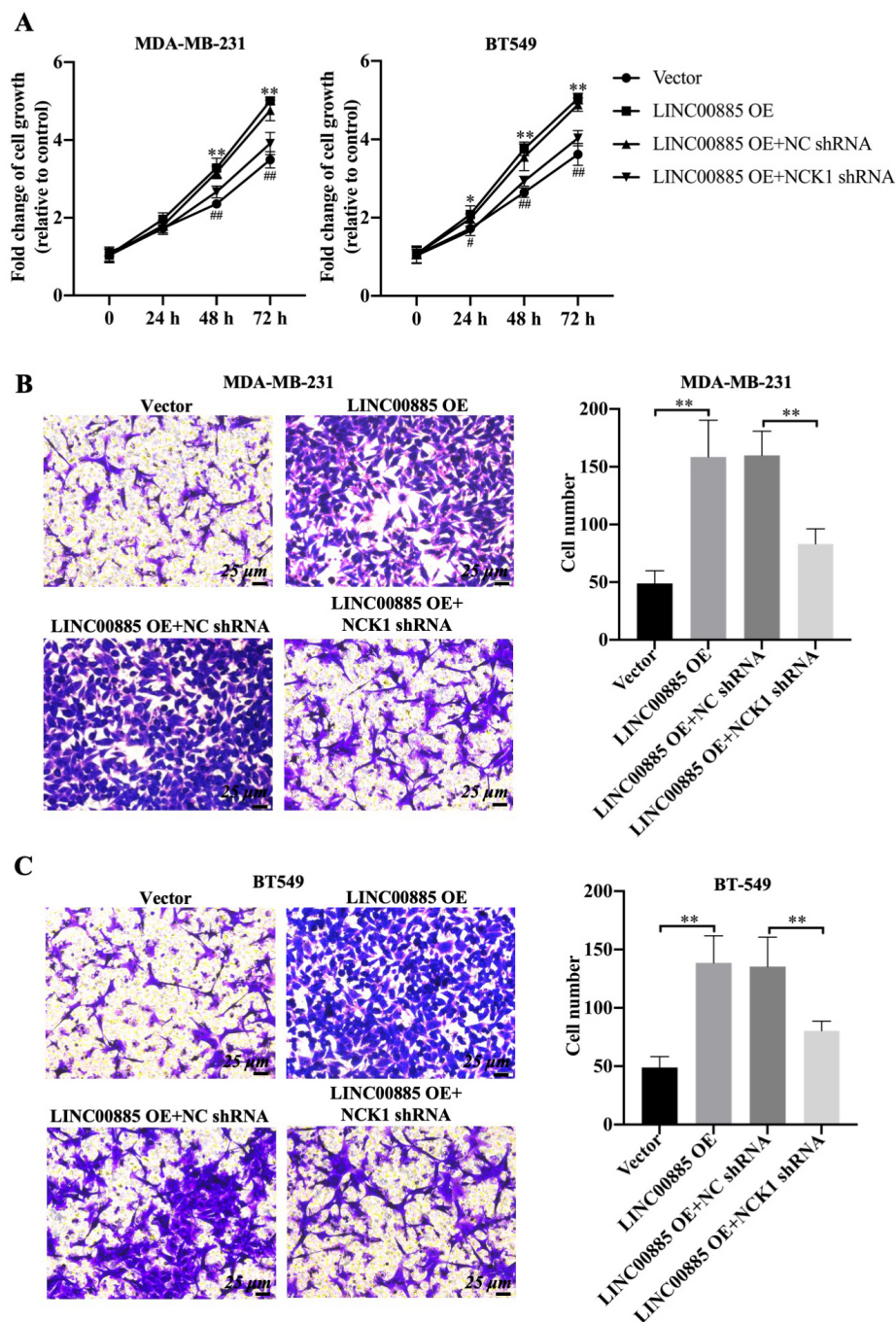


Fig. 4. Effects of NCK1 on the migration of LINC00885-mediated triple-negative breast cancer cells. MDA-MB-231 and BT549 cells were transfected with pcDNA3.1, LINC00885-overexpression plasmid, pcDNA3.1-LINC00885 + NC-shRNA and pcDNA3.1-LINC00885 + shRNA-NCK1. (A) Cell growth of MDA-MB-231 cells and BT549 cells as measured using the Cell Counting Kit-8 assay. (B) MDA-MB-231 and (C) BT549 cell migration was assessed using the Transwell assay. The relative quantification of migrated cells is illustrated in the right panel (scale bar, 25 μ m). * $P < 0.05$ and ** $P < 0.01$. NCK1, noncatalytic region of tyrosine kinase 1; NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885.

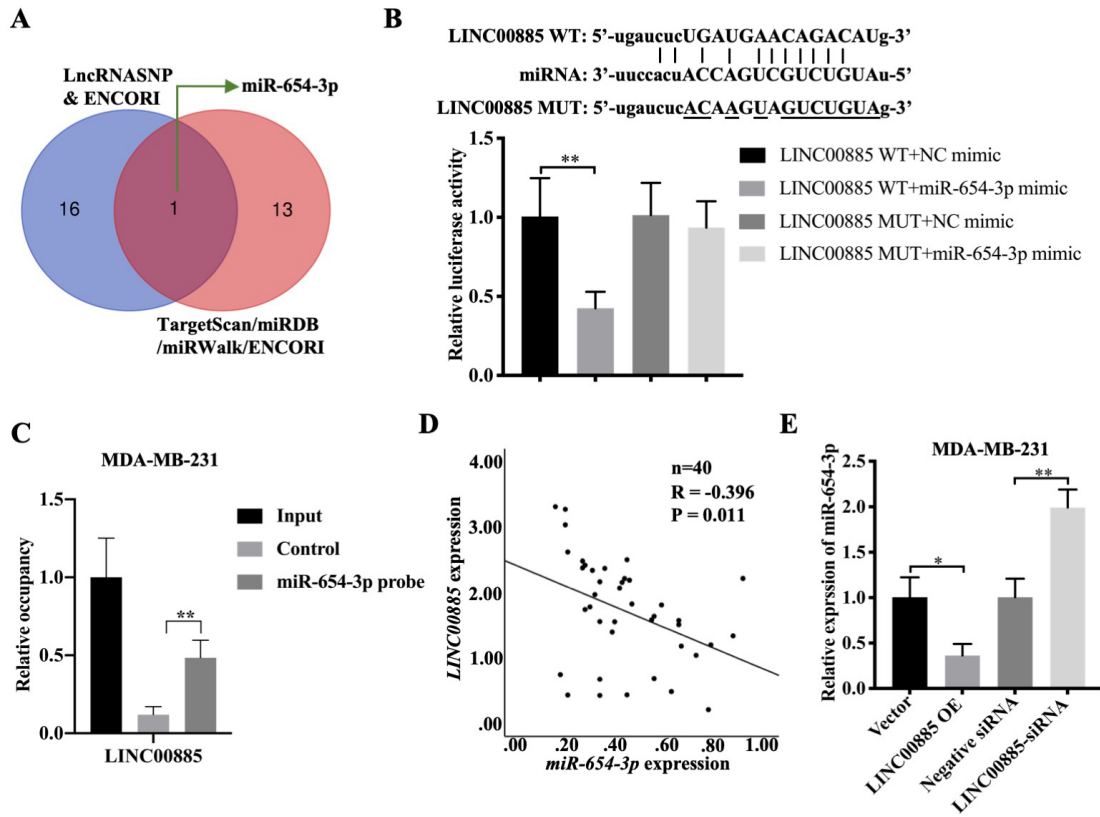


Fig. 5. Relationship between LINC00885 and miRNA. (A) Two databases predicting miRNAs that bound to LINC00885. (B) The binding sites between LINC00885 and miR-654-3p as analyzed using the ENCORI database (<http://starbase.sysu.edu.cn/>). The luciferase activity was determined using the corresponding kits following transfection with LINC00885-WT and LINC00885-MUT reporter plasmid in the presence of NC-miR or miR-654-3p mimic. ** $P < 0.01$, LINC00885-WT and miR-654-3p mimic vs. LINC00885-WT and NC-miR. (C) Cell lysates were incubated with biotinylated-miR-654-3p or control probes. The presence of LINC00885 in the RNA-binding complex was confirmed using RT-qPCR. Data are presented as mean \pm standard deviation ($n = 3$). * $P < 0.05$ and ** $P < 0.01$. (D) Pearson's correlation analysis was used to evaluate the relationship of LINC00885 and miR-654-3p in triple-negative breast cancer tissues. (E) Transfections of pcDNA3.1-LINC00885, siRNA-LINC00885 and the corresponding controls pcDNA3.1 or NC-siRNA were performed on MDA-MB-231 cells. The mRNA levels of miR-654-3p were measured with RT-qPCR in MDA-MB-231 cells. ** $P < 0.01$, pcDNA3.1-LINC00885 or siRNA-LINC00885 vs. pcDNA3.1 or NC-siRNA. * $P < 0.05$ and ** $P < 0.01$. WT, wild type; MUT, mutation; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885.

LDH was also restored by the knockdown of NCK1 in MDA-MB-231 cells (Fig. S2B). Therefore, LINC00885 upregulation promoted cell viability, migration and LDH level in TNBC cell lines, that could be rescued by NCK1 knockdown.

3.5. MiR-654-3p is negatively correlated with LINC00885 in TNBC cell lines

LncRNAs can function as ceRNAs to inhibit the degradation of mRNAs by sponging miRNAs in the cytoplasm of cells [35]. Thus, the present study investigated if LINC00885 could regulate NCK1 expression through the ceRNA mechanism by directly binding

to miRNAs in TNBC cell lines. Using LncRNASNP and ENCORI databases, 17 miRNAs that could bind to LINC00885 were predicted and screened (Fig. S3A). Meanwhile, miRNAs binding to NCK1 were predicted using TargetScan, miRDB, miRWalk and ENCORI, and 14 candidate miRNAs were identified (Fig. S3B). The overlapping gene between the 17 miRNAs and 14 candidate miRNAs was miR-654-3p. Thus, miR-654-3p was screened as the only one candidate that could bind to LINC00885 and NCK1 (Fig. 5A). Moreover, the dual-luciferase reporter assay demonstrated that the luciferase activity in the cells transfected with the luciferase reporter plasmid LINC00885-WT was inhibited by miR-654-3p mimic (Fig. 5B). However, relative

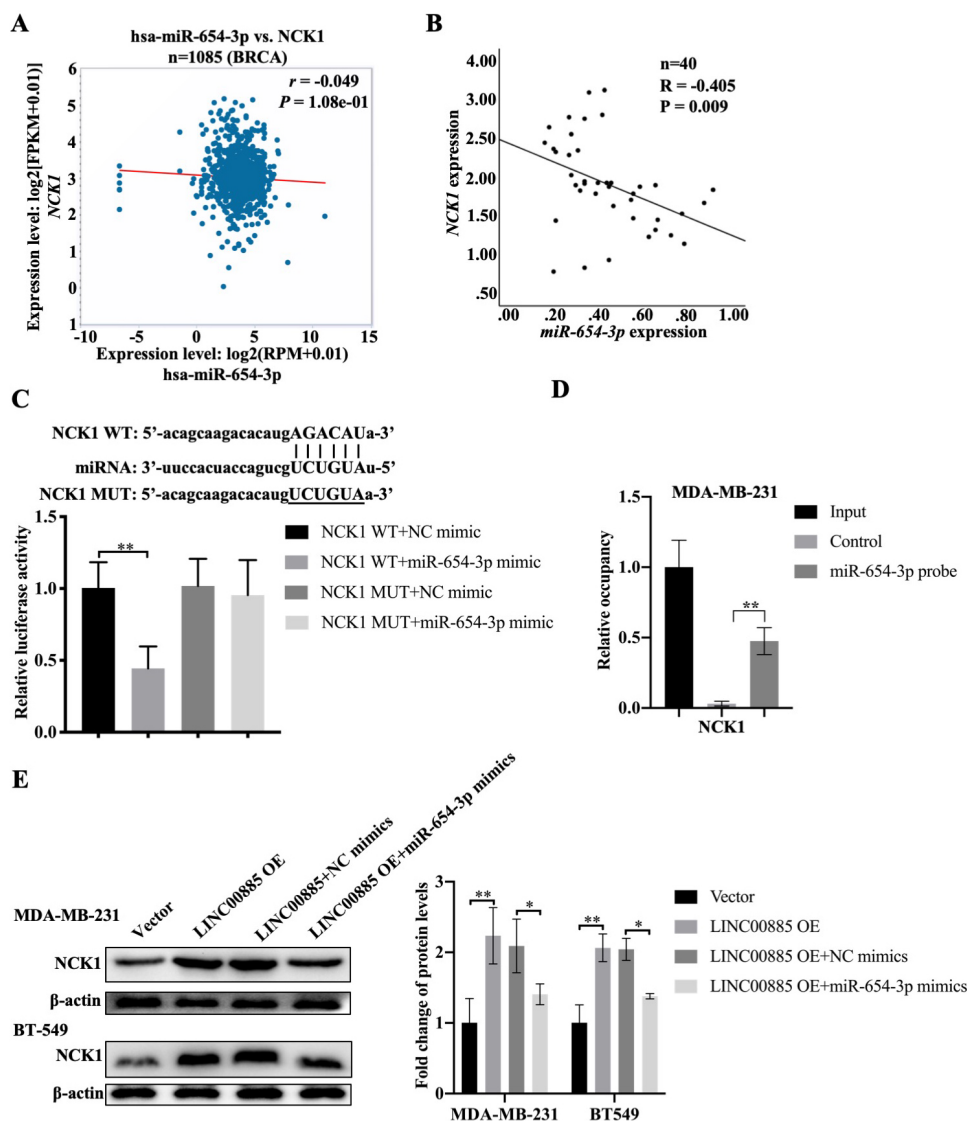


Fig. 6. Correlation among LINC00885, miR-654-3p and NCK1. (A) Study on the relationship between NCK1 and miR-654-3p in breast cancer using the ENCORI database (<http://starbase.sysu.edu.cn/>). (B) The correlation between the expression of NCK1 and LINC00885 in fresh triple-negative breast cancer specimens was studied using SPSS 22.0 software (IBM Corp.). (C) NCK1 and miR-654-3p binding site analysis using the ENCORI database. Following co-transfection of the NCK1-WT or NCK1-MUT reporter vectors with either NC-miR or miR-654-3p mimic, the respective luciferase reporter kits were used to measure luciferase activity. $**P < 0.01$, NCK1-WT and miR-654-3p mimic vs. NCK1-MUT and NC-miR. (D) Cell lysates were incubated with biotinylated-miR-654-3p or control probes. The expression of NCK1 in an RNA binding complex was then validated using reverse transcription-quantitative PCR. Data are presented as mean \pm standard deviation ($n = 3$). $*P < 0.05$ and $**P < 0.01$. (E) MDA-MB-231 cells were transfected with pcDNA3.1, pcDNA3.1-LINC00885, pcDNA3.1-LINC00885 + NCmiR, pcDNA3.1-LINC00885 + miR-654-3p mimic, NC-siRNA, LINC00885 siRNA, siRNA-LINC00885 + anti-miR-NC and siRNA-LINC00885 + anti-miR. The NCK1 protein levels of MDA-MB-231 and BT549 cells were determined by western blotting. $*P < 0.05$ and $**P < 0.01$. NCK1, noncatalytic region of tyrosine kinase 1; WT, wild type; MUT, mutation; R, Pearson's coefficient; NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885; anti-miR, miR inhibitor.

luciferase activity in cells transfected with LINC00885-MUT luciferase reporter plasmid was not affected by miR-654-3p mimic (Fig. 5B). These results, combined with the outcomes of the RNA pull-down assay, con-

firmed the direct binding of LINC00885 to miR-654-3p in MDA-MB-231 cells (Fig. 5C). In addition, miR-654-3p emerged as a potential negative correlation factor of LINC00885 in 40 pairs of TNBC and normal

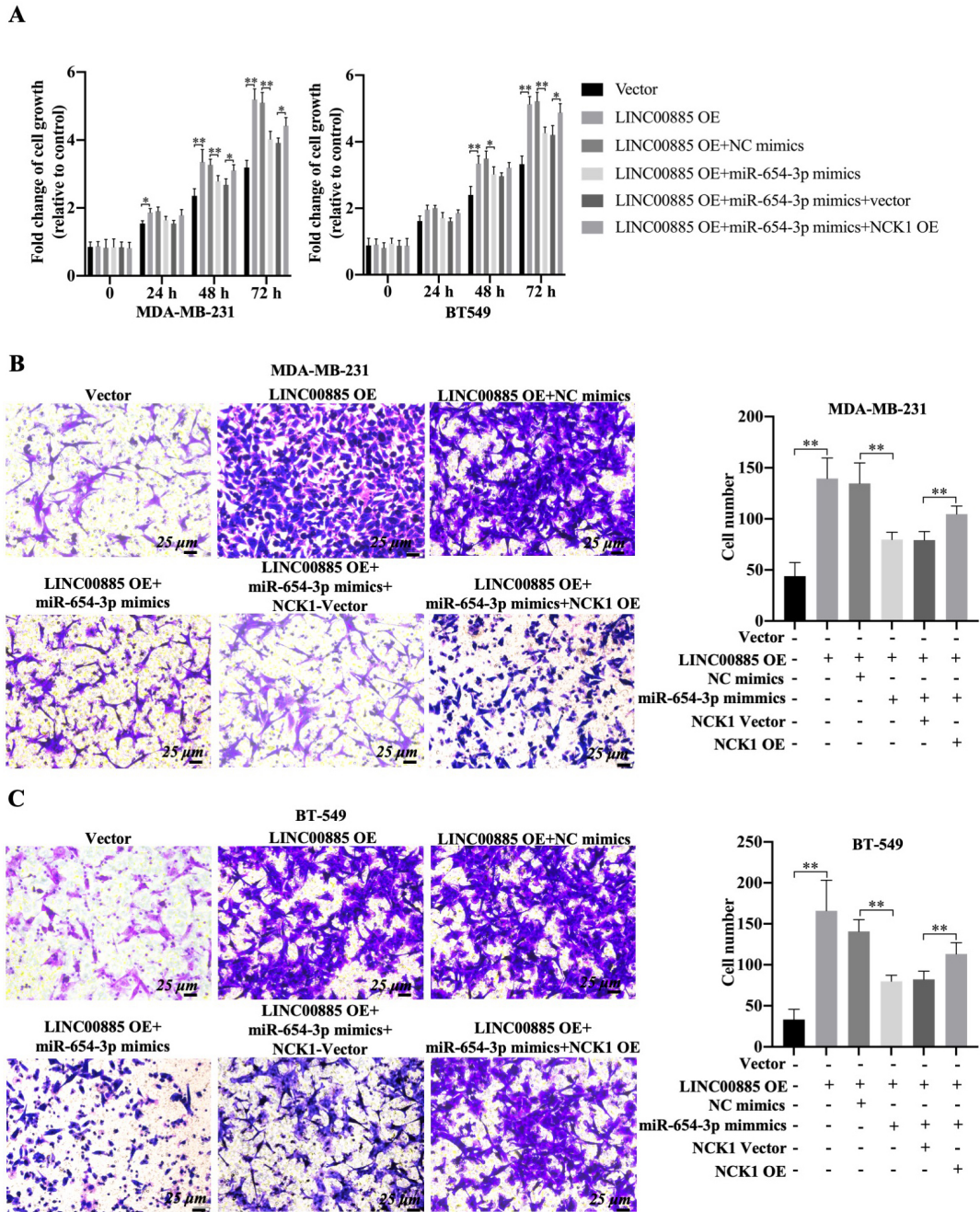


Fig. 7. Effect of LINC00885/miR-654-3p/NCK1 pathway on the migration of triple-negative breast cancer cells. MDA-MB-231 and BT549 cells were transfected with pcDNA3.1-LINC00885, pcDNA3.1-LINC00885 + NC-shRNA, pcDNA3.1-LINC00885 + mir-654-3p mimic and pcDNA3.1-LINC00885 + mir-654-3p mimic + pcDNA3.1-NCK1. (A) Reverse transcription-quantitative PCR was used to observe the growth of MDA-MB-231 and BT549 cells. Migration of (B) MDA-MB-231 and (C) BT549 cells was measured using the Transwell assay. The relative quantitation of the transferred cells is illustrated in the right panel (scale bar, 25 μ m). * $P < 0.05$ and ** $P < 0.01$. NCK1, noncatalytic region of tyrosine kinase 1; WT, wild type; MUT, mutation; R, Pearson's coefficient; NC, negative control; pcDNA3.1, plasmid NC; si-RNA, small interfering RNA; pcDNA3.1-LINC00885, LINC00885-overexpression plasmid; siRNA-LINC00885, siRNA targeting LINC00885; anti-miR, miR inhibitor; pcDNA3.1-NCK1; NCK1-overexpression plasmid.

tissue samples (Fig. 5D). Moreover, LINC00885 overexpression also inhibited miR-654-3p expression, while LINC00885 knockdown increased the expression of miR-654-3p in MAD-MB-231 cells (Fig. 5E). These results indicated that miR-654-3p was the potential binding target of LINC00885 in TNBC cell lines.

3.6. MiR-654-3p is negatively correlated with NCK1 in TNBC cell lines

Based on the ENCORI database, miR-654-3p appeared to be not associated with NCK1 in patients with BC (BRCA, $n = 1,085$; Fig. 6A). However, the association was analyzed in considering all the patients with BRCA and was not restricted to patients with TNBC. Thus, their association was also evaluated in 40 pairs of TNBC and normal tissue samples. In the fresh TNBC tissues, the negative correlation between miR-654-3p and NCK1 was further confirmed ($r = -0.405$; Fig. 6B). A reporter gene assay was then used to test for direct binding of miR-654-3p to NCK1. Notably, the relative luciferase activity of cells transfected with the NCK1-WT reporter plasmid was shown to be repressed by the miR-654-3p mimic, although the luciferase activity of cells transfected with the NCK1-MUT reporter plasmid was unaffected by the miR-654-3p mimic, indicating that miR-654-3p could downregulate NCK1 protein expression (Fig. 6C). Moreover, RNA pull-down experiment further confirmed the direct binding of miR-654-3p to NCK1 (Fig. 6D). In summary, NCK1 expression was inversely correlated with miR-654-3p expression in TNBC cell lines. Notably, the increase in NCK1 expression induced by LINC00885 overexpression was significantly counteracted by the transfection with miR-654-3p mimic in MDA-M-231 and BT549 cells (Fig. 6E). These results indicated that NCK1 was the downstream targeting gene of miR-654-3p in TNBC cell lines and NCK1 expression could be regulated by the LINC00885/miR-654-3p signaling axis.

3.7. LINC00885/miR-654-3p/NCK1 signaling axis participates in the tumor behaviors of TNBC cell lines

The functional roles of NCK1 and miR-654-3p in LINC00885-mediated alteration of cell migration were investigated. Using CCK-8 assay, overexpression of LINC00885 significantly increased the viability of BT549 cells at 48 and 72 h and promoted cell proliferation of MDA-MB-231 cells from 24–72 h (Fig. 7A). However, miR-654-3p mimic decreased the

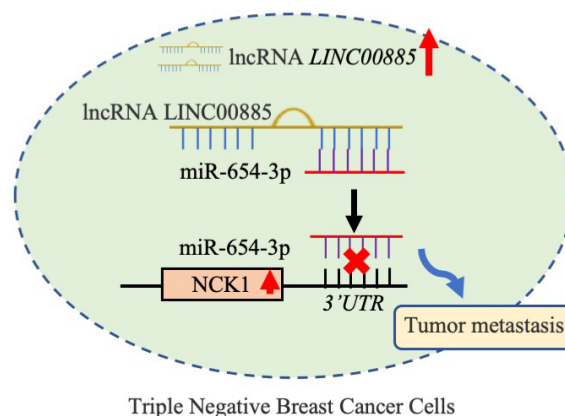


Fig. 8. Schematic diagram of the mechanism. LINC00885 upregulated NCK1 expression by sponging miR-654-3p in TNBC cell lines, thereby promoting cancer progression.

proliferation of LINC00885-overexpressing TNBC cell lines compared with that of TNBC cell lines transfected only with pcDNA3.1-LINC00885 (Fig. 7A). On the other hand, the proliferation inhibitory effect of miR-654-3p mimic was partially restored in TNBC that overexpressed both LINC00885 and NCK1 (Fig. 7A). In addition, the combined transfection of pcDNA3.1-LINC00885 and miR-NC did not affect the pro-migration role and the upregulation of LDH by LINC00885 overexpression (Fig. 7B, 7C and Fig. S2C). Once the miR-654-3p mimic was transfected, the cell migration and LDH level decreased in LINC00885-overexpressing TNBC cell lines (Fig. 7B, 7C and Fig. S2C). Upon NCK1 overexpression, the inhibitory effect of miR-654-3p mimics could be slightly restored. Therefore, LINC00885 overexpression promoted the proliferation and migration of MDA-MB-231 and BT549 cells. Possibly, the tumor-promoting properties of LINC00885 could be regulated by the miR-654-3p/NCK1 signaling axis.

4. Discussion

TNBC is a subtype of BC with a considerable migration characteristics and a high degree of malignancy and heterogeneity [36,37]. TNBC is one of the most fatal cancers in females because of its poor prognosis and limited treatment options; moreover, blockade of cell migration is the primary approach for ameliorating TNBC [38,39]. The present findings indicated that LINC00885 was highly expressed in TNBC cell lines and LINC00885 increased the tumor behaviors of TNBC cell lines through promoting NCK1 expres-

sion; therefore, LINC00885 could be a positive regulator of cell migration and aerobic glycolysis in TNBC cell lines.

LINC00885 is an oncogenic factor in cervical carcinoma (CeC) [40,41] and early-stage BC [42]. Increased levels of LINC00885 in CeC tissues may be considered prognostic biomarkers [41]. These results suggested that LINC00885 had a high level of upregulation and was related to overall survival in patients with TNBC. LINC00885 promoted tumorigenesis and invasion of CeC cells [40,43]. LINC00885 silencing in BC cells significantly decreased cell migration and motility by downregulating the expression of proliferation-related transcripts such as epiregulin, MYC proto-oncogene and cyclin D1 [42]. The present functional analysis revealed that overexpression of LINC00885 could promote the migration of TNBC cell lines. These results showed that LINC00885 was involved in the migration of TNBC cell lines. Possibly, the abnormally increased expression of LINC00885 in TNBC might be related to the increased cell migration. Previous study also suggested that LINC01605 remarkably restrained cell proliferation and migration, as well as aerobic glycolysis by inhibiting LDH in TNBC cell lines [44]. Herein, LINC00885 also positively regulated the level of LDH in TNBC cell lines. Thus, the promotive role of LINC00885 on cell migration might also be related to the process of aerobic glycolysis.

The ceRNA acts as a miRNA 'sponge' and competes with miRNA-targeted mRNAs to influence miRNA-mediated gene regulation [45]. LINC00885 promoted tumorigenesis of colorectal cancer (CoC) in *in vivo* model by upregulating MACC1 expression as ceRNA for microRNA-432-5p [46]. LINC00885 promoted CoC progression by sponging miR-3150b-3p and upregulating bromodomain adjacent to zinc finger domain 2A [41–43,46]. Thus, LINC00885 could regulate cell proliferation and migration via the ceRNA mechanism, thereby influencing the development of TNBC. MiR-654-3p inhibited non-small cell lung cancer tumorigenesis [47]. Previous study demonstrated that miR-654-3p might exert a suppressor role in hepatocellular carcinoma through inhibiting tumor cell proliferation, migration, and invasion [48]. The *in vitro* restoration of miR-654-3p decreased cell proliferation and migration and induced reprogramming of metastasis-related genes in Papillary thyroid carcinoma cells [49]. However, in the present study, miR-654-3p inhibition could reverse LINC00885-mediated the increase of proliferation and migration in TNBC cell lines. Therefore, LINC00885 induced cell viability and migration in

TNBC cell lines by sponging miR-654-3p. In addition, the present results demonstrated that NCK1 expression was positively correlated with LINC00885 expression and miR-654-3p was negatively associated with NCK1. LINC00885 regulated downstream NCK1 expression by targeting miR-654-3p through the ceRNA mechanism. A previous study indicated that NCK1 regulated EGFR-induced cell migration and tumor migration with an on-off mechanism [50]. The migration-promoting effect of NCK1 was demonstrated in several types of cancer including BC [51]. In the present study, the miR-654-3p-mediated improvement of the tumor process could be counteracted by the upregulation of NCK1. Collectively, LINC00885 upregulated NCK1 expression by sponging miR-654-3p in TNBC cell lines, thereby promoting cancer progression. The LINC00885/miR-654-3p/NCK1 pathway may play an important role in TNBC cell migration (Fig. 8).

Although the present study identified a novel lncRNA associated with an increased malignant potential in TNBC, which may become a novel biomarker and potential therapeutic target for TNBC, there are still some drawbacks to be solved in future studies. Firstly, the present findings could not be further confirmed due to the lack of *in vivo* tumor migration models. Secondly, a single lncRNAs/miRNAs signaling axis is not sufficient to prompt a clinical application and change the status quo of clinical treatment. Simultaneously, further studies are needed to determine how NCK1 affects tumor cell migration.

5. Conclusion

In summary, the current study uncovered the promotive role of LINC00885 on cell migration in TNBC, elucidating a novel ceRNA network for LINC00885/miR-654-3p/NCK1 in TNBC cell lines. LINC00885 might become a new biomarker for the prediction of cell migration and the treatment of TNBC.

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Author contributions

Conception: S.J.Y.
Interpretation or analysis of data: H.P.N., L.Z. and Q.J.X.

Preparation of the manuscript: H.P.N. and S.J.R.
Revision for important intellectual content: S.J.Y., L.Z. and Q.J.X.

Supervision: S.J.Y and H.P.N.

All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its additional files.

Ethics approval and Consent to participate

All experiments were approved by the Ethics Committee of Pingdingshan University. The data analyzed in this study all were downloaded from online database.

Patients consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no competing interests.

Supplementary data

The supplementary files are available to download from <http://dx.doi.org/10.3233/CBM-230143>.

List of abbreviations

TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor 2; OC, ovarian carcinoma; EMT,

epithelial mesenchymal transition; ECM, extracellular matrix; NCK1, Noncatalytic region of tyrosine kinase 1; TCGA, The Cancer Genome Atlas; GEPIA, Gene Expression Profiling Interactive Analysis; BRCA, breast carcinoma; CSCC, cervical squamous cell carcinoma; ceRNA, competing endogenous RNAs; CC, cervical cancer;

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