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Long noncoding RNA LINC00473 functions as a competing endogenous RNA to regulate MAPK1 expression by sponging miR-198 in breast cancer

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ABSTRACT

Breast cancer (BC) is one of the primary tumors with high incidence in women. The purpose of this study was to investigate the role of LINC00473 and underlying mechanisms in BC. Expression pattern of LINC00473 was analyzed using qRT-PCR (quantitative real-time polymerase chain reaction) assays in BC tissues and cells. Overexpression or knockdown of LINC00473 in vitro and functional experiments were performed to study its effects on BC cells. Target prediction, luciferase assays, RNA fluorescence in situ hybridization and RNA immunoprecipitation were used to verify the role of LINC00473 as a competing endogenous RNA. The impact of LINC00473 on tumor growth was also evaluated using a xenograft model. In our study, we found that LINC00473 was highly expressed in BC tissues and cells, and the elevated expression was correlated with shorter overall survival in patients with BC. Furthermore, knockdown of LINC00473 significantly inhibited the capacity of proliferation, invasion and migration of BC cells. Animal experiment suggested that silencing LINC00473 could significantly inhibit the tumor growth. Following experiments revealed that LINC00473 may function as a competing endogenous RNA to regulate the expression of Mitogen-Activated Protein Kinase 1 (MAPK1) through competition for miR-198. Thus, increased expression of LINC00473 in breast cancer tissues is linked to poor prognosis. LINC00473 may function as an endogenous completive RNA by sponging miR-198 to regulate MAPK1 expression. Findings of our study contributed to the basis for further exploring the application of LINC00473 as a prognostic and diagnostic biomarker.

1. Introduction

Breast cancer (BC) is increasingly recognized as one of the most common and aggressive malignancies in women [1]. Experience recurrence or distal metastasis is a major clinic challenge after treatments including surgery, hormonal therapy, radiation therapy and chemotherapy [2]. It is of great importance to clarify the inherent molecular mechanisms of progression, invasion, and metastasis of BC.

Long noncoding RNAs (lncRNAs) are a type of noncoding RNAs with a length of over 200 nucleotides [3]. LncRNAs were reported to be involved in fundamental biological mechanisms and documented as oncogenic and suppressive roles in tumor progression of several cancer types including cervical cancer [4], mucoepidermoid carcinoma [5], glioma [6], ovarian cancer [7]. One of the mechanisms by which lncRNAs participated in tumor progression is that they might act as a competing endogenous RNA by sponging miRNAs to regulate expression of proteins associated with tumorigenesis [8]. For example, lncRNA TINCR competitively sponged miR-375 to regulate Phosphoinositide-dependent kinase-1 (PDK1) expression in gastric cancer [9], NORAD functioned as a novel competing endogenous RNA to modulate the expression of small guanosine triphosphate (GTP) binding protein RhoA via competition for hsa-miR-125a-3p [10]. Hence, lncRNAs exhibited as promising therapeutic targets for cancer treatment [8]. LINC00473, a lncRNA, was found to be aberrantly expressed in many cancer types [4,5,11–15] and was frequently described as a marker of poor prognosis. Zhu et al. reported that LINC00473 antagonized miR-195 to mediate the pathogenesis of Wilms tumor [12]. Wang et al. presented that elevated expression of LINC00473 promoted the resistance of Taxol via sponging miR-15a in colorectal cancer [14]. Also, LINC00473 was proved to promote radioresistance of HNSCC cells through activating Wnt/β-catenin signaling pathway [15]. Accumulating studies examined the effects of LINC00473 in multiply tumors, however, the roles of LINC00473 in BC remains poorly understood.

In this study, we aimed to clarify the effects of LINC00473 in breast cancer in vitro and in vivo. We first explored the expression pattern of LINC00473 in BC tissues and cells, then the role of LINC00473 was identified in BC and the underlying molecular mechanisms were also investigated by functional experiments. Collectively, data of our study

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greatly enriched our understanding of roles and molecular mechanisms of LINC00473 in BC progression.

2. Materials and methods

2.1. Tissue samples

BC tissues and corresponding adjacent normal tissues were collected from 60 BC patients who were diagnostically confirmed by pathologic method and received treatment in The First Affiliated Hospital of Xi' an Jiaotong University between 2014 August and 2018 December. Clinicopathological data of patients was also recorded. All enrolled patients received postoperative chemotherapy and provided written informed consent and protocol of the study was authorized by the ethics committee of The First Affiliated Hospital of Xi'an Jiaotong University (No.XJTU1AF2018LSL-001).

2.2. Cell culture

Human BC cell lines (MDA-MB-231, MCF-7, SK-BR-3, MDA-MB-453) and one human mammary epithelial cell (MCF-10A) were purchased from American Type Culture Collection (ATCC). SK-BR-3, MDA-MB-453 were respectively maintained in McCoy's 5A and Leibovitz's L-15 Medium (GIBCO, USA) as recommended by ATCC, while MDA-MB-231, MCF-7 and MCF-10A were maintained in DMEM (GIBCO, USA). All cells were cultured in suggested medium supplemented with 10% fetal bovine serum (HyClone, USA) in a humidified incubator at 37 °C under 5% CO_2, while MDA-MB-453 was cultured in a free gas exchange with atmospheric air.

2.3. RNA extraction and qRT-PCR assay

Total RNA of tissues and cells was extracted using TRIzol reagent (Life Technologies Corporation, Carlsbad, CA, USA). Quality (OD_{260}/OD_{280} absorbance ratio) and concentration of total RNA was determined by a Nanodrop (Thermo, USA). cDNA was then synthesized using a Prime Script™ RT-PCR kit (Takara, Japan) on a thermal cycler (Bio-Rad, USA). QRT-PCR was performed in triplicate using a PrimeScript RT reagent kit (Takara, Japan) on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA). Relative expression levels of miRNA, mRNA and LINC00473 were calculated using $2^{-\Delta\Delta Ct}$ rate method. miRNA and LINC00473 expression were normalized to U6, while mRNA was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers were designed by Primer 5.0 as shown in Table 1.

Table 1
Sequence information of primers.

Primer	Sequence 5'-3'
GAPDH_forward	GGTGAAGGTCGGAGTCAACG
GAPDH_reverse	ACCATGTAGTTGAGGTCAATGAAGG
LINC00473 _forward	GGCAGCCTCAGGTTACAAAT
LINC00473 _reverse	AGGAGCAGGTAGGGAAATGA
P21 _forward	GACTCTCAGGGTCGAAAACG
P21 _reverse	GGATTAGGGCTTCCTCTTGG
Cyclin D1 _forward	GCTGCGAAGTGGAAACCATC
Cyclin D1_reverse	CCTCCTTCTGCACACATTTGAA
E-cadherin _forward	CGAGAGCTACACGTTCACGG
E-cadherin _ reverse	GGGTGTCGAGGGAAAAATAGG
N-cadherin _ forward	TTTGATGGAGGTCTCCTAACACC
N-cadherin _ reverse	ACGTTTAACACGTTGGAAATGTG
U6 _ forward	AAAGCAAATCATCGGACGACC
U6 _ reverse	GTACAACACATTGTTTCCTCGGA
MAPK1_ forward	AGGCTGTTCCCAAATGCT
MAPK1_ reverse	CGTCACTCGGGTCGTAAT
MiR-198_ forward	GGTCCAGAGGGGAGAT
MiR-198_ reverse	GAATACCTCGGACCCTGC

2.4. Western blot analysis

Cell lysates were prepared using RIPA lysis buffer (Cell Signaling Technology, USA) supplemented with protease inhibitor for protein extraction. Concentration of total protein was determined by the Pierce Coomassie (Bradford) Protein Assay Kit (Thermo, USA). Then protein sample were resolved on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The rinsed membranes were incubated with primary antibodies targeting human Lamin B1, actin, Ago2, GAPDH, MAPK1, Cyclin D, p21, E-cadherin or N-cadherin overnight at 4 °C overnight, all antibodies were purchased from Abcam. After washing, the membrane was incubated with goat anti-rabbit or rabbit anti-mouse IgG H&L secondary antibody conjugated with horseradish peroxidase (Abcam, UK). The signal was developed using Super Signa West Dura Extended Duration chemiluminescence substrate (Thermo, USA) and measured by ChemiDoc™ XRS + System (Bio-Rad, USA).

2.5. Vector construction and siRNA transfection

The gene encoding LINC00473 was amplified and cloned into a pGLV3/H1/GFP + Puro vector to overexpress LINC00473. A pGLV3/H1/GFP + Puro/scramble vector was used as negative control (NC). siRNA targeting LINC00473 was purchased from GenePharma Co.,Ltd (Shanghai, China). The miR-198 mimics and inhibitors were purchased from RiboBio Co., Ltd. (Guangzhou, China). Transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following recommended instructions. A short hairpin RNA (shRNA) sequences targeting LINC00473 (shLINC00473) was designed and constructed by GenePharma Co.,Ltd (Shanghai, China). The shRNA was respectively inserted into the lentiviral vector, pLKO.1-TRC cloning vector (Sigma, USA). A PLKO.1-scramble was used as a negative control (shNC). MDA-MB-231cells were transfected with the shLINC00473 or shNC vector.

2.6. Proliferation assay

Cells were cultured on a flat bottom 96-well plate at 2.5×10^3 cells per well. Cell counting kit-8 (Dojindo, China) was added into each well as user manual. OD450 nm was measured using a microplate reader (Bio-Rad, USA).

2.7. Colony formation assay

Cells were plated in 6-well plates and cultured with 2 ml complete mediun for two weeks. Then the cells were fixed with 4% paraformaldehyde for 1 h and stained with 0.1% crystal violet over night. After washing with PBS for three times, cell colonies were photographed and counted.

2.8. Migration and invasion assay

For migration assay, a total of 3×10^4 transfected cells in serum-free medium were plated into the upper chamber (Costar, USA) coated with matrigel (BD, USA) and complete medium supplementary with 10% fetal bovine serum was addedto the lower chamber. After 24 h incubation, the non-invading cells were removed using a cotton swab and the invading cells were then fixed with 95% methanol and stained with 0.5% crystal violet. Five fields per well were selected randomly to determine the cell number. For invasion assay, procedures were similar to those for the migration assay with a minor modification that 50 μL of Matrigel (BD Biosciences) was applied to the upper surface of the chamber to mimic the physiological basement membrane.

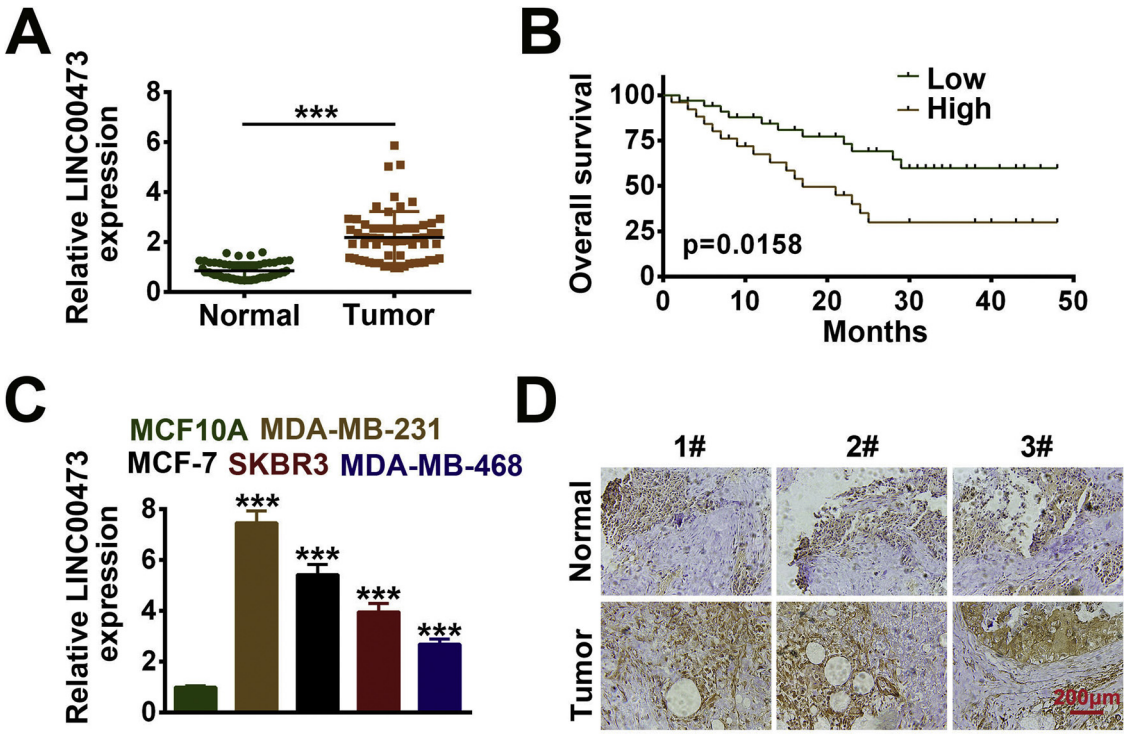

Fig. 1. Elevated expression of LINC00473 expression in human BC tissues and cells. (A) Expression of LINC00473 in 60 pairs of BC tissue and adjacent noncancerous tissue samples were analyzed by qRT-PCR. (B) Data from TCGA was collected and analyzed, BC patients were divided into two groups (Low and High) according to the LINC00473 expression. (C) LINC00473 expression was determined in BC cells using qRT-PCR. (D) RNA ISH was conducted to determined the level of LINC00473 in tissues. *** $P < 0.001$.

2.9. Luciferase assay

The wild-type (WT) miRNA response element of LINC00473 or 3′-untranslated regions (3′-UTR) of MAPK1 were seperately amplified and cloned downstream of the firefly luciferase gene in the pmiRGOL vector (Promega, USA). Subsequently the mutant (Mut) 3′-UTR plasmid or miRNA response element was created by site-directed mutagenesis. Mimics (miR-198-mimic) and negative control (NC) oligonucleotides for miR-198 were purchased from RiboBio Co. Ltd. (Gangzhou, China). 5×10^4 MDA-MB-231 and MCF-7 cells were separately seeded into a 24-well dish for 24 h. Mimics or negative control for miR-198 together with pmiRGOL vectors constructed before co-transfected into MDA-MB-231 and MCF-7 using Lipofectamine 2000 reagent following instructions. After incubation for 48 h, the activities of firefly and renilla luciferases were measured using the Dual Luciferase Assay Kit (Promega, USA) and normalized to those of firefly luciferase activity. All assays were designed in triplicate and repeated for six times.

2.10. Animal experiments

Twelve NOD/SCID mice (female, 6–8 weeks) purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai) were divided into 2 cohorts. All animals received care according to the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health, approved by The First Affiliated Hospital of Xi'an Jiaotong University (No. XJTU1AF2018LSL-001). Five million of MDA-MB-231 cells stably transfected with shLINC00473 or shNC vector were subcutaneously injected into the bilateral axilla of NOD/SCID mice. After 7 days, the tumor volume was monitored every 7 days using a caliper. After 35 days, mice were sacrificed and tumors were excised. Tumor volume was calculated using the formula: Volume = 0.5 × length × width^2.

2.11. Subcellular fractionation and RNA isolation

Cytoplasmic and nuclear RNA was isolated and purified using the PARIS Kit (Life Technologies) according to the manufacturers' instructions. RNA was isolated separately from cytoplasmic and nuclear fraction for qRT-PCR as described before.

2.12. RNA in situ hybridization (RNA ISH)

RNA ISH was performed on formalin fixed paraffin-embedded (FFPE) tissue sections with the RNAscope® 2.0 HD Detection Kit (Advanced Cell Diagnostics) and custom probe set Hs-LINC00473 as the instructions. Thesections were photographed using the Aperio Imagescope (Leica) after Hematoxylin counterstaining.

2.13. RNA fluorescence in situ hybridization (RNA-FISH)

RNA-FISH was carried out on human MDA-MB-231 and MCF-7 cells using LINC00473 Stellaris® FISH probes that were labeled with FAM dyes (Biosearch Technologies) following the instructions. Signal was observed under Leica DM6000B fluorescence microscope.

2.14. RNA immunoprecipitation (RNA-IP)

Cells were lysed using RIPA buffer containing the proteinase and Rnase inhibitors. Cell lysates were collected for immunoprecipitation and incubated with anti-Ago2 antibodies (Abcam, UK) or IgG control (Abcam, UK) and protein A/G beads overnight at 4 °C,the beads were collected by spin then washed with RIPA buffer for 5 times and protein was digested with proteinase K at 45 °C for 45 min to release the RNA from the beads. Then the TRIzol was used to isolate RNAs for qRT-PCR as previously described.

3. Statistical analysis

All statistical analyses were performed using SPSS 19.0 (SPSS, USA). Data are presented as mean ± standard deviation of three parallel experiments. *t*-test was used for difference comparison between experimental and control groups, while one-way ANOVA analysis was used for comparison of more than two groups, followed by Newman-Keuls post hoc test. Pearson analysis was used to determine the correlation between miR-198 and LINC00473 or MAPK1. $P < 0.05$ was considered to indicate a statistically significant difference.

4. Results

4.1. Expression of LINC00473 in breast cancer tissues and cell lines

To investigate the potential role of LINC00473 in BC, tumor tissues and matched adjacent non-cancerous tissues were sampled from 60 patients with BC. Levels of LINC00473 in these samples were analyzed with qRT-PCR, and the data showed that LINC00473 was significantly higher in BC tissues compared with that in the paired adjacent non-cancerous tissues (Fig. 1A, $P < 0.001$). Further analysis of LINC00473 expression using the TCGA database revealed that increased expression of LINC00473 was correlated with poor prognosis in BC patients, patients determined with higher level of LINC00473 experienced shorter overall survival (Fig. 1B). LINC00473 expression pattern in 4 breast cancer cell lines (MDA-MB-231, MCF-7, SKBR3, MDA-MB-468) were further determined using qRT-PCR. BC cell lines exhibited an evidently higher level of LINC00473 than MCF-10A (Fig. 1C), which was in accordance with what we found in tumor tissues. Subsequently, in situ expression of LINC00473 in BC tissues and normal tissues were determined using in situ RNA hybridization and results similarly indicated an increased level of LINC00473 in BC tissues (Fig. 1D). Correlations of LINC00473 level with clinicopathological features were analyzed using Chi-squared test, and results (Table 2) indicated that LINC00473 expression was significantly correlated with tumor size ($P = 0.037$), lymph node metastasis ($P = 0.005$) and TNM classification ($P = 0.007$). Together, these findings suggest that LINC00473 may function as a marker for poor prognosis in BC.

Table 2
Correlations of LINC00473 level with clinicopathological features.

Clinicopathological parameters	N2	LINC00473 expression		χ^2	P value
		H	L		
All	60	26	34		
Age(years)				0.045	0.832
< 60		16	20		
≥60		10	14		
Tumor size				4.344	0.037
< 2cm		9	21		
≥2cm		17	13		
ER status				1.086	0.297
Negative		11	19		
Positive		15	15		
PR status				1.241	0.265
Negative		10	18		
Positive		16	16		
Her-2 status				0.184	0.068
Negative		17	24		
Positive		9	10		
Lymph node metastasis				8.024	0.005
Negative		8	23		
Positive		18	11		
Ki-67				1.451	0.228
Negative		12	21		
Positive		14	13		
TNM stage				7.333	0.007
I-II		19	33		
III		7	1		

4.2. Disrupted expression of LINC00473 inhibited proliferation, invasion and migration of BC cells in vitro

To evaluate the effect of LINC00473 on proliferation, invasion and migration of BC cells in vitro. MDA-MB-231 and MCF-7 were transfected with siRNA targeting LINC00473 (si-LINC00473 1#, si-LINC00473 2#) to decrease endogenous expression of LINC00473. The knockdown efficacy of LINC00473 was measured by qRT-PCR assay (Fig. 2A), LINC00473 was successfully silenced in both cell lines by si-LINC00473 1# and si-LINC00473 2# transfection and si-LINC00473 2# was choosed for the following assays. CCK-8 assay was used to evaluate the proliferation capacity of BC cells, results suggested that deceasing LINC00473 level by si-LINC00473 transfection significantly lowered the OD450 value of MDA-MB-231 and MCF-7 after 96 h incubation (Fig. 2B), which indicated proliferation inhibition. The similar results was also obtained in the same cell lines by colony formation assay where fewer counts of colony formation was observed in groups transfected with si-LINC00473 (Fig. 2C). The above results implied that the high level of LINC00473 was of great importance for proliferation of BC cell lines in vitro. Since the capacities of invasion and migration of tumor cells was essential for tumor metastasis. Transwell assays were performed to explore the effects of LINC00473 on cells invasion and migration. Results of Transwell assay illustrated that number of migrating or invading cells were significantly decreased in groups treated with si-LINC00473 (Fig. 2D and E). Proteins associated with cell proliferation, invasion and migration were also determined by western blot and qRT-PCR. Results showed that p21 and E-cadherin were obviously upregulated in cells transfected with si-LINC00473, while Cyclin D1 and N-cadherin were downregulated in both cell lines (Fig. 2F and G). Taken together, these results implied that downregulation of LINC00473 in BC cells might impair the cell capacities of proliferation, invasion and migration.

4.3. LINC00473 expression negatively correlated with miR-198

Since it is well known that the function of lncRNAs dependents on their subcellular localization. Nucleolis were isolated from MDA-MB-231 and MCF-7 cells. Lamin B1 which limitedly express in nucleolis and Actin which is found throughout the cytoplasm were detected busing western blot analysis in separated fractions of nucleolis (N) or cytoplasm (C). Results showed that Lamin B1 was only detected in nucleolis fraction while Actin was only found in cytoplasm fraction (Fig. 3A), which indicated a complete and successful separation of nucleolis and cytoplasm RNA of nucleolis and cytoplasm were extracted separately. Then levels of LINC00473 and miR-198 in nucleolis and cytoplasm were determined by qRT-PCR. The results revealed that LINC00473 mainly accumulated in cytoplasm (Fig. 3A), also the fluorescence in situ hybridization assay indicated that LINC00473 was mostly localized in cytoplasm (Fig. 3B). Predictions of miRDB, a website for miRNA targets prediction, showed that LINC00473 contained a miRNA response element (MRE) which can bind to miR-198 (Fig. 3C). To verify the direct regulation of LINC00473 by miR-198, luciferase reporter assays were conducted. As expected, data revealed that co-transfection of miR-198 mimics and vectors carrying miRNA response element of LINC00473 evidently decreased the relative luciferase activity, while no significant difference in groups transfected with MRE of LINC00473 and negative control for miR-198. RNA immunoprecipitation (RIP) assay was performed with antibody targeting Ago2 to further confirm the interaction between LINC00473 and miR-198. Significant enrichment of LINC00473 as well as miR-198 was observed in fraction treated with the Ago2 antibody compared with IgG control (Fig. 3D). These practices demonstrated that LINC00473 physically interacted with miR-198. To dissect the regulation between LINC00473 and miR-198, we upregulated LINC00473 expression in MDA-MB-231 and MCF-7 (Fig. 3E). Interestingly, miR-198 was found to be decreased by overexpressed LINC00473 while increased by LINC00473 silence (Fig. 3F). Also, miR-

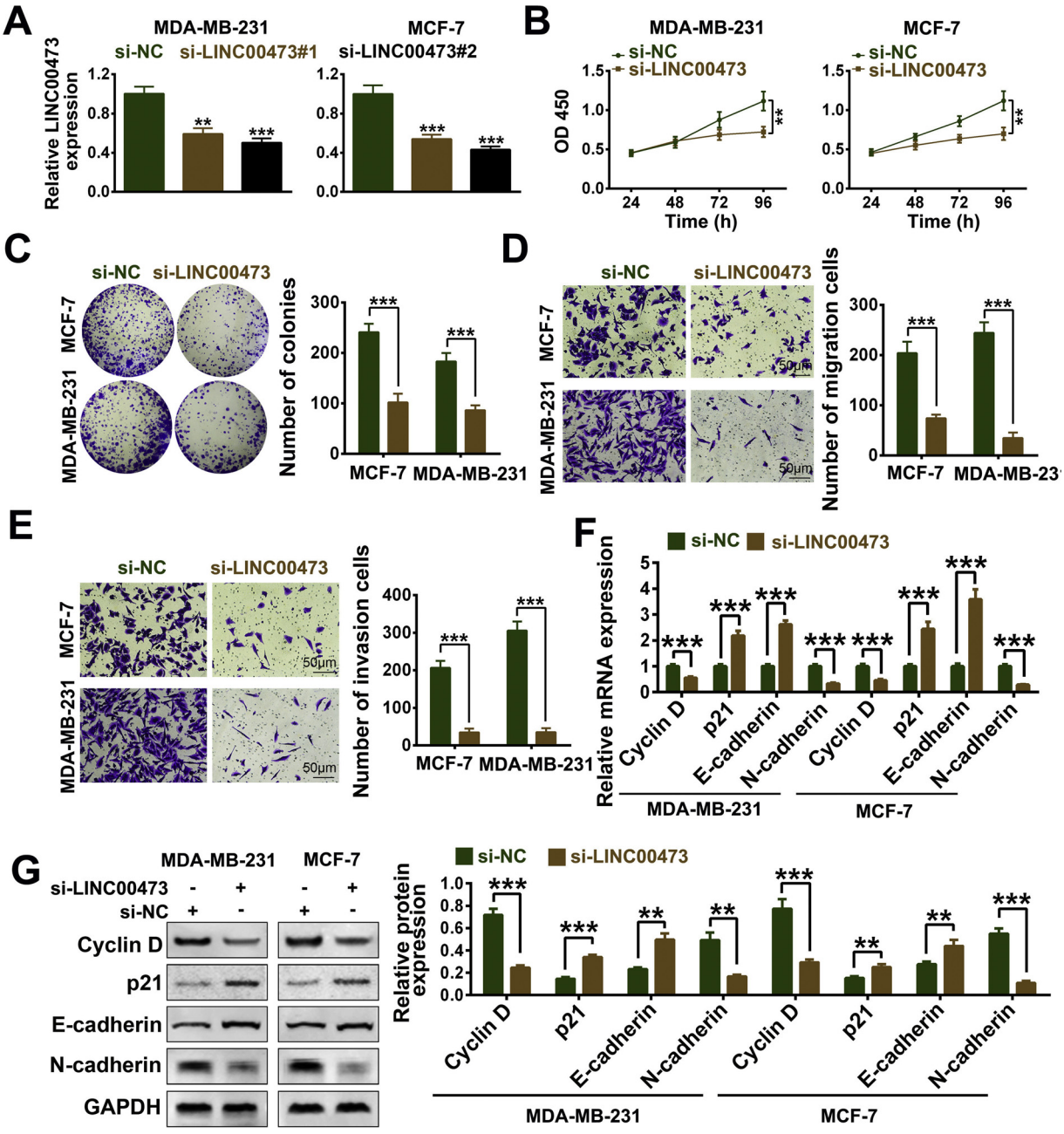

Fig. 2. Effects of LINC00473 on BC cells in vitro. (A) Two siRNAs (si-LINC00473#1 and si-LINC00473#2) were designed to successfully silence LINC00473 in MDA-MB-231 (left panel) and MCF-7 (right panel), (B and C) Effects of LINC00473 on cell proliferation were evaluated by CCK-8 assay and colony formation. (D and E) Influences of LINC00473 on invasion and migration capacities of BC cells was assessed by transwell assays, siRNA was used to downregulate LINC00473. (F and G) Proteins associated with cell proliferation, invasion and migration were determined by western blot and qRT-PCR. All results represented three independent experiments. * * P < 0.01, *** P < 0.001.

198 was detected with a comparably lower level by qRT-PCR in BC tissues than that in paired normal tissues (Fig. 3G). Furthermore, Pearson analysis suggested that the expressions of LINC00473 in BC tissues were negatively correlated with miR-198 (Fig. 3H, r = -0.5577, p < 0.0001). All these findings suggested that LINC00473 negatively regulated miR-198.

4.4. miR-198 directly targeted MAPK1

Predictions using miRDB showed that the 3′-UTR of MAPK1 contained a potential binding site for miR-198 (Fig. 4A). To dissect the direct regulation of MAPK1 by miR-198, a luciferase reporter assay was performed in MDA-MB-231 and MCF-7. We observed that co-transfection of miR-198 mimics and vectors carrying wild-type 3′-UTR of MAPK1 attenuated the relative luciferase activity which was detected with no significant difference in groups transfected with mutant 3′-UTR of MAPK1 or negative control for miR-198 (Fig. 4A). miR-198 inhibitor or miR-198 mimics were used to successfully decrease or elevate the miR-198 level in MDA-MB-231 and MCF-7 (Fig. 4B), meanwhile, MAPK1 mRNA expression in the both cells were increased by miR-198 inhibitor and impaired by miR-198 mimics (Fig. 4C). Western blot analysis similarly revealed the protein expression of MAPK1 was elevated by miR-198 silence while attenuated by miR-198 mimics (Fig. 4D). Subsequently, expression of MAPK1 was examined in tumor tissues and matched normal tissues sampled from 60 patients with BC, MAPK1 expression was significantly higher in tumor tissues compared

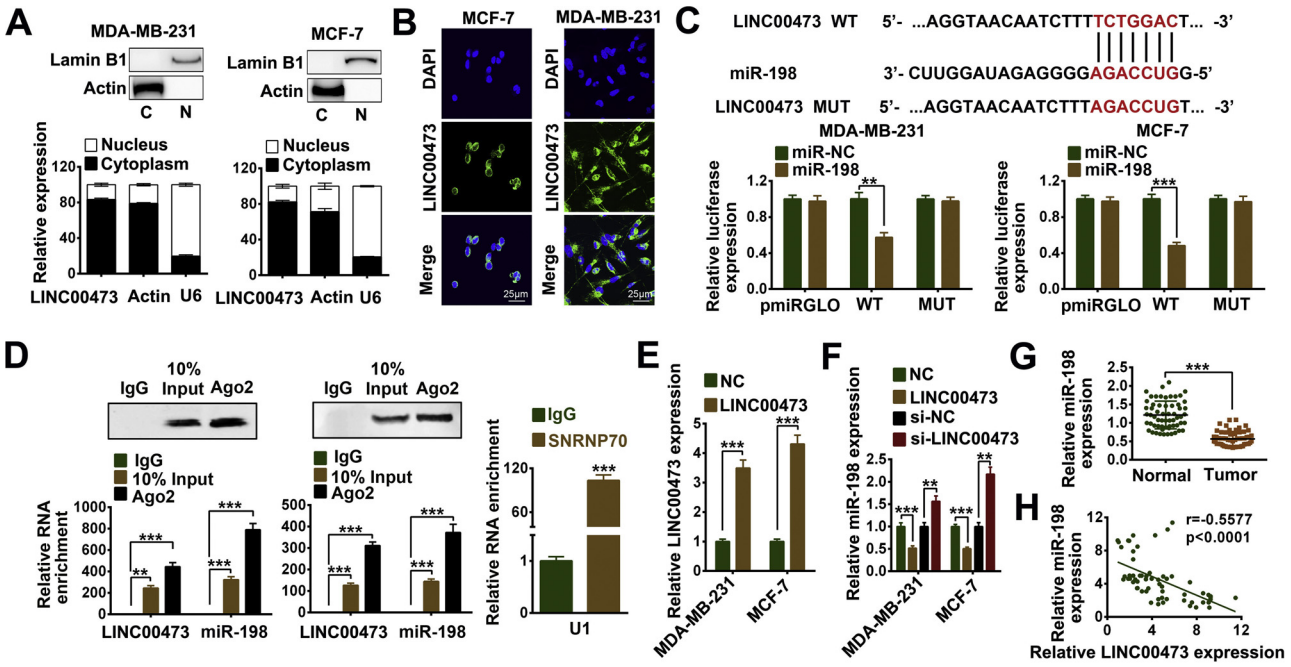

Fig. 3. LINC00473 expression negatively correlated with miR-198. (A) Determining Lamin B1 and Actin in nucleolis (N) and cytoplasm (C) fractions by western blot was to insure the complete and successful separation of these two parts, RNA of nucleolis and cytoplasm were extracted separately for determination of LINC00473 by qRT-PCR. (B) Fluorescence in situ hybridization assay was further conducted to confirm that LINC00473 was mostly localized in cytoplasm. (C) Luciferase reporter assays was performed to verify the direct interaction between LINC00473 and miR-198. (D) RNA immunoprecipitation (RIP) assay was performed to further confirm the interaction between LINC00473 and miR-198, qRT-PCR was used to detectt LINC00473 and miR-198 in groups treated with Ago2 antibody compared with IgG control. (E and F) LINC00473 was ectopically expressed in MDA-MB-231 and MCF-7 by trancfection of vectors carrying LINC00473 gene, expression of miR-198 was determined by qRT-PCR. (G and H) MiR-198 was detected by qRT-PCR in BC tissues, pearson analysis was used to uncover the correlation between LINC00473 and miR-198 in BC tissues. All results represented three independent experiments. * * P < 0.01, *** P < 0.001.

to normal tissues (Fig. 4E and F, P < 0.001). Pearson analysis revealed that the expressions of MAPK1 were negatively correlated with miR-198 (Fig. 4G, r = -0.4662, P < 0.0002).Since we demonstrated that LINC00473 in BC tissues were negatively correlated with miR-198, it can be easily proposed that LINC00473 was positively correlated with MAPK1 expression, which was further confirmed by the Pearson analysis (Fig. 4G, r = 0.8527, P < 0.001). All these results proved that MAPK1 was overexpressed in BC and directly regulated by miR-198.

4.5. LINC00473 influenced the proliferation, invasion and migration of BC cell via direct modulation of miR-198

Since we have proved that silencing LINC00473 might hinder the proliferation, invasion and migration of MDA-MB-231. We proposed that the inhibition effects induced by LINC00473 silence was mediated by its effects on miR-198. As expected, CCK-8 and transwell assays indicated that inhibition effects on MDA-MB-231 caused by LINC00473 silence were compromised by downregulation of miR-198 (Fig. 5A, B, C and D). Simultaneously, results of western blot and qRT-PCR showed that p21 and E-cadherin were upregulated while Cyclin D1, N-cadherin and MAPK1 were downregulated by LINC00473 silence, which was similar to what we observed before, however, the effects of LINC00473 silence on expression of these proteins were completely reversed by treatment with miR-198 inhibitor (Fig. 5E and F). Taken together, influences of LINC00473 on proliferation, invasion and migration of BC cell were partially mediated through modulating miR-198.

4.6. Knockdown of LINC00473 suppressed tumorigenesis of v MDA-MB-231

The influence of LINC00473on BC tumorigenesis in vivo was evaluated using a xenograft model. MDA-MB-231 transfected with negative control shRNA (shNC) or shRNA targeting LINC00473 (shLINC00473) were subcutaneously injected into NOD/SCID mice. It was observed that tumor growth was significantly slowed down in shLINC00473 group than that in shNC group (Fig. 6A, P < 0.001), indicating that silencing LINC00473 significantly inhibits the tumorigenesis of MDA-MB-231. LINC00473 level and MAPK1 expression in these tumors were also detected. Data exhibited that LINC00473 level and MAPK1 expression were lower in tumors from shLINC00473 group (Fig. 6B and C, P < 0.001), which was in line with what we obtained in vitro experiments.

5. Discussion

Breast cancer (BC) is globally recognized as one of the most common and aggressive malignancies in women [1,16,17]. Patients often experience recurrence or distal metastasis after traditional treatments [1]. LncRNAs has been documented as oncogenic and suppressive roles in tumor progression [8]. This article mainly explored the roles of LINC00473 in BC progression and the underlying mechanisms.

At present study we demonstrated that LINC00473 was significantly highly expressed in BC tissues and cell lines compared to adjacent noncancerous tissues and normal cells respectively, which was in line with the results of an published investigation [18]. And we found patients with higher level of LINC00473 experienced shorter overall survival, and the occurrence of LINC00473 was positively correlated with tumor size, lymph node metastasis and TNM classification. Similar results were previously reported in cervical cancer [4], lung cancer [11], and gastric cancer [13]. Combining the results we observed with conclusions from previous works [8,12,13], thus we proposed that LINC00473 exerted as a promoter and poor prognosis marker during BC progression. Subsequently, the effects of LINC00473 on BC cell proliferation, invasion and migration were evaluated in vitro. And we revealed that knockdown of LINC00473 evidently impaired the proliferation, invasion and migration capacities of MDA-MB-231 and MCF-

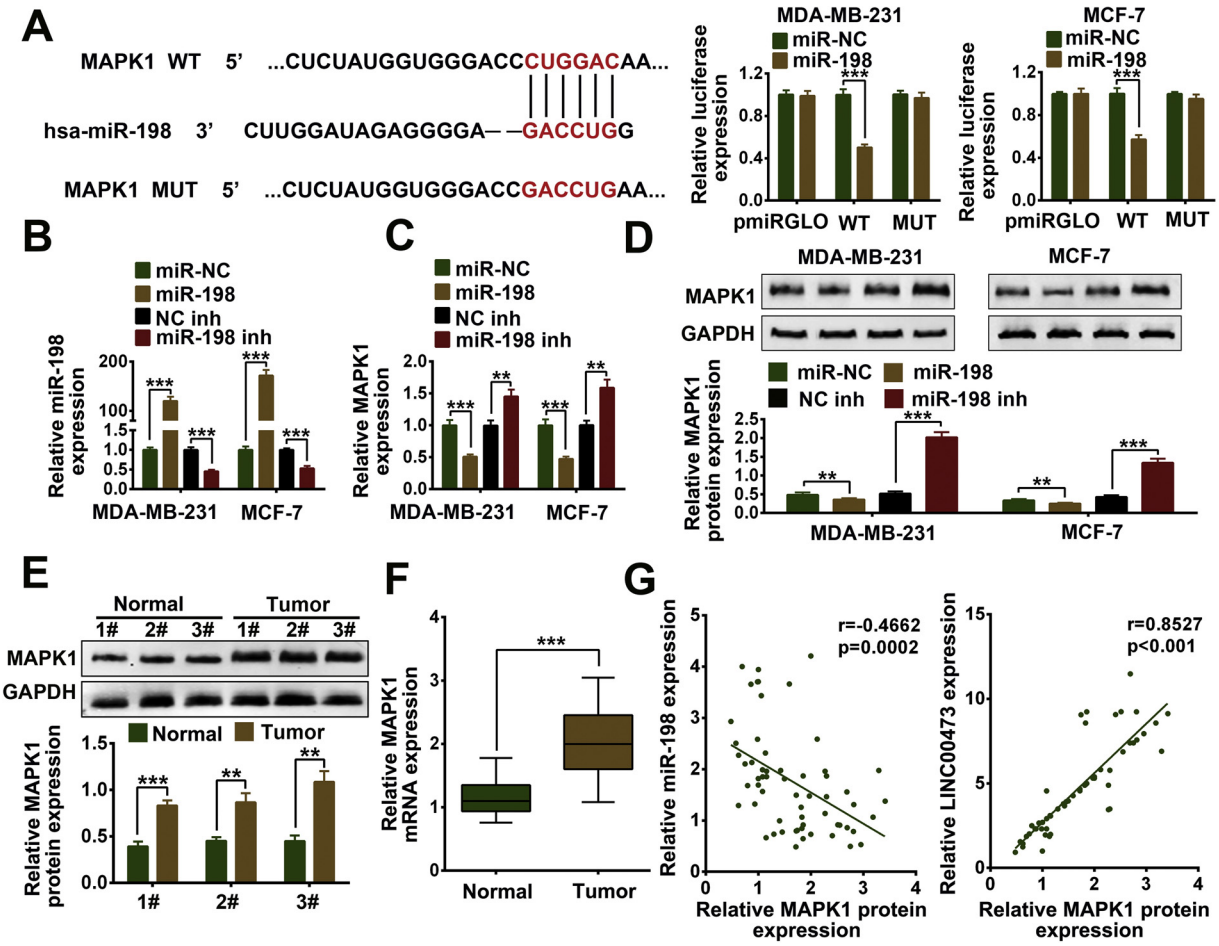

Fig. 4. miR-198 directly targeted MAPK1. (A) Binding site prediction showed that MAPK1 was a direct target of miR-198, luciferase reporter assay was performed in MDA-MB-231 and MCF-7. (B) miR-198 inhibitor or miR-198 mimics were used to successfully decrease or elevate the miR-198 level in MDA-MB-231 and MCF-7, (C and D) MAPK1 expression in the both cells were examined by qRT-PCR and western blot analysis after miR-198 inhibitor ormiR-198 mimics treatment. (E and F) Expression of MAPK1 was examined in tumor tissues and matched normal tissues sampled from 60 patients with BC. (G) Pearson analysis was used to dissect the correlation between MAPK1 and miR-198 or LINC00473 in BC tissues. All results represented three independent experiments. * * P < 0.01, *** P < 0.001.

7, in vivo experiment also indicated that tumorigenesis of MDA-MB-231 was inhibited by suppressing LINC00473. The critical role of LINC00473 on tumor cells was also verified in cervical cancer [4], mucoepidermoid carcinoma [5], lung cancer [11], gastric cancer [13] and colorectal cancer [14] in several researches. Chen et al. also revealed that LINC00473 was involved in modulation of radioresistance via targeting SPIN1 in esophageal squamous cell carcinoma [19]. The underlying molecular mechanisms of LINC00473 mediated proliferation, invasion and migration were briefly explored here. Our data depicted that Cyclin D1 and p21 expression was significantly influenced by LINC00473 alteration. Cyclin D1 was well recognized as a tumor promoter while p21 was found to be a protective role, both of the proteins were involved in cell cycle regulation [20,21]. Hence the significant role of LINC00473 on BC cell proliferation was speculatively associated with its potential regulation on cell cycle. N-cadherin is a hallmark of epithelial-to-mesenchymal transition, and its occurrence always leads to the appearance of an aggressive tumor phenotype [22], while loss of E-cadherin was associated with distal metastasis [23]. Previously published studies have reported that E-cadherin might be regulated by diverse lncRNAs in multiply cancers including colon cancer [24], lung cancer [25] and gallbladder cancer [26]. The regulation of lncRNAs on N-cadherin was also described in several cancers [27,28]. The regulation of LINC00473 on these two factors partially represented the molecular mechanisms that mediated its effect on BC cell invasion and migration. Otherwise, matrix metalloproteinase (MMP) played vital role in tumor progression and was closely associated with tumor metastasis, recent studies have demonstrated that MMP might be regulated by lncRNA [29,30]. The relation between LINC00473 and MMP will deserve more attention in following researches.

Some lncRNAs have been documented as competitive endogenous RNA by binding miRNAs and reducing their posttranscriptional effects on their mRNA targets [8]. To dissect whether LINC00473 functions as a competitive endogenous RNA in BC cells, we examined the distribution of LINC00473 in cytoplasm and nucleus. As expected, LINC00473 mainly occurred in cytoplasm. Prediction by miRDB revealed a putative binding site of miR-198 in LINC00473, which verified by luciferase assay. And RIP assay demonstrated a direct interaction between LINC00473 and miR-198. Hence, we established a negative relationship between LINC00473 and miR-198 expression in BC. MiR-198 has previously been reported as a tumor suppressor in several studies in regard to prostate cancer [31], lung cancer [32], hepatocellular carcinoma [33] and breast cancer [17]. Following, we uncovered that MAPK1 was a direct target of miR-198. The regulation of miR-198 on MAPK1 expression was also observed in MDA-MB-231 and MCF-7 cells as well as a negative correlation between MAPK1 and miR-198 expression in BC tissues. In reality, the regulation of miR-198 on MAPK1 has already been described in recent investigation focusing on prostate cancer [34]. Since MAPK1 signal pathway was well known as a classic signal pathway which was frequently involved in regulation of cell survival,

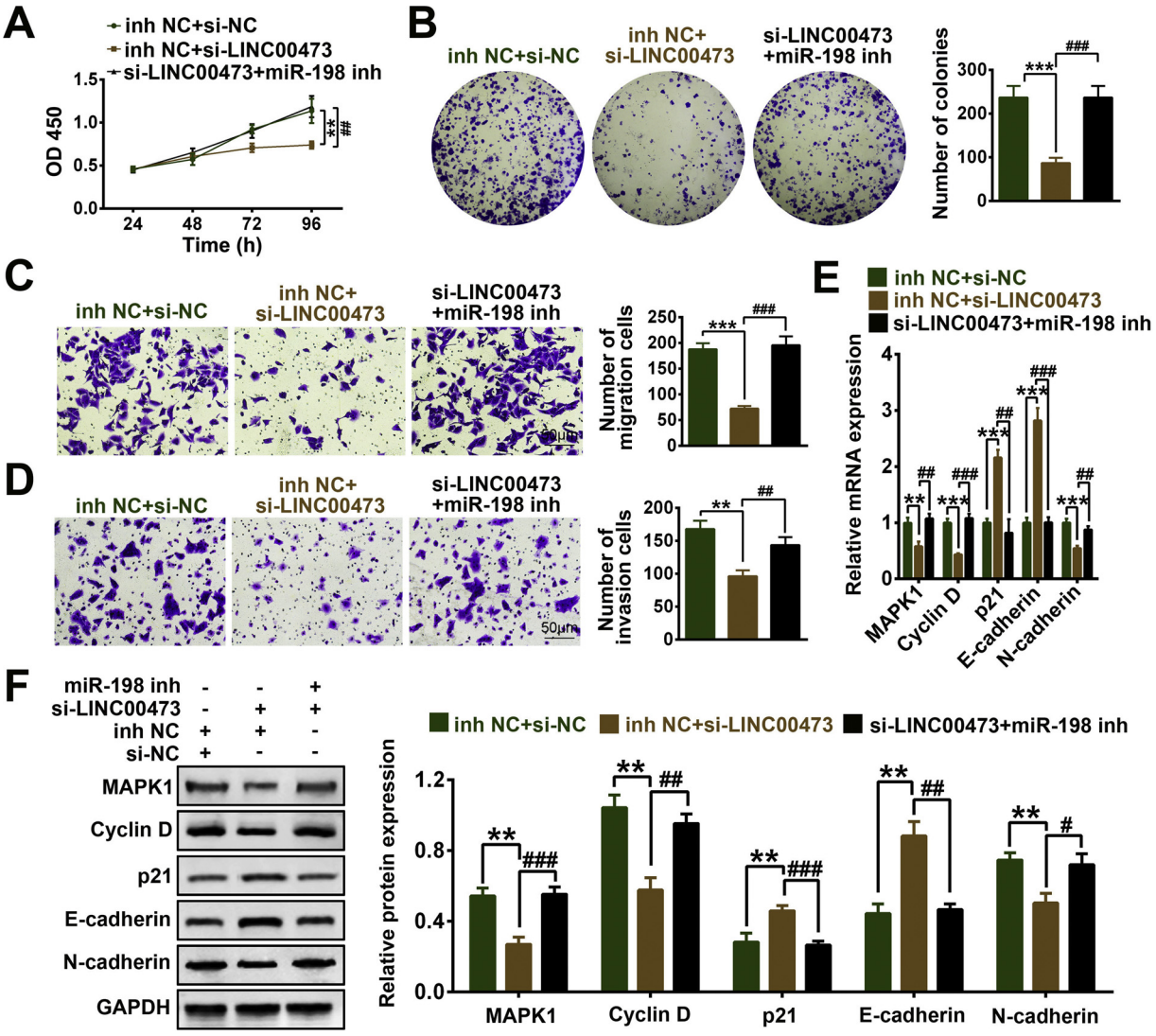

Fig. 5. LINC00473 influenced the proliferation, invasion and migration of BC cell via direct modulation of miR-198. (A and B) CCK-8 and colony formation assays were conducted to prove that inhibition effects on proliferation and colony formation of MDA-MB-231 caused by LINC00473 silence were compromised by miR-198 inhibitor (miR-198 in.). (C and D) Invasion and migration of MDA-MB-231 were assessed by transwell assay to verify whether miR-198 inhibitor might recover the invasion and migration capacities impaired by LINC00473 silence. (E and F) p21, E-cadherin, Cyclin D1 N-cadherin and MAPK1 were determined by western blot and qRT-PCR in cells treated with miR-198 inhibitor or si-LINC00473. All results represented three independent experiments. * * P < 0.01, *** P < 0.001.

migration and invasion [35], we hypothesized that LINC00473 might regulate the MAPK1 expression via functioning as an endogenous completive RNA by sponging miR-198. To confirm the hypothesis, si-LINC00473 and miR-198 inhibitor were co-transfected to MDA-MB-231. Meanwhile, we found that the siLINC00473 induced inhibitive effects in cell proliferation, invasion and migration were reversed by miR-198 inhibitor. The siLINC00473 induced altered expressions of Cyclin D1, p21, N-cadherin, E-cadherin and MAPK1 were all rescued by miR-198 inhibitor. MAPK1 was frequently described as a poor prognosis indicator in diverse tumors, for instance, a miR-20a / MAPK1 / c-Myc cascade was previously proposed by Si et al. to regulate breast carcinogenesis and chemoresistance [1], Xu et al. reported that Linc00161 regulated the drug resistance of ovarian cancer by sponging microRNA-128 and modulating MAPK1 [7]. From the above, an axis of LINC00473 / miR-198 / MAPK1 was established in BC cells. Interestingly, MAPK1 is not the only target of miR-198 and more downstream targets of miR-198 have been reported, such as livin in prostate cancer [31], serine hydroxymethyltransferase 1 in lung adenocarcinoma [32] and HGF/c-MET pathway in hepatocellular carcinoma [33]. The various targets of miR-198 complicated the molecular mechanisms by which LINC00473 regulated tumor progression including BC. Likewise, LINC00473 was also proved to regulate other miRNAs like miR-34a [4] and miR-195 [12]. Cellular activity are generally regulated by multiply cellular signals, for instance, a published study proposed that the regulation of LINC00473 on Cyclin D1 expression was a net effect of activation and repression of related signal pathways in breast cancer cells [36], making the underlying mechanisms more elusive.

6. Conclusion

This study revealed that LINC00473 participated in the proliferation, invasion and migration of BC cells and functioned as an endogenous completive RNA by sponging miR-198 to regulate MAPK1 expression. Findings of our study provided the basis for further exploring the application of LINC00473 as a prognostic and diagnostic biomarker.

Authors' contributions

All authors contributed equally to this work.

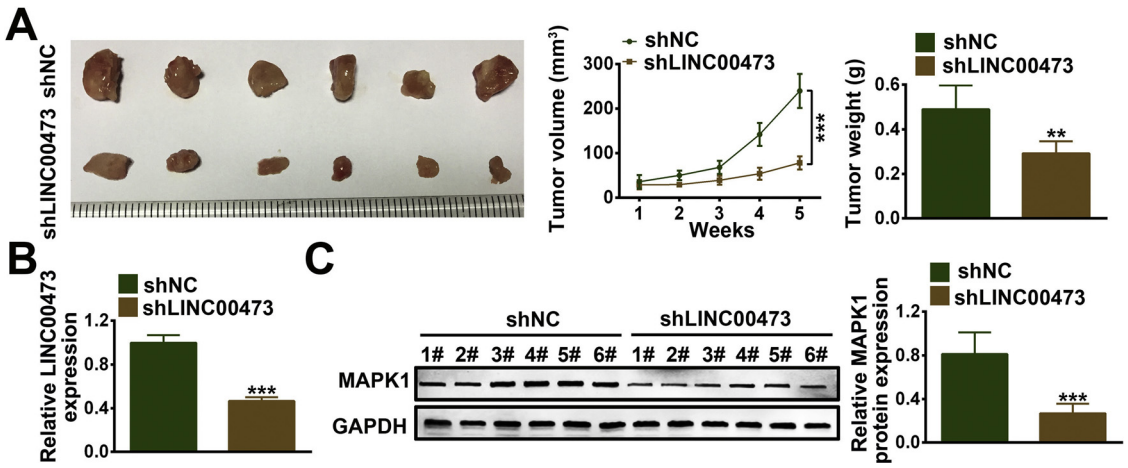

Fig. 6. Knockdown of LINC00473 suppressed tumorigenesis in a xenograft model.
(A) Image of tumor formation in the MDA-MB-231 shNC group or LINC00473-depleted group (shLINC00473) in NOD/SCID mice, the tumor size and weight of final dayin shLINC00473 and shNC groups were recorded. (B and C) LINC00473 level and MAPK1 expression in these tumors were also detected with qRT-PCR and western blot assays. * * P < 0.01, *** P < 0.001.

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Ethics approval and consent to participate

Written informed consent was obtained from all enrolled patients, and the study was approved by the ethics committee of The First Affiliated Hospital of Xi'an Jiaotong University. All animal experiments were approved by the institutional animal care and use committee of The First Affiliated Hospital of Xi'an Jiaotong University. All procedures were in accordance with the animal care and use committee of the institution and conformed to legal mandates and national guidelines for the care and maintenance of laboratory animals.

Competing interests

The authors of this manuscript declare no conflicts of interest.

Availability of data and materials

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

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