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LncRNA MORT negatively regulates FGF1 to suppress malignant progression of breast cancer

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Abstract. – OBJECTIVE: To explore the biological function of long non-coding RNA (lncRNA) MORT in the malignant progression of breast cancer (BCa) and the underlying mechanism, and to provide a novel strategy for clinical treatment.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was conducted to detect differential level of MORT in BCa specimens and cell lines. The correlation between MORT level and pathological indexes of BCa patients was analyzed. After intervening MORT level in SKBR-3 and MCF-7 cells, cell viability, migratory rate and wound closure were examined through Cell Counting Kit-8 (CCK-8), transwell and wound healing assay, respectively. Dual-Luciferase reporter assay and rescue experiments were conducted to uncover the regulatory effect of MORT on its target gene FGF1. *In vivo* function of MORT in mediating tumor growth of BCa was finally assessed by generating a xenograft model in nude mice.

RESULTS: MORT was downregulated in BCa tissues and cell lines. Low level of MORT predicted higher rate of distant metastasis in BCa patients. Overexpression of MORT in SKBR-3 cells reduced proliferative and migratory rates, while knockdown of MORT in MCF-7 enhanced them. Moreover, *in vivo* overexpression of MORT slowed down tumor growth of BCa in nude mice. MORT could negatively regulate its target gene FGF1, which was responsible for the anti-cancer role of MORT in BCa progression.

CONCLUSIONS: MORT is downregulated in BCa specimens, which suppresses proliferative and migratory potentials of BCa cells by negatively regulating FGF1. MORT can be an effective target for precision treatment of BCa.

Key Words:
LncRNA MORT, FGF1M, Breast cancer, Malignant progression.

Introduction

Breast cancer (BCa) is a malignant tumor originating from breast epithelial tissues, which is a highly prevalent malignant disease in women[1,2]. The recent incidence of BCa shows an evident upward trend[3,4]. The number of newly diagnosed cases of BCa worldwide has exceeded 1.3 million each year. In our country, BCa covers about 15% of new tumor cases in women[3-6]. Due to the promotion of screening and health management of BCa, the mortality has sharply decreased[2,7]. However, some BCa patients have poor responses to therapeutic strategies due to malignant proliferation, recurrence, and metastasis[8]. It is still a serious threat to the lives and health of women globally[9]. In-depth understanding of the molecular mechanism of BCa, and finding key molecules that can effectively control the malignant phenotypes of BCa cells are beneficial to develop targeted therapies[10,11].

Long non-coding RNAs (lncRNAs) belong to a type of noncoding RNAs with 200-1000 bp long. Their biological functions have been well concerned nowadays[12,13]. In mammalian genome sequences, about 4-9% of the transcripts are lncRNAs[14]. The number of lncRNAs far exceeds that of protein-coding genes. With the rapid development of RNA sequencing, epigenome technology and computational prediction, more and more lncRNAs have been discovered[15,16]. Abnormally expressed lncRNAs have been identified in BCa profile, and they can be of significance in the carcinogenetic process[17,18]. LncRNA MORT is overexpressed in mantle cell lymphoma and oral squamous cell carcinoma, which is able to mediate tumor cell phenotypes[19,20]. Its potential role in BCa, however, is unclear.

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According to the bioinformatic analysis, MORT can target FGF1. In the present study, how MORT influences both *in vitro* and *in vivo* progression of BCa by targeting FGF1 is mainly explored.

Patients and Methods

Patients and BCa Samples

Fifty BCa and paired non-tumoral tissues (5 cm away from tumor lesion) were surgically resected, frozen in liquid nitrogen and preserved at -80°C. Their clinical data were completely recorded. Inclusion criteria were newly diagnosed BC and the enrolled patients confirmed by histopathological exams. Exclusion criteria were recurrent cases, patients complicated with other clinical disorders and therapies were performed. This study was approved by the research Ethics Committee of The First Affiliated Hospital of University of South China and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell Lines and Reagents

BCa cell lines (MCF-7, MDA-MB-231 and SKBR-3) and mammary epithelial cell line (MCF-10A) were purchased from Cell Bank, Chinese Academy of Science. They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Rockville, MD, USA). Cell passage was conducted at 90% confluence using trypsin + EDTA (ethylenediaminetetraacetic acid).

Transfection

Transfection plasmids were synthesized by GenePharma (Shanghai, China). Cells were cultured to 40-50% density in a 6-well plate, and transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h cell transfection, cells were collected for verifying transfection efficacy and functional experiments.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells/well. At day 1, 2, 3 and 4, optical density at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

Cell suspension was prepared at 5×10^5 cells/mL and applied on the top of the transwell insert (200 μL). Meanwhile, 700 μL of medium containing 20% FBS was added per well. After 48-h migration, bottom cells were fixed and dyed using 0.2% crystal violet. Migratory cells were captured and counted in 5 randomly selected fields of each sample.

Wound Healing Assay

Cell suspension in serum-free medium was prepared at 5×10^5 /mL, and implanted in 6-well plates. Cells were cultivated to 90% density, followed by creating an artificial scratch using a sterilized pipette tip. Cells were washed in PBS for 2-3 times and cultured in the medium containing 1% FBS. 24 hours later, wound closure percentage was calculated.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used for isolating RNAs and they were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using AMV reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) was utilized for qRT-PCR. β-actin was the internal reference. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$ method[21]. LncRNA MORT: Forward: 5'-TTTGAGGTGACCGTTGCGTA-3', Reverse: 5'-ACACAAACGCGCGATCAAAA-3'; FGF1: Forward: 5'-GTGGATGGGACAAGGGACAG-3', Reverse: 5'-ATTTGGTGTCTGTGAGCCGT-3'; β-actin: Forward: 5'-CCTGGCACCCAGCACAAT-3', Reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'.

Western Blot

Cells were digested and centrifuged at 14000×g, 4°C for 15 min. Protein concentration was calculated by bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). After adjusting the concentration of each sample to the same value, they were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). It was blocked in 5% skim milk for 2 h and immunoblotted with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

HEK293 cells were co-transfected with pcDNA-NC/pcDNA-FGF1 and wild-type/mutant-type MORT vector using Lipofectamine 2000. After 48 h, Luciferase activity was determined (Thermo Fisher Scientific, Waltham, MA, USA).

In Vivo Xenograft Model

Tumorigenesis assay in nude mice was approved by the Committee of University of South China Animal Center. Fifteen female nude mice with 8 weeks old were randomly divided into three groups, and they were administrated with SKBR-3 cells transfected with NC+pcDNA-NC, pcDNA-MORT+pcDNA-NC or pcDNA-MORT+pcDNA-FGF1. Each group had five mice. Tumor width and length were weekly recorded. Mice were sacrificed at the sixth week. Tumor volume (mm^3) was calculated using the formula: Tumor width $(mm)^2$ × tumor length (mm)/2.

Statistical Analysis

GraphPad Prism 5 V5.01 (La Jolla, CA, USA) was used for statistical analyses and data were expressed as mean ± standard deviation. Differences between groups were compared by the *t*-test. Clinical significances of MORT in BCa were analyzed by Chi-square test. $p<0.05$ was considered as statistically significant.

Results

MORT Was Lowly Expressed in BCa

MORT was detected to be lowly expressed in BCa tissues than that of normal ones (Figure 1A, 1B). In the meantime, MORT was downregulated in BCa cell lines as well (Figure 1C). MCF-7 and SKBR-3 cells were used to generate *in vitro* knockdown and overexpression models of MORT, respectively because they expressed the most evident differential level of MORT. Subsequently, clinical significance of MORT in BCa patients was analyzed. The incidence of distant metastasis was significantly different between BCa patients expressing high and low level of MORT (Table I). In particular, BCa patients with distant metastasis had the lower level of MORT in comparison to non-metastatic patients (Figure 1D).

MORT Inhibited Proliferative and Migratory Abilities in BCa Cells

To explore the influence of MORT on BCa cell functions, transfection efficacy of pcDNA-MORT and anti-MORT was tested in SKBR-3 and MCF-7 cells, respectively (Figure 2A). In SKBR-3 cells overexpressing MORT, cell viability, migratory cell number and wound closure percentage were all reduced. On the contrary, knockdown of MORT promoted proliferative and migratory abilities in MCF-7 cells (Figure 2B-2D).

FGF1 Was the Target Gene of MORT

Protein level of FGF1 was downregulated in SKBR-3 cells overexpressing MORT, and it was upregulated in MCF-7 cells with MORT knockdown (Figure 3A). A binding site was predicted in FGF1 3'UTR that was paired to MORT 3'UTR. Furthermore, Dual-Luciferase reporter assay confirmed their binding relationship between FGF1 and MORT (Figure 3B). FGF1 was highly expressed in BCa tissues and cell lines (Figure 3C, 3D).

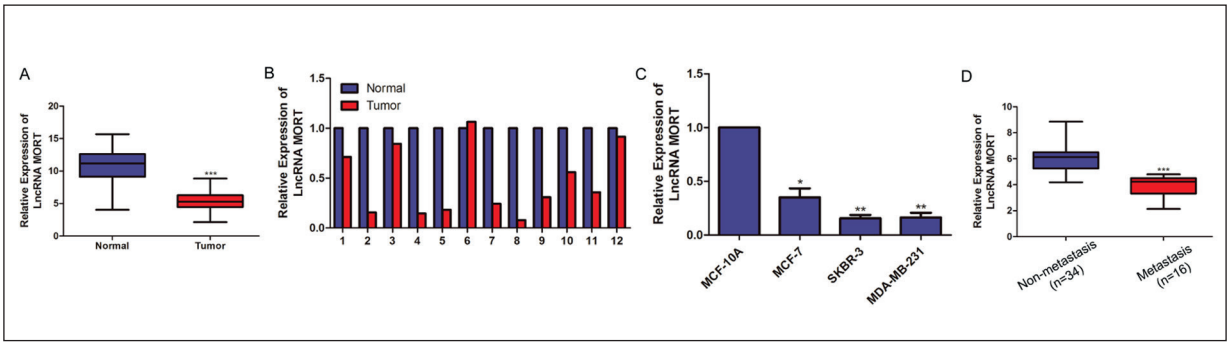

Figure 1. MORT was lowly expressed in BCa. **A,** Differential level of MORT in BCa and non-tumoral tissues; **B,** Differential level of MORT in 12 pairs of BCa and paracancerous tissues; **C,** MORT levels in BCa cell lines; **D,** MORT levels in BCa patients either with distant metastasis or not. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table I. The relationship between LncRNA MORT level and clinicopathologic characteristics of breast cancer.

Parameters	No. of cases	LncRNA MORT expression		*p*-value
		Low (n = 26)	High (n = 24)	
Age (years)				0.395
< 60	25	14	11	
≥ 60	25	12	13	
T stage				0.412
T1-T2	28	16	12	
T3-T4	22	10	12	
Lymph node metastasis				0.164
No	32	19	13	
Yes	18	7	11	
Distance metastasis				0.044
No	34	21	13	
Yes	16	5	11	

MORT Negatively Regulated FGF1 to Inhibit Malignant Progression of BCa

Next, we constructed pcDNA-FGF1 and si-FGF1 aiming to uncover the biological function of FGF1 in BCa progression. Their transfection efficacy of pcDNA-FGF1 and si-FGF1 was tested in SKBR-3 and MCF-7 cells, respectively (Figure 4A). Compared with SKBR-3 cells overexpressing MORT, those co-overexpressing MORT and FGF1 presented higher viability, migratory cell number and wound closure (Figure 4B-4D). In addition, co-silence of MORT and FGF1 resulted in lower proliferative and migratory rates in MCF-7 cells than those with MORT knockdown only.

MORT Inhibited the Tumorigenicity of BCa In Vivo

SKBR-3 cells transfected with NC+pcDNA-NC, pcDNA-MORT+pcDNA-NC or pcDNA-MORT+pcDNA-FGF1 were administrated

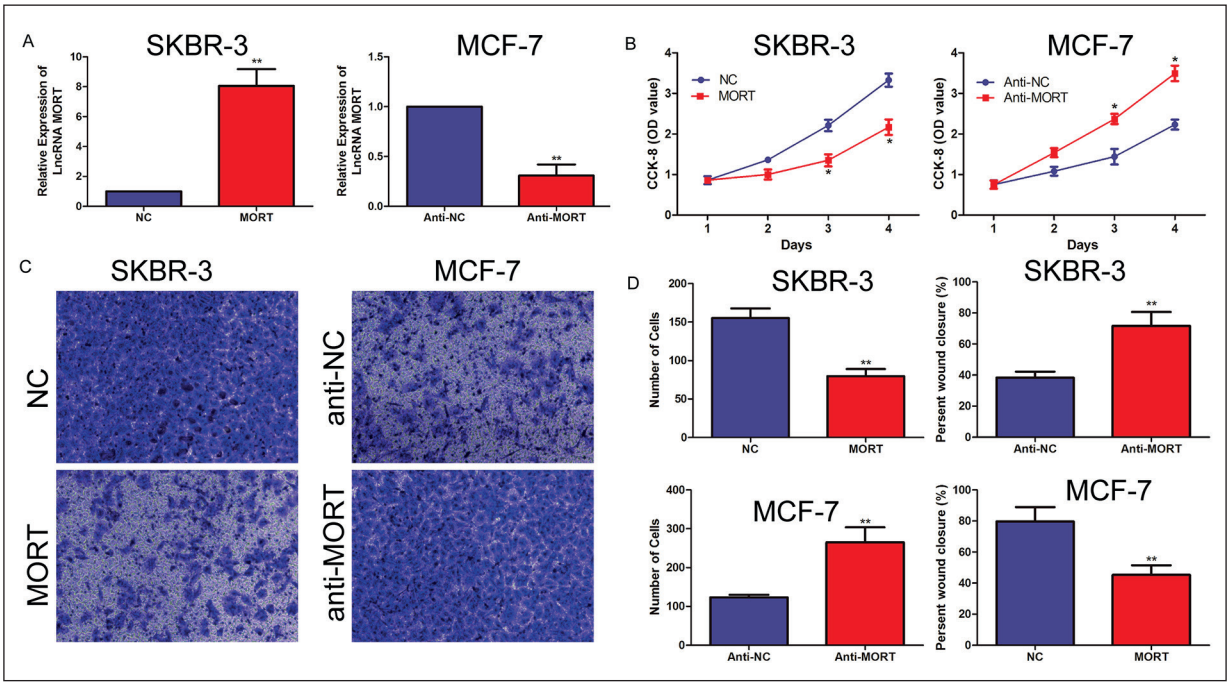

Figure 2. MORT inhibited proliferative and migratory abilities in BCa cells. **A,** Transfection efficacy of pcDNA-MORT and anti-MORT in SKBR-3 and MCF-7 cells, respectively; **B,** Viability in SKBR-3 and MCF-7 cells regulated by MORT; **C,** Migration in SKBR-3 and MCF-7 cells regulated by MORT (magnification: 40×); **D,** Wound closure in SKBR-3 and MCF-7 cells regulated by MORT. *$p < 0.05$, **$p < 0.01$.

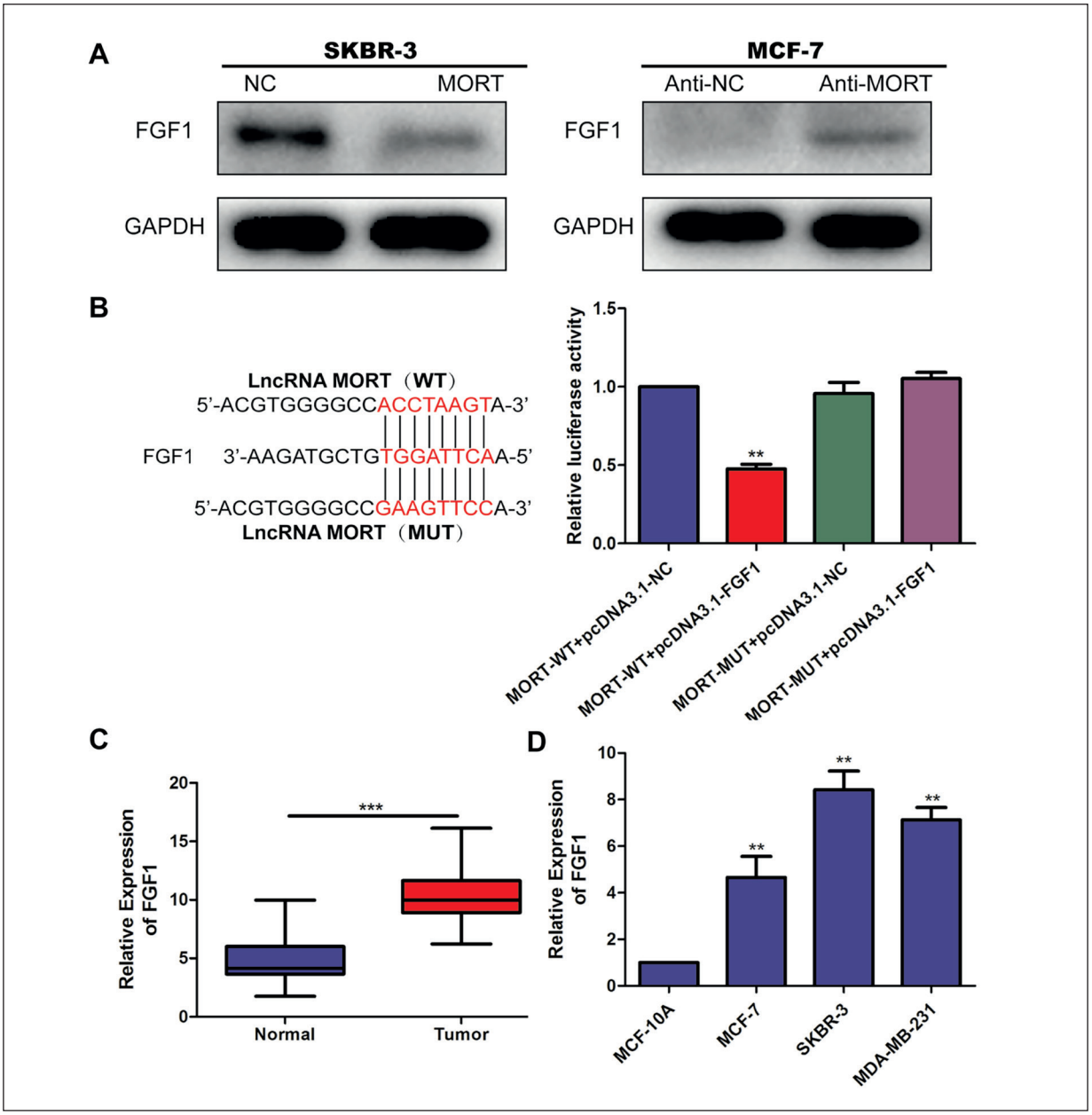

Figure 3. FGF1 was the target gene of MORT. **A,** Protein level of FGF1 in SKBR-3 and MCF-7 cells regulated by MORT; **B,** Binding relationship between FGF1 and MORT; **C,** Differential level of FGF1 in BCa and non-tumoral tissues; **D,** FGF1 levels in BCa cell lines. $^{**}p < 0.01$, $^{***}p < 0.001$.

to nude mice for establishing xenografted BCa tissues. *In vivo* overexpression lowered tumor growth and tumor weight than control mice and those with co-overexpressing MORT and FGF1 (Figure 5A, 5B). As expected, MORT level was lower in BCa tissues of mice with co-overexpression of MORT and FGF1 than those with overexpression of MORT only, and positive expression of FGF1 had the opposite result (Figure 5C, 5D).

Discussion

BCa is the common cause of death in women and its incidence and mortality are rising in recent years[1-5]. In China, the mortality of BCa is on the top place, accounting for 9.2% of global deaths[6]. It is a serious killer for women's health and lives[9]. Malignant growth of BCa is the major reason for the poor prognosis[8,9]. At present,

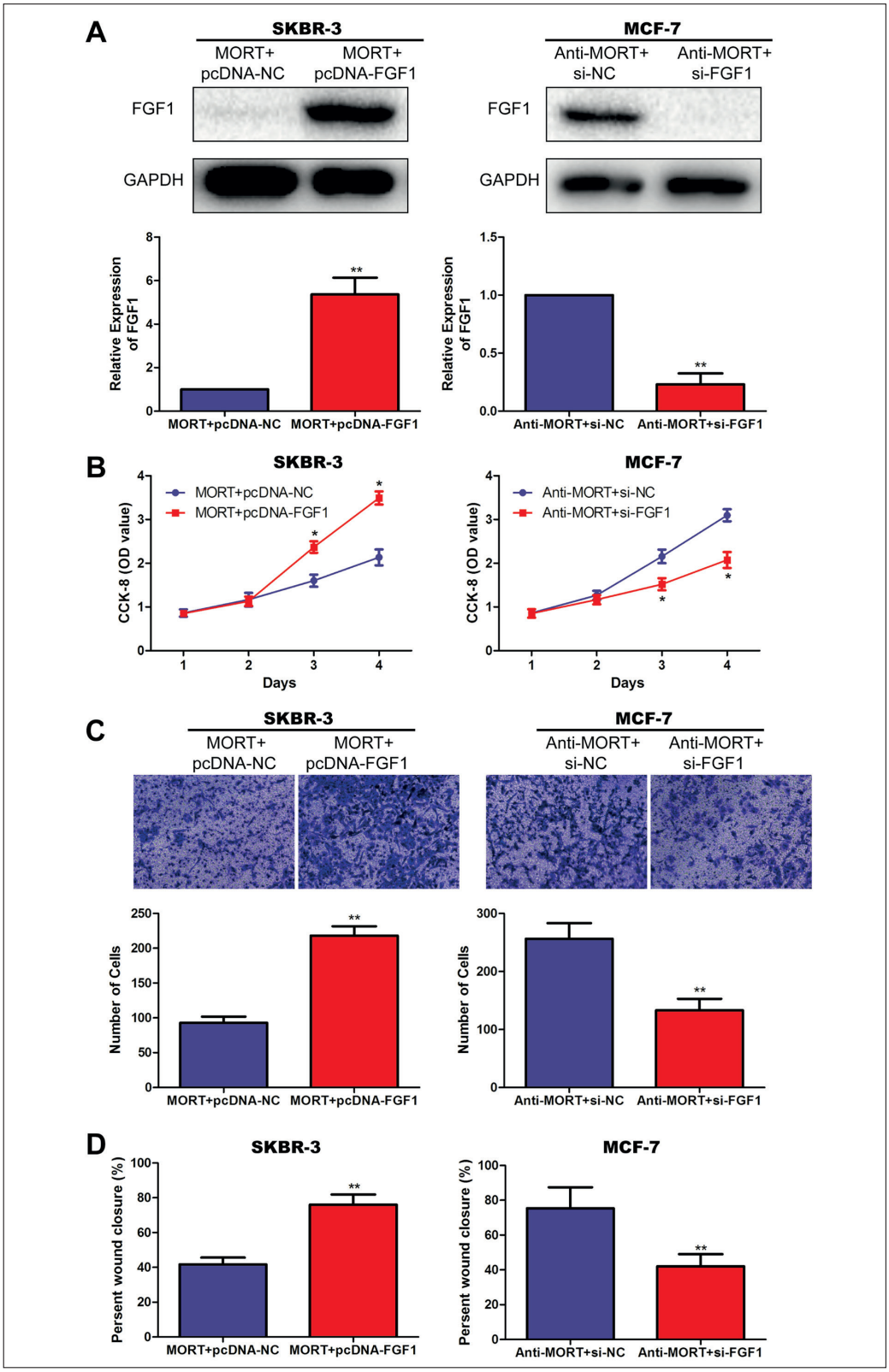

Figure 4. MORT negatively regulated FGF1 to inhibit malignant progression of BCa. **A,** Transfection efficacy of pcDNA-FGF1 and si-FGF1 in SKBR-3 and MCF-7 cells, respectively; **B,** Viability in SKBR-3 and MCF-7 cells co-regulated by MORT and FGF1; **C,** Migration in SKBR-3 and MCF-7 cells co-regulated by MORT and FGF1 (magnification: 40×); **D,** Wound closure in SKBR-3 and MCF-7 cells co-regulated by MORT and FGF1. *$p < 0.05$, **$p < 0.01$.

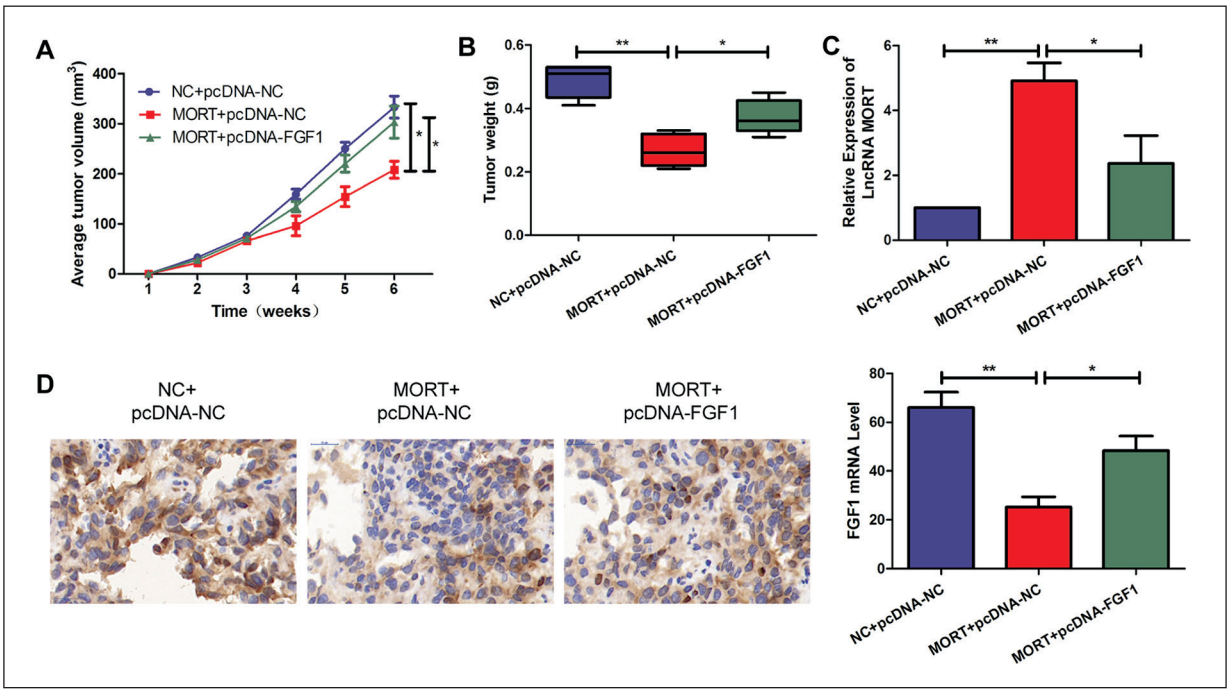

Figure 5. MORT inhibited the tumorigenicity of BCa *in vivo*. **A,** Tumor volume in nude mice; **B,** Tumor weight in nude mice; **C,** Relative level of MORT in xenografted BCa tissues; **D,** Positive expression of FGF1 in xenografted BCa tissues. (magnification: 400×) *$p < 0.05$, **$p < 0.01$.

molecular targeted therapy has achieved positive results due to the most deepen understanding of the pathogenesis of BCa[10]. Seeking for key molecules that are able to affect malignant phenotypes of BCa contributes to enhance the clinical outcome[10,11].

The human transcriptome includes both protein-encoding mRNAs and noncoding RNAs without protein-encoding function (i.e., lncRNAs, miRNAs and circRNAs)[12]. LncRNAs can participate in regulating the biological functions of tumor cells by regulating gene expressions, changing subcellular distribution of proteins in cancer cells or forming complexes with proteins or subcellular structural components *via* altering their own structures[12-14]. LncRNAs are differentially expressed in tumor specimens[15,16]. In comparison to normal breast epithelial tissues, multiple abnormally expressed lncRNAs in BCa tissues have been discovered[17,18]. Previous studies[19,20] have demonstrated the anti-cancer role of MORT in tumor progression. Our results uncovered that MORT was lowly expressed in BCa specimens and cell lines. Moreover, MORT was able to reduce proliferative and migratory rates in BCa cells *in vitro*, suggesting its anti-cancer role in BCa. The previous study[22] reported that overexpression of MORT could inhibit cell proliferation in oral squamous cell carcinoma, which was the same as our result. We thereafter explored the *in vivo* role of MORT in BCa by generating a xenograft model in nude mice. Compared with the control group, mice administrated with SKBR-3 cells overexpressing MORT presented a slower rate of BCa growth, further identifying its protective role.

FGF1 is one of the fibroblast growth factor family. FGF1 has strong mitogenic effects on a variety of cell types in various stages of development, morphogenesis and angiogenesis in neoplastic or non-neoplastic tissues[23,24]. FGF1 was proven to be the target gene of MORT. Overexpression of MORT markedly downregulated protein level of FGF1, and on the contrary, knockdown of MORT upregulated its level. Notably, rescue experiments showed that FGF1 could abolish the regulatory effect of MORT on BCa cell phenotypes and tumorigenicity of BCa.

Taken together, our results demonstrated the anti-cancer role of MORT in BCa progression relied on its target gene FGF1. MORT could be a promising biomarker for diagnosis, treatment and prognosis assessment of BCa.

Conclusions

Taken together the above results demonstrated that MORT is downregulated in BCa specimens, which suppresses proliferative and migratory potentials of BCa cells by negatively regulating FGF1. MORT can be an effective target for precision treatment of BCa.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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