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Down-regulation of lncRNA Linc00152 suppressed cell viability, invasion, migration, and epithelial to mesenchymal transition, and reversed chemo-resistance in breast cancer cells

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Abstract. – OBJECTIVE: Breast cancer is one of the most common cancer types in women, and long non-coding RNAs (lncRNAs) were found to play important roles in breast cancer progression. The present study examined the effects of Linc00152 on the breast cancer progression and explored the underlying molecular mechanisms.

MATERIALS AND METHODS: The expression levels of relevant genes in tissues and cells were detected by quantitative Real-time PCR (qRT-PCR) assay. Cell viability, growth, invasion, and migration were measured by CCK-8, colony formation, transwell invasion, and migration assays, respectively. Western blot was used to detect the expression levels of proteins.

RESULTS: The results showed that Linc00152 was highly expressed in the breast cancer tissues compared to their adjacent normal tissues, and Linc00152 was also up-regulated in the breast cancer cell lines compared to normal cell lines. Knock-down of Linc00152 by using siRNAs in breast cancer cell lines (MDA-MB-231 and MCF-7) significantly suppressed cell viability, cell growth, cell invasion and migration as measured by the CCK-8, colony formation, transwell invasion, and migration assays. The qRT-PCR and Western blot results showed that knock-down of Linc00152 suppressed epithelial-mesenchymal transition in breast cancer cell lines. In addition, CCK-8 assay showed that knock-down of Linc00152 in MCF-7/ADR cells reversed the chemo-resistance to doxorubicin.

CONCLUSIONS: Our results suggested the oncogenic role of Linc00152 in the breast cancer progression. Understanding the role of Linc00152 in breast cancer progression may provide a novel therapeutic target for the treatment of breast cancer.

Key Words:
Breast cancer, Linc00152, Cell viability, Invasion and migration, Epithelial-mesenchymal transition, Chemo-resistance.

Introduction

Breast cancer is one of the most common cancer types in women and has become one of the leading causes of cancer deaths in women worldwide[1]. Although significant advances have been made in breast cancer screening and treatment including surgical methods, chemotherapy, and targeting agents against estrogen receptors and human epidermal growth factor receptor 2, a poor prognosis and early metastasis are found in patients with advanced-stage invasive breast carcinoma after treatment[2,3]. Moreover, chemo-resistance in breast cancer to various classes of chemotherapeutic agents has become a major obstacle to the successful treatment of breast cancer[4,5]. In this regard, to further understand the

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molecular mechanisms underlying breast cancer progression may aid us to identify novel therapeutic targets for the treatment of breast cancer.

Long non-coding RNAs (lncRNAs) are a type of transcripts with more than 200 nucleotides and had no protein-coding capacity[6]. A growing number of studies has suggested the important roles of lncRNAs in various biological processed by regulating gene expression at chromatin organization, transcriptional and post-transcriptional levels[7]. For example, lncRNA HOX transcript antisense RNA functioned as microRNA (miRNA) sponge to promote the epithelial to mesenchymal transition (EMT) in esophageal cancer[8]. LncRNA colorectal neoplasia differentially expressed was found to promote colorectal cancer cell viability via epigenetically silencing dual-specificity phosphatase 5/cyclin dependent kinase inhibitor 1A expression[9]. In addition, a growing number of lncRNAs has been identified to involve in the breast cancer progression. The forkhead box N3-nuclear abundant transcript 1 (NEAT1)-SIN3A repressor complex was reported to promote progression of hormonally responsive breast cancer[10]. Targeting lncRNA differentiation antagonizing non-protein coding RNA was found to suppress triple negative breast cancer progression[11]. LncRNA H19 could confer chemo-resistance in estrogen receptor α-positive breast cancer through epigenetic silencing of the pro-apoptotic gene BCL2 interacting killer[12]. The newly discovered lncRNA Linc00152 was reported to regulate cancer progression in several types of cancers, such as gastric cancer, lung cancer, tongue squamous cell carcinoma, liver cancer, pancreatic cancer, and colorectal cancer[13-18]. Linc00152 was reported to involve in cell cycle progression mitosis in Hela cells[19]. However, the exact molecular mechanisms of Linc00152-involved breast cancer progression are still unclear.

In the present investigation, a series of *in vitro* experiments were performed to investigate the potential actions of Linc00152 in breast cancer. The data suggested that Linc00152 was up-regulated in breast cancer tissues and cell lines, and knock-down of Linc00152 suppressed cell viability, cell growth, cell invasion and migration, and EMT, and also promoted chemo-sensitivity in breast cancer cells.

Materials and Methods

Clinical Samples

Breast cancer tissues and adjacent normal tissues were obtained by surgical resection from 40 patients in Shenzhen People's Hospital. All the patients had no preoperative chemotherapy, radiotherapy or molecular targeting treatment. The signed informed consent was obtained from all the patients. The studies were approved by the Ethics Committee of Shenzhen People's Hospital. The re-sectioned tissues were snap-frozen in liquid nitrogen and stored at -80°C for further experimentation.

Cell Lines and Cell Culture

The normal human mammary epithelial cell (MCF-10A), human breast cancer cell lines (SK-BR3, MDA-MB-231, and MCF-7), and MCF-7/adriamycin (ADR) (human breast adenocarcinoma multidrug-resistant cell line selected against doxorubicin) were obtained from Shanghai Cell Institute of Chinese Academy of Sciences (Shanghai, China). MCF-10A cells were cultured in a 1:1 ratio of Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) and Ham's F-12 nutrient mixture (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). SKBR3, MDA-MB-231, and MCF-7 cells were cultured in DMEM medium supplemented with 10% FBS. MCF-7/ADR cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS. All cultures were grown at 37°C in 5% CO_2 atmosphere.

Small Interfering RNA Transfection and Doxorubicin Treatment

MDA-MB-231 and MCF-7 cells were transfected with scrambled siRNA (si-NC) or siRNAs targeting Linc00152 (si-Linc00152 #1, #2, and #3), and the siRNAs were designed and synthesized by RiboBio (Guangzhou, China). Cells were cultured in 6-well plates until 50%-60% confluence, transfected with siRNAs at a final concentration of 100 nM by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and after 48 h, the transfected cells were processed for further experiment. Doxorubicin was purchased from Sigma, and MCF-7 and MCF-7/ADR cells were treated with different concentrations (2, 4, 6, 8, 10, 20, 40 μg/ml) for 24 h, and the treated cells were processed for further experimentation.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from cells or tissues by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The cDNA was

generated by using the Reverse Transcript System kit. Real-time PCR reactions were performed by using SYBR green PCR Master Mix (TaKaRa, Dalian, China) and an ABI7500 System (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control for Linc00152, E-cadherin, N-cadherin, and vimentin. The primers for Linc00152: forward, 5'- TGAGAATGAAGGCTGAGGTGT-3', reverse, 5'-GCAGCGACCATCCAGTCATT-3'; the primers for E-cadherin: forward, 5'-CACCTGGAGAGAGGCCATGT-3', reverse, 5'-TGGGAAACA TGAGCAGCTCT-3'; the primers for N-cadherin: forward, 5'-TGGGAAACATGAGCAGCTCT-3', reverse, 5'-TACACCGTGCCGTCCTCGTC-3'; the primers for vimentin: forward, 5'-CTTGAACGGAAAGTGGAATCCT-3', reverse, reverse, 5'-GTCAGGCTTGGAAACGTCC-3'; the primers for GAPDH: forward, 5'-CGTATTGGGCGCCTGGTCAC-3' and reverse, 5'-ATGATGACCCTTTTGGCTCC-3'. The relative expression of the genes was calculated by comparative Ct method.

Cell Viability Assay

The transfected cells or doxorubicin-treated cells were seeded into 96-well plated. At 0, 24, 48, 72, and 96 h post-transfection, or at 24 h after doxorubicin treatment, cell viability was assessed by Cell Counting Kit-8 (CCK-8) assay (Beyotime, Beijing, China). The cell viability was determined by optical density (OD) values at 450 nm by using a microplate reader (Hitachi, Tokyo, Japan).

Colony Formation Assay

The transfected cells were trypsinized. 1000 viable cells were sub-cultured in six-well plates. Cells were allowed to grow on the six-well plates for two weeks, and the medium was refreshed every 3 d. To visualize the colonies, the cells were fixed in 70% ethanol and, then, stained with crystal violet. The number of colonies was counted under a light microscope.

Transwell Invasion and Migration Assays

For the transwell invasion assay, the transfected cells were plated in serum-free medium in the top chamber with a Matrigel-coated membrane (8 μm pore size; Millipore, Billerica, MA, USA) and the medium supplemented with 10% FBS was filled in the lower chamber. For the transwell migration assay, the transfected cells were plated in serum-free medium in the top chamber with a non-coated membrane (8 μm pore size; Millipore). After 48 h culture, the invaded or migrated cells at the bottom of the chamber inserts was fixed with ethanol and stained with crystal violet. The number of invaded or migrated cells were counted under a light microscope.

Western Blot Assay

The proteins were extracted from transfected cells by using the RIPA lysis buffer (Sigma-Aldrich, St. Louis, MO, USA). The isolated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The membranes were blocked by 5% skim milk at room temperature for 1 hour and, then, incubated with the primary antibodies at 4°C overnight. The primary antibodies including: anti-E-cadherin (dilution, 1:1500), anti-N-cadherin (dilution, 1:1000), anti-vimentin (dilution, 1:1500) and anti-β-actin (dilution, 1:2000; Abcam, Cambridge, MA, USA). The membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody. The bands were visualized by the enhanced chemiluminescence kit (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

Statistical analysis was performed using the software of GraphPad Prism Version 6.0 (GraphPad Software, La Jolla, CA, USA). All the *in vitro* experiments were performed at least in triplicates. Student's *t*-tests or one-way ANOVA followed by Bonferroni's post-hoc comparison tests was used to determine the statistical significance of differences between experimental groups. $p < 0.05$ was considered statistically significant.

Results

Up-Regulation of Linc00152 in Breast Cancer Tissues and Cell Lines

Studies have indicated that Linc00152 was up-regulated in several types of cancers and contributed to tumor progression. In the present study, we first assessed the expression levels of Linc00152 in clinical samples from patients with breast cancer, and qRT-PCR results showed that the expression level of Linc00152 was higher in cancerous breast tissues than that in normal adjacent tissues (Figure 1A). Consistently, the expression levels of Linc00152 were significantly higher in the breast cancer cell lines (SK-BR3, MDA-MB-231, and MCF-7) than that in the normal cell line (MCF-10A). Furthermore, we selected the MDA-MB-231 and MCF-7 cells

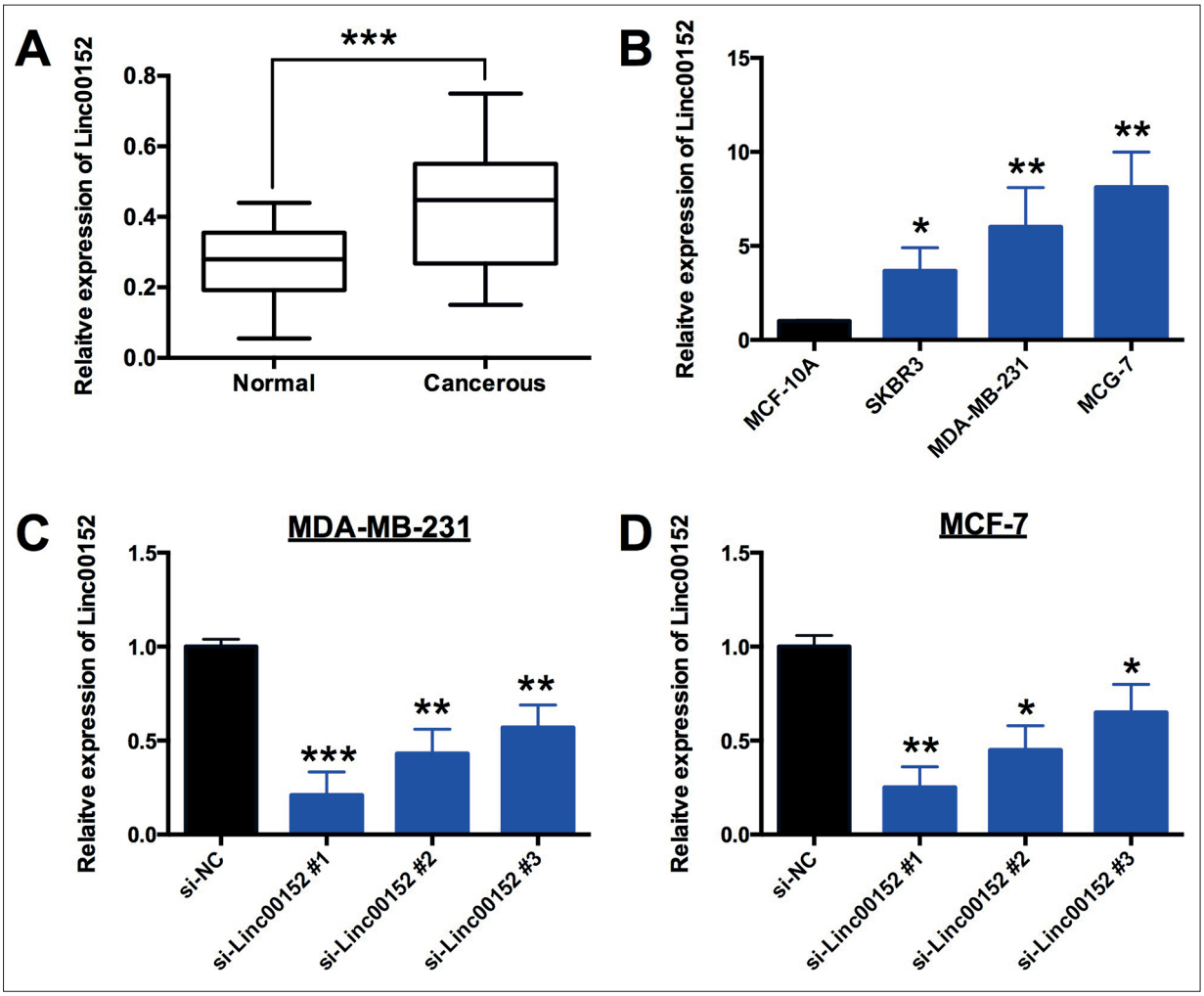

Figure 1. Up-regulation of Linc00152 in breast cancer tissues and cell lines. **(A)** The expression levels of linc00512 in normal adjacent tissues (n = 40) and cancerous tissues (n = 40) were detected by qRT-PCR. **(B)** qRT-PCR assays for Linc00152 expression in MCF-10A, SKBR3, MDA-MB-231 and MCF-7 cells. **(C)** MDA-MB-231 cells and **(D)** MCF-7 cells transfected with si-NC (scrambled siRNA) and Linc00152 siRNAs (si-Linc00152 #1, si-Linc00152 #2, and si-Linc00152 #3). After 48 h, qRT-PCR assay was performed to detect Linc00152 expression. Data are shown as mean ± standard error of the mean (SEM), n = 3. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs.* relative controls.

for further functional studies, as the Linc00152 was highly expressed in the two cell lines. The transfection results showed that MDA-MB-231 and MCF-7 cells transfected with Linc00152 siRNAs (si-Linc00152 #1, si-Linco00152 #2, and si-Linco00152 #3) had lower expression levels of Linc00152 compared to that transfected with si-NC (scrambled siRNA) (Figure 1C and 1D).

Knock-Down of Linc00152 Suppressed Cell Viability and Growth in Breast Cancer Cells

The CCK-8 assay was performed to investigate the knock-down effects of Linc00152 on cell viability in MDA-MB-231 and MCF-7 cells, and the results showed that si-Linc00152 #1 transfection in MDA-MB-231 and MCF-7 cells decreased the cell viability compared with si-NC transfection (Figure 2A and Figure 2B). Further colony formation assay obtained the similar results, in which the number of colonies in MDA-MB-231 and MCF-7 cells transfected with si-Linc00152 #1 was markedly reduced compared with control group (Figure 2C and 2D).

Knock-Down of Linc00152 Suppressed Cell Invasion and Migration in Breast Cancer Cells

Next, the transwell invasion and migration assays were performed to determine the effects

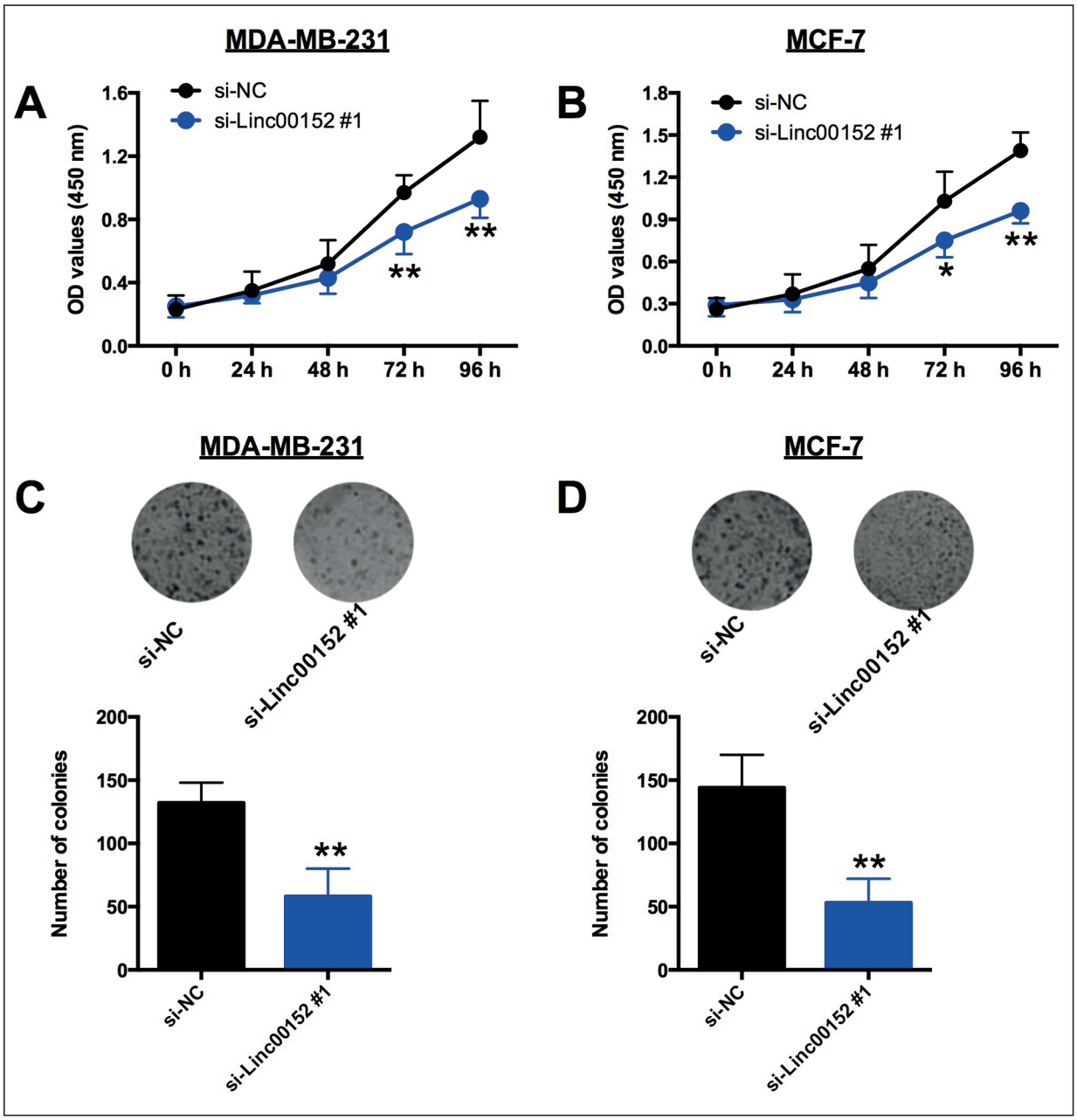

Figure 2. Effects of Linc00152 on cell viability and growth in breast cancer cell lines. **(A)** MDA-MB-231 cells and **(B)** MCF-7 cells transfected with si-NC and si-Linc00152 #1. At 0, 24, 48, 72, and 96 h post-transfection, the CCK-8 assay was performed to measure the cell viability. **(C)** MDA-MB-231 cells and (B) MCF-7 cells transfected with si-NC and si-Linc00152 #1. After 48 h, colony formation assay was performed to determine the cell growth ability. Data are shown as mean ± SEM, n = 3. *p < 0.05 and **p < 0.01 *vs.* relative controls.

of Linc00152 knock-down on invasive and migratory cell abilities in MDA-MB-231 and MCF-7 cells. The transwell invasion assay results showed the invasive cell abilities were markedly reduced in cells transfected with si-Linc00152 #1 compared with that in the control group (Figure 3A and 3B). Also, the transwell migration assay showed consistent results found in the transwell invasion assay, and si-Linc00152 #1 transfection in MDA-MB-231 and MCF-7 cells significantly inhibited cell migration compared with the control group (Figure 3C and 3D).

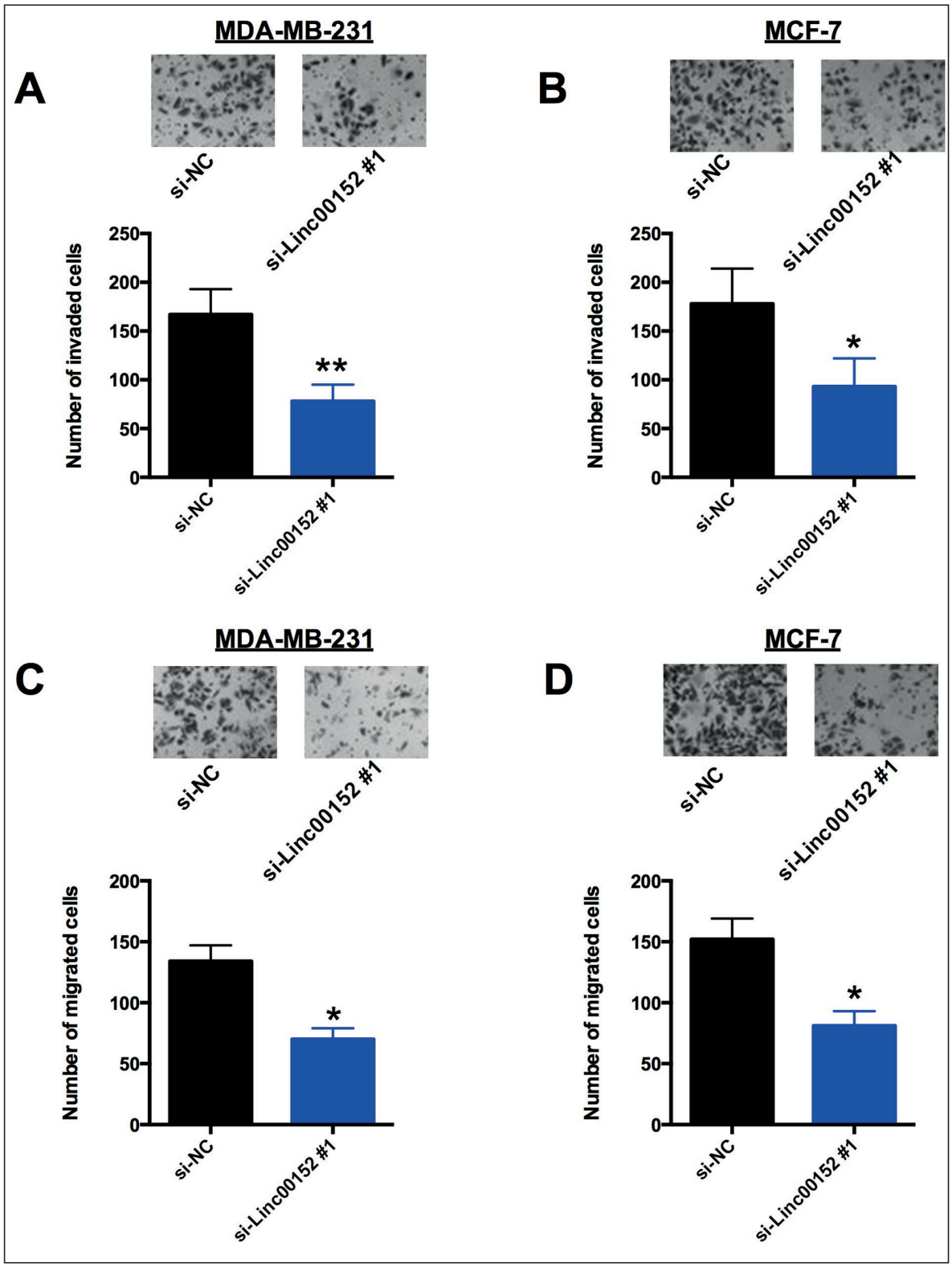

Figure 3. Effects of Linc00152 on cell invasion and migration in breast cancer cell lines. **(A)** MDA-MB-231 cells and **(B)** MCF-7 cells transfected with si-NC and si-Linc00152 #1. After 48 h, transwell invasion assay was performed to determine the invasive cell ability. **(C)** MDA-MB-231 cells and **(D)** MCF-7 cells transfected with si-NC and si-Linc00152 #1. After 48 h, transwell migration assay was performed to determine the cell migratory ability. Data are shown as mean ± SEM, n = 3. *p < 0.05 and **p < 0.01 *vs.* relative controls.

Knock-Down of Linc00152 Suppressed EMT in Breast Cancer Cells

EMT is an important cellular process and it has been indicated to be involved in the tumor metastasis. In the present study, qRT-PCR and Western blot assays were performed to determine the expression of the EMT-related markers (E-cadherin, N-cadherin, and vimentin). The qRT-PCR results showed that si-Linc00152 #1 transfection in MDA-MB-231 and MCF-7 cells significantly increased mRNA expression of E-cadherin, and decreased the mRNA expression of N-cadherin and vimentin compared with control group (Figure 4A and 4B). Consistently, si-Linc00152 #1 transfection in MDA-MB-231 and MCF-7 cells also significantly increased protein expression of E-cadherin and decreased the protein expression of N-cadherin and vimentin compared with control group (Figure 4C and 4D).

Knock-Down of Linc00152 Promoted Chemo-sensitivity in Breast Cancer Cells

Firstly, we compared the expression of Linc00152 between MCF-7 and MCF-7/ADR cells, and the expression of Linc00152 in MCF-7/ADR was significantly higher than that in MCF-7 cells (Figure 5A). In addition, the cell viability of doxorubicin-treated MCF-7/ADR cells was significantly higher than that of doxorubicin-treated MCF-7 cells (Figure 5B). The qRT-PCR assay confirmed that si-Linc00152 #1 transfection significantly reduced the expression of Linc00152 in MCF-7/ADR cells when compared to the control group (Figure 5C). The CCK-8 assay showed that si-Linc00152 #1 transfection significantly suppressed the cell viability of doxorubicin-treated MCF-7/ADR cells when compared to control group (Figure 5D).

Discussion

The present work for the first time reports the expression level changes and biological actions of Linc00152 in breast cancer tissues and cell lines. The results showed that Linc00152 was up-regulated in both breast cancer tissues and cell lines. The *in vitro* functional assays showed that knock-

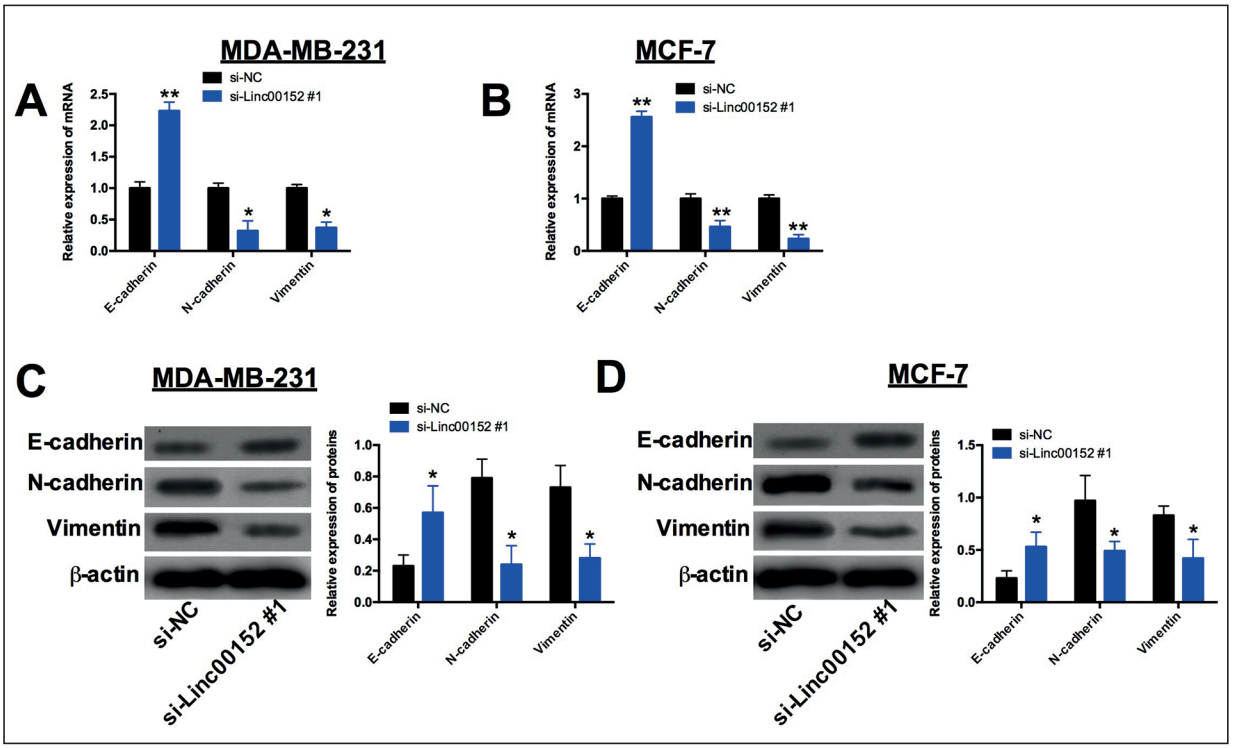

Figure 4. Effects of Linc00152 on EMT in breast cancer cell lines. **(A)** MDA-MB-231 cells and **(B)** MCF-7 cells transfected with si-NC and si-Linc00152 #1. After 48 h, the qRT-PCR assay was performed to detect the mRNA expression of E-cadherin, N-cadherin, and vimentin. **(C)** MDA-MB-231 cells and **(D)** MCF-7 cells transfected with si-NC and si-Linc00152 #1. After 48 h, the qRT-PCR assay was performed to detect the protein expression of E-cadherin, N-cadherin, and vimentin. Data are shown as mean ± SEM, n = 3. *$p <$ 0.05 and **$p < 0.01$ *vs.* relative controls

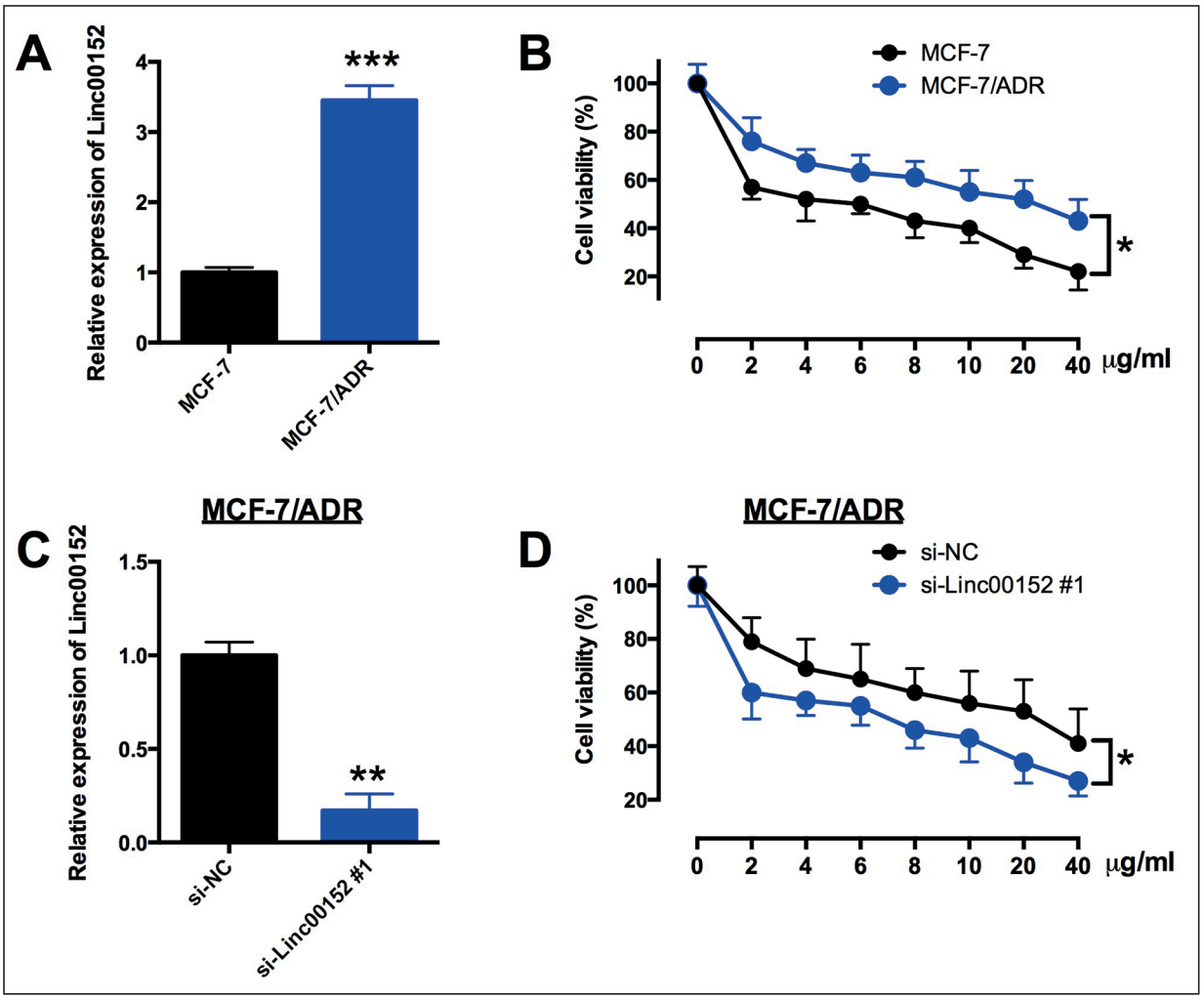

Figure 5. Effects of Linc00152 on chemo-sensitivity in breast cancer cell lines. **(A)** The expression levels of Linc00152 in MCF-7 cells and MCF-7/ADR cells were detected by qRT-PCR. **(B)** MCF-7 and MCF-7/ADR cells were treated with the indicated concentrations of doxorubicin for 24 h, followed by the CCK-8 assay to detect cell viability. **(C)** MCF-7/ADR cells transfected with si-NC and si-Linc00152 #1. After 48 h, the qRT-PCR assay was performed to detect the expression of Linc00152. **(D)** MCF-7/ADR cells transfected with si-NC and si-Linc00152 #1 were treated with indicated concentrations of doxorubicin for 24 h. Data are shown as mean ± SEM, n = 3. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* relative controls.

down of Linc00152 suppressed cell viability, cell growth, cell invasion and migration as well as EMT in breast cancer cells, and suppression of Linc00152 also promoted chemo-sensitivity in MCF-7/ADR cells.

Linc00152 was a newly discovered lncRNA, and it is located on the 2p11.2[20]. The oncogenic roles of Linc00152 have been revealed in various types of cancers, including gastric cancer, lung cancer, tongue squamous cell carcinoma, liver cancer, pancreatic cancer, and colorectal cancer[13,15,17,18,20]. Up-regulation of Linc00152 in these types of cancerous tissues was positively correlated with poor prognosis in the cancer patients. In gastric cancer, Linc00152 was found to promote cell viability via the epidermal growth factor receptor (EGFR)-dependent pathway[21] and promote tumor cell cycle progression by binding to the enhancer of zeste homolog 2 (EZH2) and repressing p15 and p21[22]. In the lung cancer, up-regulation of Linc00152 promoted lung cancer cell viability and invasion, and also predicted poor prognosis in cancer patients, and down-regulation of Linc00152 suppressed the biological activity of lung cancer (cell viability, invasion, and migration) via EGFR/PI3K/ATK signaling pathway[23]. In colorectal cancer, Linc00152 was found to be down-regulated by miR-376c-3p, which in turn

suppressed cell viability and promoted apoptosis in colorectal cancer cell lines[18]. The microarray analysis of several types of cancers showed that Linc00152 was up-regulated in the breast cancer tissues, and was essential for cell cycle progression in HeLa cells[19]. Consistently, our qRT-PCR results showed that Linc00152 was highly expressed in both breast cancer tissues and cell lines, and knock-down of Linc00152 suppressed cell viability, growth, invasion, and migration in breast cancer cells. Collectively, the data suggest that Linc00152 may play an oncogenic role in the breast cancer.

EMT is a process that endows epithelial tumor cells with mesenchymal properties including reduced adhesion and increased motility, and EMT has been suggested to be essential in driving the early phase of cancer metastasis. Lots of lncRNAs have been identified to regulate the EMT in breast cancer, such as plasmacytoma variant translocation 1, NEAT1, HOXA11 Antisense RNA, anti-differentiation ncRNA and so on[24-27]. In terms of Linc00152, knock-down of Linc00152 reduced the EMT in gastric cancer cells[28], and overexpression of Linc00152 promoted EMT in the gallbladder cancer cells[29]. In the liver cancer, Linc00152 overexpression was found to suppress E-cadherin expression via interacting with EZH2 and promoted EMT in liver cancer cells[13]. As expected, our results showed that knock-down of Linc00152 suppressed EMT in breast cancer cells, suggesting that Linc00152 may regulate the cell invasion and migration possibly via the EMT process.

Adriamycin is one of the most commonly used chemotherapy agents used for breast cancer[30]. However, repeated adriamycin treatment usually developed drug resistance, which hurdles the clinical cure of breast cancer. Recently, the lncRNAs were found to be involved in the chemo-resistance in different types of cancers[31]. In breast cancer, inhibition of lncRNA regulator of reprogramming reversed the resistance to tamoxifen by inducing autophagy[32]. The lncRNA adriamycin resistance associated was found to be up-regulated in MCF-7/ADR cells and contributed to the adriamycin resistance in the breast cancer cells[33]. In terms of Linc00152, studies have found that Linc00152 functioned as a competing endogenous RNA to confer oxaliplatin resistance in colon cancer[34]. In agreement with previous findings, we showed that knock-down of Linc00152 reversed the chemo-resistance in MCF-7/ADR cells, suggesting that Linc00152 may have enhanced effects on the chemo-resistance in the breast cancer cells. However, the underlying molecular mechanisms require further investigation.

Conclusions

We demonstrated the highly abnormal expression of Linc00152 in breast cancer tissues and cells, and knock-down of Linc00152 impaired cell viability, invasion, migration and EMT, and also reversed chemo-resistance in the breast cancer cells. However, as we only performed the *in vitro* experiments, the current investigation on Linc00152 is still incomplete and further *in vivo* experiments may be required to clarify the roles of Linc00152 in breast cancer progression.

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Conflict of Interest
The Authors declare that they have no conflict of interests.

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