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Long non-coding RNA Loc554202 regulates proliferation and migration in breast cancer cells

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ABSTRACT

Data derived from massive cloning and traditional sequencing methods have revealed that long non-coding RNAs (lncRNA) play important roles in the development and progression of cancer. Although many studies suggest that the lncRNAs have different cellular functions, many of them are not yet to be identified and characterized for the mechanism of their functions. To address this question, we assay the expression level of lncRNAs–Loc554202 in breast cancer tissues and find that Loc554202 is significantly increased compared with normal control, and associated with advanced pathologic stage and tumor size. Moreover, knockdown of Loc554202 decreased breast cancer cell proliferation, induced apoptosis and inhibits migration/invasion in vitro and impeded tumorigenesis in vivo. These data suggest an important role of Loc554202 in breast tumorigenesis.

1. Introduction

Breast cancer is now the most frequently diagnosed cancer in women worldwide [1], and also is one of the well explored human cancers with genome-wide technologies [2]. Current treatment of breast cancer includes hormonal therapy, cytotoxic chemotherapy, immunotherapy and targeted therapy [3]. Despite survival advantages achieved by using such therapies, many breast tumors are not eradicated completely due to acquiring resistance, significant toxicities, or relapse following an initial response, thus resulting in metastatic disease at later stages that leads to patient death [8]. Therefore, genomic information can be combined with clinicopathological characteristics to create novel diagnostic and therapeutic strategies remains an important component in the current management of this malignancy [4].

Recent improvements in high-throughput transcriptome analysis in the last few years, have led to the discovery that the human transcriptome comprises largely of non-coding RNAs (ncRNAs) which are with limited or no protein-coding capacity [5,6]. The ncRNAs can be broadly divided into short ncRNAs (typically up to 200 nucleotides in length) and long ncRNAs (lncRNAs; from 200 nucleotides up to ca. 100 kb) [7–9]. lncRNAs players in several biological processes [10], including X chromosome inactivation, nuclear structure, genomic imprinting and development [11]. Dysfunction of lncRNAs has been strongly associated with cell fate determination and human disease pathogenesis, including cancer [12]. Examples include modulation of melanoma cell apoptosis and invasion by SPRY-IT1 [13], promotion of bladder cancer proliferate by UCA1a [14], control of breast cancer cell apoptosis by GAS5 [15], regulation of hepatocellular cancer cell growth and apoptosis by MEG3 [16].

Given the importance of lncRNAs in cancers, in the current study, we explored the lncRNAs–Loc554202 expression level in breast cancer tissues and uncovered marked upregulation of lncRNAs–Loc554202 in tumor samples compared with matched normal samples by performing quantitative qRT-PCR. We further discovered that lncRNAs–Loc554202 up-regulation also correlated with tumor size and advanced pathologic stage. Functional analyses of lncRNAs–Loc554202 indicated that siRNA-mediated knockdown of lncRNAs–Loc554202 results in diminished cell proliferation, migration, invasion and increased apoptosis. Taken together, these findings suggest that LOC554202 participates as a non-coding oncogene in breast tumorigenesis.

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2. Materials and methods

2.1. Tissue collection

Breast cancer tissues and normal tissues were obtained from 20 patients who had undergone surgical resection of breast cancer between 2009 and 2011 at Second Affiliated Hospital of Nanjing Medical, China. Local or systemic treatment had not been conducted in these patients prior to the operation. All the tissues were washed with sterile phosphate-buffered saline before being snap frozen in liquid nitrogen and then stored at −80 °C until total RNA was extracted. The pathological stage, grade and nodal status were appraised by an experienced pathologist. Clinicopathologic characteristics including tumor, node-metastasis (TNM) staging were also collected. The non-tumorous tissues were 5 cm from the edge of the tumor and there were no obvious tumor cells which were evaluated by the pathologist. All of the experiments were approved by the Research Ethics Committee of Nanjing Medical University, China.

2.2. Cell line and culture conditions

The human breast cancer cell lines (MD-MB-231, MD-MB-435S) were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). MCF-10A and MCF-7 were provided by Dr. Jianwei Zhou (the Molecular Toxicology Laboratory, Nanjing Medical University, China). MD-MB-231 and MD-MB-435S were cultured in Leibovitz's L-15 Medium (L-15; gibco) in humidified air at 37 °C with 5% CO_2. MCF-10A and MCF-7 were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) in humidified air at 37 °C with 5% CO_2. All Medium were supplemented with 10% fetal bovine serum (10% FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Invitrogen, Shanghai, China).

2.3. RNA extraction and qRT-PCR analyses

Total RNA was extracted from tissues or cultured cells with TRIzol reagent (Invitrogen Life Technologies) according to the manufacturer's protocols. For qPCR, RNA reverse transcribed to cDNA from 1 μg of total RNA was reverse transcribed in a final volume of 20 μl using random primers and a Reverse Transcription Kit (Takara). According to the manufacturer's instructions, the reverse transcription was performed at 37 °C for 15 min, then 85 °C for 5 s. Real-time PCR (RT-PCR) analyses were performed using a standard protocol from Power SYBR Green (Takara). All protocols were performed according to the manufacturer's instructions. ΔCt values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primer sequences were as follows:

- GAPDH:

  Forward: 5′-AGAGGCAGGGATGATGTTCTG-3′;
  Reverse: 5′-GACTCATGACCACAGTCCATGC-3′;
- Loc554202:

  Forward: 5′-TCTCTGGTGCTTCCCTCCTT′;
  Reverse: 5′-GATCTAAGCTTGAGCCCCCA-3′.

QPCR and data collection were performed using an ABI 7500. Each sample was analyzed in triplicate.

2.4. Small interfering RNA transfection

Antihuman siRNAs which were purchased from Life Technologies (Carlsbad, California, USA) were used for Loc554202 knockdown. The target sequences for the si-LOC554202 included: si-Loc554202-1 (GCAGGUAGAGAUGGAUUCCUGGAAA); si-LOC554202-2 (GGAGCGCUUUGUGUGAGAAGUUGAA); and si-LOC554202-3 (CCUGGUUGAGCUGAGGUCUUCAUAG), and the experiments showed that the later had the highest inhibition efficiency. Synthetic sequence-scrambled siRNA was used as a negative control siRNA. Cells were plated and cultured in growth media until cell density reached 50–60% prior to siRNA transfection using Lipofectamine2000 (Invitrogen, Shanghai, China) according to the manufacturer's instructions. Cells were harvested after 48 h for qRT-PCR and Western blot analyses.

2.5. Cell proliferation assays

Forty-eight hours after siRNA transfection, 3000 cells per well were allowed to grow in 96-well plates with five replicate wells. After 6 h of culture, as well as at hours 24, 48, 72 and 96, cells were treated with 100 μg 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) by adding it to the medium. The cells were incubated at 37 °C for another 4 h, then the medium was removed, dimethylsulfoxide (DMSO) was added for 10 min to lyse the cells. Finally, the absorbance was measured at 490 nm. All experiments were performed in triplicates.

2.6. Colony formation and clonogenic assays

Cells were trypsinised into single-cell suspension 48 h after transfection. For the colony formation assay, 1000 cells were plated into each well of a 6-well plate and maintained in media containing 10% FBS to allow colony formation, replacing the medium every 4 days. After 2 weeks, colonies were fixed with methanol and stained with 0.1% crystal violet (Sigma) in PBS for 15 min. Visible colonies were manually counted. Triplicate wells were measured in each treatment group.

2.7. Cell migration/invasion assay

3×10^4 cells were suspended in 200 μl of L-15 with 1% FBS and seeded on top chamber of the transwell (Millicell, PIEP12R48) with a membrane pore with an 8 μm diameter at 48 h after siRNA transfection. The medium containing 10% FBS was immediately placed into the lower well of the chamber as a chemoattractant. The cells were allowed to migrate for 24 h, and the cells that did not migrate through the pores of the transwell inserts were manually removed with a cotton swab. Cells which presented at the bottom of the membrane were fixed and stained using Giemsa stain and counted under the microscope. Cell numbers were calculated in five random fields for each chamber, and the average value was calculated. Each experiment was conducted in triplicate. Matrigel invasion assays were performed as described previously [17].

2.8. Flow cytometry

Cells transiently transfected with siRNA were harvested 48 h after transfection by trypsinisation, washed with ice-cold phosphate-buffered saline, and fixed with 75% ethanol overnight. Cells for cell-cycle analysis were stained with propidium oxide (100 μg/mL) using the Cycle Test Plus DNA Reagent Kit (BD Biosciences) following the protocol and analyzed by flow cytometry (FACScan; BD Biosciences) equipped with a CellQuest software (BD Biosciences).The percentages of the cells in G0–G1, S, and G2–M phases were counted and compared.

Cells for apoptosis analysis were harvested 48 h after transfection, and stained for 15 min with fluorescein isothio-cyanate (FITC)–Annexin V and propidium iodide (PI) in the dark at room temperature, according to the manufacturer's recommendations.

Then they were examined by flow cytometry (FACScan®; BD Biosciences) equipped with a CellQuest software (BD Biosciences) [18], and were discriminated into viable cells, dead cells, early apoptotic cells, and apoptotic cells. The percentage of early apoptotic cells were compared to control groups from each experiment. All of the samples assayed were in triplicates.

2.9. Western blot assay and antibodies

Western blot analysis was performed as previously described [19]. Briefly, cells were lysed using the mammalian protein extraction reagent RIPA (Beyotime) supplemented with a protease inhibitor cocktail (Roche) and PMSF (Roche). Protein were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and then transferred to 0.22 mm nitrocellulose (NC) or polyvinylidene difluoride membranes (Sigma). The membranes were washed, blocked, and incubated with the specific primary antihuman antibodies. The secondary antibody was horseradish peroxidase-conjugated goat anti-rabbit IgG. ECL chromogenic substrate was used to visualize the bands and the intensity of the bands was quantified by densitometry (Quantity One software; Bio-Rad). Mean ± SD was calculated from 3 individual experiments. We used 1:1000 cyclinD2 rabbit monoclonal antibody (#3741, CST, USA), 1:500 cyclinD1 rabbit antibody (bs-0623R, Bioss, China), 1:1000 caspase-9 rabbit monoclonal antibody (#9502, CST, USA), 1:1000 bcl-2 rabbit monoclonal antibody (#2872, CST, USA), 1:10,000 β-actin rabbit monoclonal antibody (#3741, CST, USA) and 1:10,000 goat anti-rabbit IgG (bs-0293Gs, Bioss, China).

2.10. Tumor formation assay in a nude mouse model

Four-week-old female BALB/c nude mice were obtained from Shanghai Laboratory Animals Center of the Chinese Academy of Sciences (Shanghai, China), housed under pathogen-free conditions with a 12 h light/12 h dark schedule, fed with an autoclaved diet ad libitum , and injected subcutaneously with 5×10^6 cells. Tumor growth was examined every 3 days, and tumor volumes were calculated using the following formula: $0.5 \times \text{length} \times \text{width}^2$. At 2 weeks after injected, mice were euthanized, and the subcutaneous weight of each tumor was measured and also used for further analysis. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Nanjing medical University (Permit Number: 200933).

2.11. Statistical analysis

All the data were expressed as mean ± SD (standard deviation, SD), and were analyzed using Student's *t* test between two groups in vitro and in vivo data using SPSS 17.0 software. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Expression of LOC554202 in breast cancer tissues and breast cancer cell lines

QRT-PCR analysis was used to examine the expressions of LOC554202 in 20 breast cancer tissues and matched breast normal tissues. Significantly, the expressions of LOC554202 were up-regulated in breast cancer tissues compared with normal tissues (Fig. 1A). Moreover, correlation analysis of LOC554202 expression with clinical pathological features of breast cancer patients, revealed that the higher expression of LOC554202 correlated with larger tumor size (Fig. 1B) and an advanced pathologic stage (Fig. 1C).

Next, we examined the differential expressions of LOC554202 in 3 human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435S) versus normal breast cancer cells (MCF-10A), revealed that LOC554202 was over expressed in MDA-MB-231 and MDA-MB-435S by 2.5-fold and 7-fold (Fig. 1D).Therefore, we chose MDA-MB-435S for further studies.

3.2. siRNA-mediated knock down of LOC554202 inhibits breast cancer cell proliferation and causes apoptosis

Because LOC554202 was highly expressed in breast cancer tissues compared with normal tissues, we hypothesized that the biological functions of LOC554202 may involve in the control of cell proliferation. To examine this possibility, MTT assays were used to detect the impact of LOC554202 knockdown on proliferation of breast cancer cell MDA-MB-435S. According to the results, we found that siRNA transfection-mediated LOC554202 knockdown decreased cell growth by more than 50% relative to negative control scrambled siRNA transfection at 96 h in MDA-MB-435S cell lines ($p < 0.05$; Fig. 2A). To further examine the effect of LOC554202 on the proliferative of MDA-MB-435S, colony formation assay was performed. The results revealed that the colony numbers of breast cancer cells transfected with siRNA were obvious lower than those negative control scrambled siRNA transfection ($P < 0.05$; Fig. 2B, Supplementary 1).These findings suggest that LOC554202 is involved in the promotion of breast cancer cell proliferation.

To assess potential mechanisms of LOC554202 in the breast cancer cells proliferation, we tested cell cycle and apoptosis in MDA-MB-435S cells by flow cytometry. The results demonstrated that LOC554202 knockdown led to a significant accumulation of cells at G0/G1-phase ($p < 0.05$) and a significant decrease in cells in S-phase ($p < 0.05$; Fig. 2C). Furthermore, the results of Western blot analysis showed that the expressions of cyclinD1 and cyclinD2 were significantly decreased in MDA-MB-435S cells transfected with siRNA compared to negative control scrambled siRNA transfection ($p < 0.05$ Fig. 2D and E). All the results consistented with the results of cell proliferation assays.

Next, we investigated the effects of LOC554202 knockdown on apoptosis. As shown in Fig. 2F, the proportion of apoptotic cells following a 48-h treatment by LOC554202–siRNA versus scrambled siRNAs had a significant increase ($p < 0.05$; Fig. 2G), and the results of Western blot analysis showed that the expressions of caspase-9 and bcl-2 were significantly increased in MDA-MB-435S cells transfected with siRNA compared to scrambled siRNA ($p < 0.01$, $p < 0.05$ respectively, Fig. 2D and E). These findings suggest that LOC554202-mediated promotion of breast cancer cell proliferation seems to be mediated by modulation of the G1–S checkpoint and apoptosis.

3.3. siRNA-mediated knockdown of LOC554202 inhibits breast cancer cell migration/invasion

We also examined the effect of LOC554202 knockdown on breast cancer cell invasion/migration (Fig. 3A). Knockdown of LOC554202 inhibited MDA-MB-435S cell invasion/migration by 30% and 50% respectively ($p < 0.05$, Fig. 3B and C). These data imply that LOC554202 involves mechanisms relevant to the promotion of breast cancer cell invasion and migration.

3.4. LOC554202 inhibits tumorigenesis of breast cancer in vivo

We next determined whether knockdown of LOC554202 could inhibit tumor proliferation in vivo. siRNA–LOC554202 and scrambled siRNA transfected MDA-MB-435S cells were inoculated into male nude mice. Fifteen days after injection, we found that the

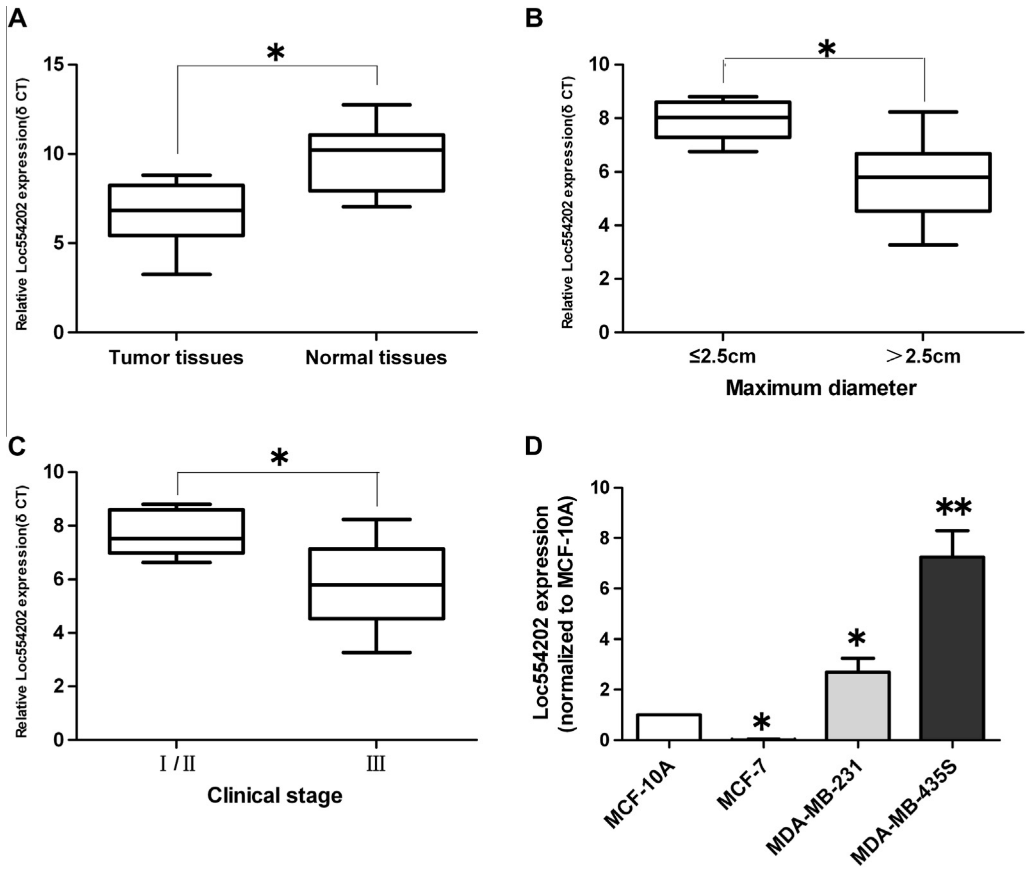

Fig. 1. qRT-PCR analysis of lncRNA LOC554202 in breast cancer tissues and breast cancer cell lines. (A) LOC554202 expression was significantly upregulated in breast cancer tissues relative to their corresponding breast normal tissues in 20 breast cancer patients (shown as ΔCT). (B and C) Data are presented as relative expression level in tumor tissues. LOC554202 expression was significantly higher in patients with a higher pathological stage and larger tumor size (shown as ΔCT). (D) Expression of LOC554202 in 3 breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-435S) and 1 normal breast epithelial cell line (MCF-10A). The expressions of LOC554202 are normalized to MCF-10A. $^{*}p < 0.05$ and $^{**}p < 0.01$.

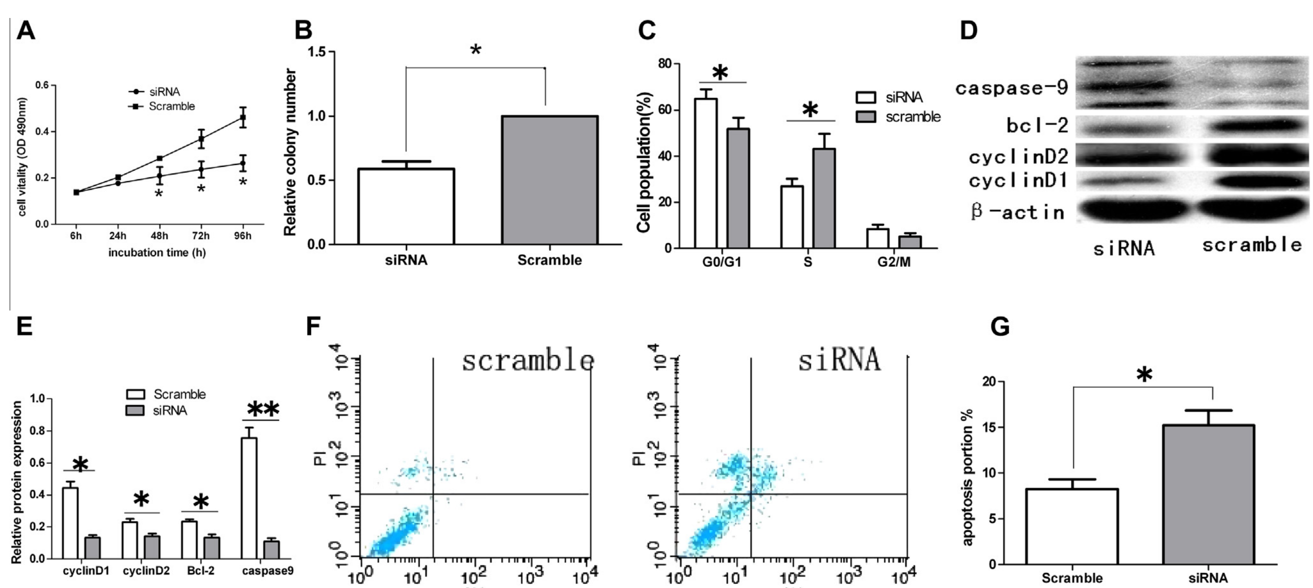

Fig. 2. siRNA-mediated knockdown of LOC554202 inhibited breast cancer cells proliferation and causes apoptosis. (A) MTT assay was performed to determine the proliferation of MDA-MB-435S cells. (B) Colony formation assays were performed to determine the proliferation of MDA-MB-435S cells. The colonies were captured and counted. (C) The bar chart represented the percentage of cells in G0/G1, S, or G2/M phase, as indicated. (D and E) Western blot analysis of cyclinD1, cyclinD2, caspase-9 and bcl-2 after siRNA or scrambled siRNA transfection in breast cancer cell lines. β-Actin protein was used as an internal control. (F and G) The apoptotic rates of cells were detected by flow cytometry. Data represented the mean ± SD from three independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$.

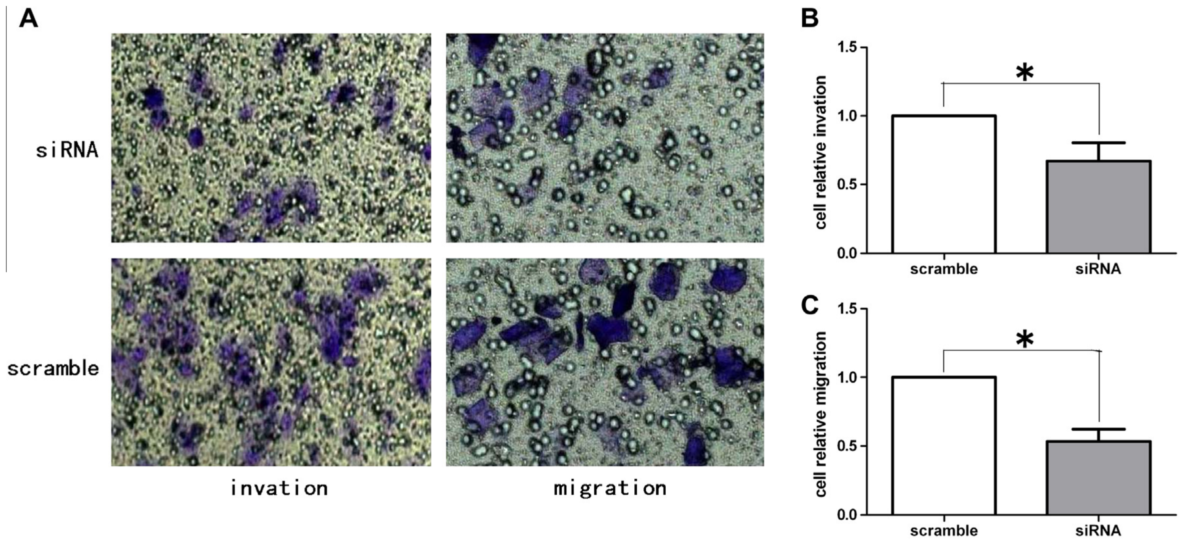

Fig. 3. Invasion/migration assay using matrigel transwel chambers for MDA-MB-435S. (A) Representative pictures of invasion/migration assay. (B and C) Knockdown of LOC554202 inhibited MDA-MB-435S cell invasion/migration by 30% and 50% respectively ($^{*}p < 0.05$).

tumors formed in siRNA-LOC554202 group were dramatic smaller than those in the scrambled group (Fig. 4A–C). In addition, the average tumor weight was markedly lower in the siRNA–LOC554202 group (0.08 ± 0.02 g) compared to the control group (0.30 ± 0.03 g) at the end of the experiment ($p < 0.01$, Fig. 4D). QRT-PCR analysis of LOC554202 expression was then performed in selected tumor tissues. The results showed that the expressions of LOC554202 in tumor tissues in the group of siRNA–LOC554202 cells were lower than those in control group ($p < 0.01$, Fig. 4E). These results indicate that LOC554202 knockdown could inhibit tumor growth in vivo.

4. Discussion

Recently, several studies have revealed that at least 90% of the genome is actively transcribed with the advent of tiling resolution genomic microarrays and whole genome and transcriptome sequencing technologies [9,20], and these transcripts are emerging as critical regulators of gene expression and cell fate. One such transcript, the newly discovered long non-coding RNA (lncRNA) genes, may contribute a significant portion of the 'dark matter' of the human transcriptome [21,22]. lncRNAs are non-coding RNAs greater than 200 nucleotides in length, they can be transcribed by RNA polymerase II (RNA pol II) and are polyadenylated [23,24]. Increasing reports that differential expression between normal and tumor states suggest that similar to protein-coding oncogenes, lncRNAs can also promote cellular pathways that lead to tumorigenesis [25], although their mechanisms of function remain less well understood [5]. For example, a new study has found that HOTAIR is significantly overexpressed in breast tumors, expression level is a powerful predictor of patient outcomes such as metastasis and death [26]. GAS5 (growth arrest-specific 5) represents another example of lncRNAs, which has been observed to be downregulated in breast cancer, perhaps to keep cancer cells active even under low nutrient conditions [27,15].

The LOC554202 gene which does not contain an open reading frame is located at chromosome 9p21.3. Studies have

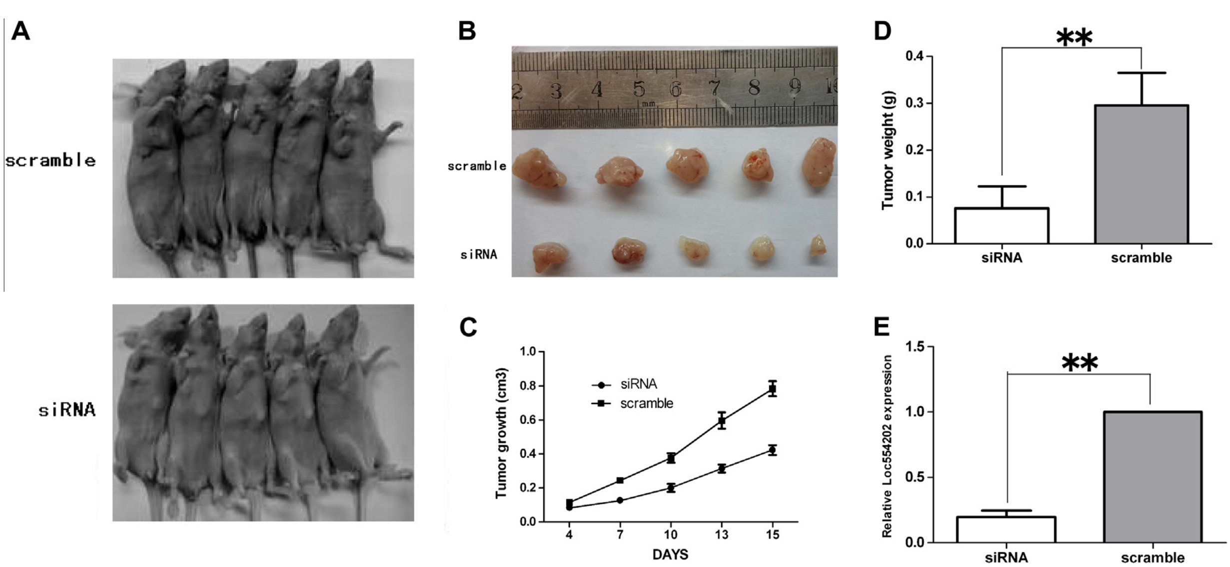

Fig. 4. Effects of LOC554202 on tumor growth in vivo. (A and B) tumors formed in siRNA group were dramatic smaller relative to controls. (C) The tumor volume was calculated once every three days after injection of MDA-MB-435S cells stably transfected with siRNA or scrambled siRNA. Points, mean ($n = 3$); bars indicate SD. (D) Tumor weights are represented as means of tumor weights ± SD. (E) qPCR analysis of LOC554202 expression in tumor tissues formed from MDA-MB-435S/LOC554202, MDA-MB-435S/control. $^{**}p < 0.01$.

demonstrated that the LOC554202 is transcribed from 4 exons resulting in a spliced transcript of ~2.2 kb [28]. In their study (Katarzyna Augoff), the authors identified LOC554202 is down-regulated in the TNBC cell lines of basal subtype and over-expressed in the luminal counterparts, CpG island methylation plays an important role in silencing of the LOC554202 genes, but they did not study the function of LOC554202 in breast cancer.

In our study, we focused on the identification and characteristics of LOC554202 in breast cancer and its role in proliferation, apoptosis, migration and invasion. However, we found LOC554202 was overexpressed in MDA-MB-231 and MDA-MB-435S, which was not consistent with the article reported by Katarzyna. Further studies shown that LOC554202 was upregulated in 20 human breast cancer tissues when compared to normal tissues. Specifically, higher expression of Loc554202 in breast cancer tissues was associated with larger tumor size and a more advanced clinical stage, suggesting that the expression of Loc554202 could be used as a diagnostic and prognostic breast cancer marker. In order to highlight the function of Loc554202, antihuman siRNAs was used for Loc554202 knockdown, functional analyses of Loc554202 indicated that siRNA-mediated knockdown of lncRNAs–Loc554202 results in diminished cell proliferation, migration, invasion and increased apoptosis in MDA-MB-435S breast cancer cells. Finally, we demonstrated that LOC554202 knockdown could inhibit tumor growth in vivo. Unfortunately, we were unable to further verify the downstream pathways. Further studies are needed to elucidate the precise mechanisms by which LOC554202 modulates its targets.

In summary, in this study, for the first time, we have shown that upregulated of the Loc554202 in breast cancer tissues. The effects of this lncRNA on cell proliferation, cell cycle, cell apoptosis, migration and invasion suggest that it promotes tumorigenesis in breast cancer. However, the exact downstream regulatory mechanisms of Loc554202 remain to be elucidated in order to provide a new target for and early diagnosis and treatment of breast cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.144.

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