miR-506 attenuates methylation of IncRNA MEG3 to inhibit migration and invasion of breast cancer cell lines via targeting SP1 and SP3

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Abstract
Background: Breast cancer has been the first death cause of cancer in women all over the world. Metastasis is believed to be the most important process for treating breast cancer. There is evidence that IncRNA MEG3 functions as a tumor suppressor in breast cancer metastasis. However, upstream regulation of MEG3 in breast cancer remain elusive. Therefore, it is critical to elucidate the underlying mechanism upstream MEG3 to regulate breast cancer metastasis.

Methods: We employed RT-qPCR and Western blot to examine expression level of miR-506, DNMT1, SP1, SP3 and MEG3. Besides, methylation-specific PCR was used to determine the methylation level of MEG3 promoter. Wound healing assay and transwell invasion assay were utilized to measure migration and invasion ability of breast cancer cells, respectively.

Results: SP was upregulated while miR-506 and MEG3 were downregulated in breast tumor tissue compared to adjacent normal breast tissues. In addition, we found that miR-506 regulated DNMT1 expression in an SP1/SP3-dependent manner, which reduced methylation level of MEG3 promoter and upregulated MEG3 expression. SP3 knockdown or miR-506 mimic suppressed migration and invasion of MCF-7 and MDA-MB-231 cells whereas overexpression of SP3 compromised miR-506-inhibited migration and invasion.

Conclusions: Our data reveal a novel axis of miR-506/SP3/SP1/DNMT1/MEG3 in regulating migration and invasion of breast cancer cell lines, which provide rationales for developing effective therapies to treating metastatic breast cancers.

Keywords: Breast cancer, Migration and invasion, miR-506, SP3, DNMT1, MEG3, Methylation

Background
Breast cancer is the most common cause of cancer-related death in women worldwide [1–3]. Similar to other solid tumors, distant metastasis (especially lung metastasis) is the leading cause of breast cancer-associated death and resistance to various treatments [4]. Therefore, it is essential for us to elucidate the molecular mechanism underlying breast cancer progression.

MicroRNAs are around 22-nucleotide-long non-coding RNAs [5]. They modulate gene expression through targeting 3′-UTR of mRNAs, leading to mRNA degradation or translational block [6, 7]. Many studies have revealed that miRNAs are involved in cell growth, migration, apoptosis and cancer metastasis [8–10]. Arora et al. [11] reported that miR-506 had a role in regulating EMT in breast cancer cell lines. As a validation, Yu et al. [12] has shown that miR-506 overexpression inhibits proliferation and metastasis of breast cancer cells. However, the mechanism of miR-506 inhibition of breast cancer metastasis remains elusive.

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MEG3 is identified as an imprinted gene with maternal expression and encodes a long non-coding RNA [13]. Dysregulation of MEG3 has been found in various human tumors, including bladder cancer, hepatocellular carcinoma, lung cancer and ovarian cancer [14–16]. More interestingly, MEG3 has been implicated into tumorigenesis and progression of breast cancer [17, 18]. Previous studies have revealed that overexpression of MEG3 could induce cell growth arrest and increase cell apoptosis in human breast cancer cells. In addition, downregulated MEG3 regulates proliferation, migration and invasion of breast cancer in a p53-dependent manner [17]. Whether miR-506 cooperates with MEG3 to regulate the metastasis of breast cancer remains unclear. In pituitary tumors, hypermethylation of the MEG3 regulatory region is identified as an important mechanism associated with the loss of MEG3 expression [19]. miR-29a was shown to regulate methylation of MEG3 via DNA methyltransferase (DNMT) 1 and 3b, thus contributing to hepatocellular carcinoma (HCC) growth [20]. Likewise, Li et al. [18] demonstrated that MEG3 was epigenetically repressed by DNMT1 to suppress the p53 pathway in glioma. Based on these findings, we hypothesized that MEG3 may be regulated in a DNA methylation-dependent manner in breast cancer cells.

SP1 and SP3 transcription factors are expressed in almost all mammalian cells. They belong to the specificity protein/Kruppel-like factor (SP/KLF) transcription factor family and are involved in regulation of DNMTs [21]. Davie et al. [22] showed SP1 and SP3 could either enhance or repress the activity of promoters of genes implicated in differentiation, cell cycle progression, and oncogenesis. Although SP1 and SP3 has been investigated in breast cancer, the detailed mechanism by which SP1 and SP3 regulate progression of breast cancer requires to be further investigated [23].

Here, we show that miR-506 inhibits migration and invasion of breast cancer cell lines through the SP3/DNMT1/MEG3 axis. Our findings reveal the detailed mechanism by which miR-506 regulates metastasis of breast cancer, which facilitates the development of therapeutic strategies for treating breast cancer.

Materials and methods

Patients and samples

The present study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University. A total of 20 breast tumor samples and 20 adjacent normal tissue samples were obtained from patients aged 20–70 in 2016–2017. No patients had received chemotherapy or radiotherapy prior to surgery. Breast cancer was validated by histological examination in all cases according to World Health Organization criteria. Breast tumors and normal tissue specimens excised surgically from patients were immediately snap-frozen and stored in liquid nitrogen until use.

Cell lines

Human breast cancer cells (MCF-7, MDA-MB-231, SKBR3) and Human Embryonic Kidney (HEK) 293T cells were purchased from ATCC and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Hyclone) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin at 37 °C, 5% CO2. Human breast epithelial MCF10A cells were grown in the base medium for this cell line (MEBM) along with the appropriate additives (MEGM, Lonza/Clonetics Corporation, CC-3150). HEK 293T cells were employed in lentivirus packaging.

Plasmid generation and lentivirus package

SP3 cDNA was cloned into pcDNA4 vector. The short hairpin RNA (shRNAs) targeting SP3 (target sequence showed blow) were purchased from GenePharma, Shanghai, China and cloned into PLKO.1 vector. To generate lentiviruses, the packaging vectors (pPAX2 and pVSIG) and transfer vector were co-transfected into 293T cells. The supernatant was harvested at 24 h and 48 h after transfection and filtered through 0.45 μm membrane. For virus infection, the virus supernatant was added to medium at 1:3 ratio, 24 h later, 2ug/ml puromycin was used to select stable cell lines. scramble shRNA and empty pcDNA4 vector was used as negative control respectively.

shRNA targeting sequences of SP3: shSP3#1: GCA AGAACTGTGGTGTTGTTGG; shSP3#2: CCTTCTGCT AACATCCAGAAT; shSP3#3: CGCGAGATGATACTTTGATTA; shSP3#4: GTGGTGATTCTACCTTGAAATA.

Transfection

For NC and miR-506 mimic transfection, we used LipofectamineVR LTX with PlusTM Reagent (Life Technologies) according to manufacturer’s instructions. mimic NC and miR-506 mimics were synthesized by GenePharma.

Wound healing assay

Migration of cells was measured by a wound healing assay in vitro. Briefly, 2 × 10^5 MCF-7 and MDA-MB-231 cells were seeded onto 6-well plates, and incubated in appropriate complete culture medium for 16 h under normal conditions at 37 °C. The monolayer was scratched and incubated in fresh medium without FBS for 24 h. Finally, the wound width was measured. Three different locations were visualized and photographed under inverted microscope.
Invasion assay

Invasion assays were performed using chambers containing 8.0-μm pore membranes (Millipore) with matrigel basement membrane matrix. Breast cancer cells (1 x 10^5 cells) were resuspended in 200 μl of FBS-free medium, and then seeded into the top chamber with Matrigel-coated membrane. Next, 500 μl medium with 10% FBS was added to the bottom chamber as a chemoattractant. After 48 h of incubation, the non-invaded cells were removed from the upper surface of the membrane with a cotton-tipped swab, and the invaded cells were fixed, stained with crystal violet, finally, 5 fields of the stained cells per sample were counted under the inverted microscope.

Western blot

The cells were harvested and lysed by RIPA buffer. The lysates were boiled at 100 °C for 5 min and centrifuged at 10,000 rpm for 1 min. About 50 μg of total protein were loaded onto SDS-PAGE gel. After that, the proteins were transferred to PVDF membrane at 300 mA for 2.5 h. The membrane was blocked with 5% non-fat milk in 1 x TBST for 1 h at room temperature, then incubated with primary antibodies at 4 °C overnight. Then, the membrane was washed with 1 x TBST for 3 times, 5 min each time, and incubated with secondary antibodies at room temperature for 1 h. Finally, the membrane was incubated with ECL and exposed. The following antibodies were used: anti-SP3 (Santa Cruz, USA), anti-DNMT1 (Cell Signaling Technology, USA), anti-β-actin (Proteintech).

RT-qPCR

Cells were harvested and RNA was extracted by Trizol method, then chloroform was added to the mixture. The sample was centrifuged at 12,000 rpm for 10 min and transferred to new RNase-free EP tubes, mixed with an equal volume of isopropanol and centrifuged. Supernatant was then removed and 75% ethanol added to wash the pellet. Finally, ethanol was discarded and the pellet dried and resuspended in 20–30 μl Rnase-free H2O.

For reverse transcription, ~ 1 μg of total RNA was used for reverse transcription according to manufacturer’s instruction (TAKARA PrimeScript Kit).

For real time PCR, we used SYBR as a probe dye to detect the signal, with GAPDH used as internal control. The Ct value was calculated using the ΔΔCt method and normalized to GAPDH levels. The following primers were used:

**P < 0.01)

Luciferase assay

The 3′-UTRs of SP1 and SP3 were fused to the luciferase gene using the XhoI/NotI restriction sites in the psiCHECK2 vector. Mutations in the miR-506 target site in these UTRs were generated using the QuikChange Multi Site-directed Mutagenesis kit (Stratagene, LaJolla, CA). Luciferase assays were performed using the Dual-Luciferase assay (Promega). Renilla expression was normalized to the luciferase gene on the psiCHECK2 vector.
Methylation-specific PCR
DNA methylation status was examined by the methylation-specific PCR with genomic DNA treated with sodium bisulfite using the EZ DNA MethylationDirect kit (Zymo Research). Two primer sets were used to amplify the promoter region of the MEG3 gene containing a number of CpG sites, one for the methylated sequence (forward, 5′-TATGAGTTGTAACGGTACAGGTTC-3′; reverse, 5′-TACGAACCTAAGCAGAAAATCAT-3′) and the other for the unmethylated sequence (forward, 5′-GAA TATGAGTTGTAAGGTAGAGTTT-3′; reverse, 5′-TACAACTTACAAAAACATCAT-3′).
Statistical analysis
Each experiment was performed for three times independently. All values were presented as mean ± SD. Comparisons were performed using student’s t-test for two groups or one-way ANOVA for multiple groups. *P < 0.05 was considered statistically significant.

Results
SP3 depletion suppresses migration and invasion of breast cancer cells
We firstly attempted to examine the effect of SP3 on metastasis of breast cancer. We probed the mRNA level of SP3 in normal and breast tumor tissues and observed that expression of SP3 was upregulated in breast tumors (n = 20) relative to normal breast tissues (n = 20) (Fig. 1a).

Next, we employed RT-qPCR and Western blot to detect mRNA and protein level of SP3 in normal and breast cancer cell lines, respectively. In agreement with results in tissues, expression levels of SP3 were higher in breast cancer cells (MDA-MB-231, MCF-7 and SKBR3) compared to MCF10A (Fig. 1b, c).

Next, we sought to explore the influence of SP3 on migration and invasion of MCF-7 and MDA-MB-231 cells. We examined the knockdown efficiency of 4 designed shRNAs against SP3 and found that shSP3#4 was the validated one at both mRNA and protein levels (Fig. 1d, e). Next, we performed wound healing assay to evaluate migration ability of human breast cancer cells with or without SP3. The results revealed that SP3 knockdown markedly reduced the migration ability of MCF-7 and MDA-MB-231 cells (Fig. 1f, g). Besides, we assessed whether SP3 had a role in the invasion ability of MCF-7 and MDA-MB-231 cells by transwell invasion assay. Expectedly, SP3 depletion suppressed invasion of the two breast cancer cells (the invaded cell number was decreased by ~ 50% in SP3-silenced cells) (Fig. 1h, i).
SP3 knockdown downregulates DNMT1 expression and decreases methylation level of MEG3 promoter. SP3 cooperates with p300 to control DNMT1 expression [24]. To explore the mechanism underlying SP3 knockdown-attenuated metastasis of MCF-7 and MDA-MB-231 cells, we attempted to confirm whether SP3 also impacted the expression of DNMT1 in breast cancer cells. Consistent with previous reports, SP3 knockdown led to decrease in DNMT1 expression in MCF-7 and MDA-MB-231 cells (Fig. 2a, b). Furthermore,
we found that SP3 silencing decreased DNMT1 levels but also upregulated MEG3 (Fig. 2c). As DNMT1 has been known as a DNA methyltransferase, we hypothesized that SP3 knockdown reduced DNMT1 levels, which in turn altered MEG3 methylation level and increased MEG3 expression. To validate this hypothesis, we carried out methylation-specific PCR to examine MEG3 methylation level and observed that, in both MCF-7 and MDA-MB-231 cells, SP3 knockdown substantially decreased the methylation level of MEG3, whereas unmethylated MEG3 levels was increased in SP3-depleted cells (Fig. 2d, e). Taken together, SP3 silencing could result in decreased expression of DNMT1 and then reduce methylation level of MEG3, finally thereby increasing MEG3 levels.

**Reduced expression of miR-506 and MEG3 in breast tumors and cell lines**

To elucidate the clinical role of miR-506 and MEG3 in breast cancer progression, we determined the expression level of these two genes in normal (n = 20) and breast tumor tissues (n = 20), as well as in normal human breast epithelial cells and three breast cancer cell lines. The RT-qPCR results showed that miR-506 and MEG3 levels were significantly lower (about half) in breast tumor tissues than in adjacent normal tissues (Fig. 3a, b). In addition, we found that the levels of miR-506 and MEG3 were remarkably decreased (~ 50%) in breast cancer cell lines (MDA-MB-231, MCF-7 and SKBR3) compared to immortalized human breast epithelial cell (MCF10A) (Fig. 3c, d). These data indicated that dysregulation of miR-506 and MEG3 might be involved in breast cancer.

**miR-506 overexpression downregulates SP1, SP3, DNMT1 and upregulates MEG3 expression**

To clarify the mechanism by which miR-506 played a tumor suppressive role in migration and invasion of breast cancer cell lines, we transfected MDA-MB-231 with miR-506 mimic to overexpress miR-506 (Fig. 4a). The Western blot analysis demonstrated that miR-506 overexpression led to remarkably decreased expression of DNMT1, SP1 and SP3 compared to NC mimic group (Fig. 4b). In parallel, the RT-qPCR assay revealed that expression levels of SP1 and SP3 were declined in miR-506 overexpressed breast cancer cells relative to that in NC mimic cells (Fig. 4c). Since microRNAs function to regulate gene expression by targeting 3’-UTR region of mRNAs, we hypothesized that miR-506 was likely to bind 3’-UTR of SP1 and SP3. The bioinformatic prediction revealed that miR-506 could target 3’-UTR of SP1 and SP3 by complementary sequences, which was confirmed by luciferase assay. The results showed that miR-506 only caused significant decrease (by ~ 60%) in wild-type 3’-UTR-fused luciferase reporter, not mutant 3’-UTR-fused luciferase reporter (Fig. 3d–g).

Finally, we also observed that MEG3 level was upregulated (~ twofold) by miR-506 mimic compared to NC mimic (Fig. 4h). Moreover, we utilized methylation-specific PCR to measure methylation level of MEG3 upon miR-506 overexpression. The data demonstrated that MEG3 methylation level decreased in MCF-7 and MDA-MB-231 cells transfected with miR-506 mimic, whereas unmethylated MEG3 was higher (Fig. 4i, j).

**SP3 compromises miR-506 overexpression-attenuated migration and invasion of breast cancer cells**

Since SP3 is a downstream target of miR-506 and promotes migration and invasion of breast cancer cells, we sought to ask if miR-506 regulates migration and invasion of breast cancer cell lines in an SP3-dependent manner. First, we confirmed that miR-506-regulated SP3, DNMT1 and MEG3 expression levels could be reverted by SP3 overexpression in mRNA level (Fig. 5a). Wound healing assays revealed that miR-506 mimic significantly inhibited the migration of MCF-7 and MDA-MB-231 cells compared to NC mimic group (Fig. 5b, c). More importantly, we found that SP3 had the ability to largely rescue miR-506 overexpression-impaired migration. Similarly, transfection of miR-506 alone in MCF-7 or MDA-MB-231 cells led to decreased invasion ability compared to NC mimic cells; however, cells co-transfected with both miR-506 and SP3 displayed invasion ability comparable to cells transfected with NC mimic (Fig. 5d, e).
Discussion
The major finding of the present study is that miR-506 inhibits migration and invasion of breast cancer cell lines through an undescribed pathway SP1/SP3/DNMT1/MEG3. We revealed a novel epigenetic mechanism of how miR-506 and SP3 play a role in breast cancer progression.

Many studies have shown that miR-506 functions as tumor suppressor in different types of malignant tumors [25, 26]. For instance, Chen et al. [27] reported that miR-506 inhibits colorectal cancer progression by targeting DNMT1 and DNMT3b. In the present study, we further uncover SP1 and SP3 as novel targets by which miR-506 regulates DNMT1 expression. A previous meta-analysis revealed that miR-506 is associated with the survival of breast cancer patients [28]. Recently, miR-506 has been shown to regulate TGFβ1-induced EMT of breast cancer cells through targeting EMT-related gene expression [11]. Although it has been found that miR-506 has the ability to repress IQGAP1 and MAPK signaling pathway to influence breast cancer metastasis, other downstream targets may exist, which prompted us to search by bioinformatic prediction and for the first time, find out SP1 and SP3 as a direct target of miR-506 [29], thereby regulating breast cancer metastasis via DNMT1/MEG3 axis. SP1 and SP3 expression level are often greater in cancer cells than in normal cells [23]. Compared to SP3, SP1 has been extensively studied in breast cancer, thyroid cancer, hepatocellular cancer, pancreatic cancer, colorectal cancer, gastric cancer and lung cancer [30–32]. Hence, we primarily focused on the role of SP3 in present study, bridging miR-506 and DNMT1/MEG3.

It was found that the demethylation of MEG3 promoter and the change of gene region are the main reasons for the abnormal expression of MEG3 in tumors [33]. Consistently, another study has shown that MEG3 expression is closely regulated by DNA methylation with the treatment of DNA methylation inhibitor (5′-Aza-2′-deoxycytidine) [18]. The further work can be focused on assessing whether other members of DNMT (e.g., DNMT3a and DNMT3b) or DNA demethylases (e.g., TET1-3) are implicated in regulating the expression of MEG3 mediated by miR-506. Besides, another interesting question is to search for other miRNAs responsible for upregulating MEG3 through targeting DNMT1.

A number of previous studies have identified MEG3 as a classical tumor suppressor [34, 35]. The mechanism of how MEG3 exerts its effects on tumorigenesis is almost fully understood. To date, two groups have confirmed that MEG3 suppresses tumorigenesis and progression of breast cancer and gliomas by p53 pathway [17, 18]. In addition, by comparing gene expression profiles in embryonic brains between Meg3 KO mice and wild-type mice using microarray techniques, researchers found that Meg3 deletion could lead to elevated VEGFA and VEGFR1 [36]. Based on these findings, we do not draw much attention on the downstream signaling regulated by MEG3 or function of MEG3.

Conclusion
In conclusion, our study highlighted a novel regulation axis responsible for miR-506-attenuated migration and invasion of breast cancer cell lines. Of which, for the first time, we reveal miR-506 has a role in targeting and regulating SP1 and SP3 expression, down-regulating methylation level of MEG3 promoter in a DMNT1 dependent manner. Our findings provide new mechanism for explaining the breast cancer progression as well as potential candidate for treating breast cancer in future.

Abbreviations
DNMT: DNA methyltransferase; SP/KLF: specificity protein/Kruppel-like factor; DMEM: Dulbecco’s Modified Eagle’s Medium; shRNAs: short hairpin RNAs.

Authors’ contributions
XXW designed the study, prepared and edited the manuscript. GCG and XKQ did literature research and clinical studies. DWD, ZZ and XDX did data analysis and statistical analysis. XD and XHP edited and revised the manuscript.

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Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate
The present study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University.

Consent for publication
Informed consent for publication were obtained from all patients.

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