



MiR-519d-5p modulates the sensitivity of breast cancer to chemotherapy by forming a negative feedback loop with RELA

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Background: The chemoresistance of breast cancer (BC) has become the main cause of treatment failure. MicroRNAs (miRNAs) play a critical role in tumorigenesis, development, and chemoresistance, but the underlying mechanism of miR-519d in BC development and chemotherapy sensitivity remains to be elucidated.

Methods: The levels of miR-519d-5p in BC samples and cell lines were measured by quantitative reverse transcription polymerase chain reaction (RT-qPCR). Cell viability was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The in vivo effect of miR-519d-5p on tumor formation and doxorubicin response were investigated in a xenograft study. Bioinformatic analysis, luciferase reporter assay, RT-qPCR, and western blotting were conducted to validate RELA as a target gene of miR-519d-5p. We performed RT-qPCR, western blotting, chromatin immunoprecipitation (ChIP), and DNA pull down to verify miR-519d-5p as a transcriptional target of RELA.

Results: This study found that miR-519d-5p was expressed at lower levels in BC cells and tissues, and overexpression of miR-519d-5p sensitized BC to chemotherapy both in vitro and in vivo. Meanwhile, the expression of RELA was negatively correlated with miR-519d-5p. We then showed that RELA is one of the targets of miR-519d-5p: miR-519d-5p inhibited RELA expression by directly binding to its 3'-untranslated region (3'-UTR). Conversely, it was verified that miR-519d-5p is one of the targets of transcription factor RELA, and RELA repressed miR-519d-5p by binding to the promoter region of miR-519d-5p, which forms a feedback loop.

Conclusions: Overall, the results provide a novel therapeutic strategy for the combinational use of miR-519d-5p and chemotherapeutic agents to overcome chemo-resistance by forming a negative feedback loop with RELA.

Keywords: microRNA-519d-5p; breast cancer (BC); chemosensitivity; RELA; feedback loop

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Introduction

MicroRNAs (miRNAs) have 18-25 nucleotides that are a kind of endogenous, small non-coding RNAs. They regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of target messenger RNAs (mRNAs) (1). We know that miRNAs are involved in almost every biological process in mammals (2). In cancer, miRNA regulates the expression of important cancer-associated genes, thereby acting as a tumor suppressor or oncogene (3).

Breast cancer (BC) is the most widespread malignant pathology affecting women globally, with an incidence rate of 24.2%, and a mortality rate of 15.0% (4). Over the last decade, miRNAs' function in BC has been thoroughly investigated. Previous studies have confirmed that miRNA expression profiles are different between BC and normal breast tissue (5). In BC cells, miRNA can regulate the proliferation, cell cycle and apoptosis (6), invasion and metastasis (7), differentiation (8), angiogenesis (9), immune regulation (10), and stem cell phenotype (11). Clinically, a variety of chemotherapy drugs [doxorubicin (Dox), cisplatin, and so on] are effective against BC, but resistance is inevitable (12). Previous research has verified abnormal miRNA expression may be related to drug resistance in BC, and miRNA is involved in regulating the sensitivity of tumor cells to chemotherapy drugs (13,14). Clinical trials of miRNAs are still in progress, but have focused on practical issues, such as toxicity, off-target effects, and systemic delivery. In BC, further studies are required that focus on the specific regulatory mechanisms of miRNA expression, and their inter-relationships in its occurrence and development.

The miRNA, miR-519d belongs to the miRNA cluster of chromosomes 19 (C19MC), which is the largest human miRNA cluster (15). It often acts as a negative regulator and serves an important role in a variety of tumors (16). Studies have shown that miR-519d inhibits the occurrence and metastasis of BC by targeting matrix metalloproteinase-3 (MMP3) (17), which suppresses proliferation and accelerates apoptosis by targeting sirtuin 7 in human hypertrophic scar fibroblasts (18). It has also been shown that miR-519-d inhibits nasopharyngeal carcinoma cells proliferation by targeting p53 and DNA damage-regulated protein 1 (PDRG1) (19); however, the underlying mechanism of miR-519d in BC development and chemotherapy sensitivity remains to be elucidated. A specific miRNA can target multiple genes, so it is of particular importance to investigate the miR-519d targets associated with BC chemotherapy sensitivity.

The nuclear factor kappa light chain enhancer of activated B cells (NF- κ B) signaling pathway is involved in tumorigenesis, tumor development, and the inflammation-related response, which mediates cell proliferation, metastasis, and anti-apoptosis by regulating the transcription of downstream genes (20). The NF- κ B transcription factor is an important and complex cytokine for the survival and development of BC cells. The protein RELA is an important subunit of NF- κ B, and abnormal activation of its signaling pathway is associated with the abnormal expression of BC-related genes, which can promote proliferation and inhibit apoptosis and tumor metastasis (21). Studies have shown that miRNA is not only involved in the invasion and metastasis of BC, but also participates in regulation of the NF- κ B pathway activity, at the same time, the expression of miRNA is also regulated by NF- κ B (22).

In this study, we investigated the function of miR-519d-5p in modulating the sensitivity of BC to chemotherapy. Overexpression of miR-519d-5p sensitized BC to chemotherapy both *in vitro* and *in vivo*. In addition, a novel miR-519d-5p/RELA negative feedback loop was discovered, which may be involved in the chemosensitivity of BC. We present the following article in accordance with the ARRIVE reporting checklist (available at <https://dx.doi.org/10.21037/atm-21-3241>).

Methods

Cell culture

The BC cell lines (MCF-7, SKBR3, MDA-MB-231, and HCC1937), a normal human normal breast cell line (Hs 578bst), and the 293T cell line were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), where they were characterized by DNA fingerprinting, mycoplasma, and cell vitality detection. The cells were cultured in complete Iscove's Modified Dulbecco Medium (IMDM) and Roswell Park Memorial Institute (RPMI) 1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Each medium was supplemented with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin. The cells were maintained in a humidified incubator at 37 °C with 5% CO₂.

Patients and specimens

We obtained 17 pairs of human breast tumor and para

tumor tissues from BC patients who underwent surgery at the Henan Cancer Hospital (Zhengzhou, China) from June 2020 to September 2020. All of the tissue samples were frozen in liquid nitrogen for further experiments. The research was approved by the Ethics and Scientific Committees of Henan Cancer Hospital (2020240). Informed consent and approval were provided by all participants. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013).

Transfection and transduction

The oligonucleotides of miR-519d-5p mimic and inhibitor, mimic negative control (nc) and inhibitor nc, the plasmid of vector (RELA overexpression negative control), RELA OE (RELA overexpression), short hairpin (sh)NC, shRELA were purchased from RiboBio (Guangzhou, China). The recombinant lentiviruses for the miR-519d-5p overexpression and miR-519d-5p vector were purchased from GeneChem (Shanghai, China). Cells were transfected using LipoFiter 3.0 reagent purchased from Hanbio Biotechnology (Shanghai, China). To generate stable cell lines, MCF-7 and SKBR3 cells were infected with lentiviruses, and the cells were screened with 1 µg/mL puromycin for 2 weeks.

RT-qPCR

Total RNA samples, including miRNAs, were extracted using BIOzol Reagent (BioFlux, Singapore; #BSC52M1). A reverse transcription kit (Takara Bio, Shiga, Japan; #RR047A) was used to reverse transcribe mRNA into complementary DNA (cDNA), and the Mir-XTM miRNA First-Strand Synthesis Kit (Takara Bio, #636313) was used to reverse transcribe miRNA into cDNA. Quantitative polymerase chain reaction (qPCR) was performed in triplicate on a LightCycler 96 Real-Time PCR System (Roche, Basel, Switzerland; G10120-100G) with SYBR Premix Ex Taq (Takara Bio, #RR420A). Relative expression was normalized to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or U6 by the $2^{-\Delta\Delta C_t}$ method. The miRNA-specific primers synthesized by Takara Bio are shown in Table S1.

Proliferation assay

The viability of the treated cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium

bromide (MTT) assay. Briefly, miR-519d-5p mimic or mimic NC was transfected into the tumor cells in 96-well plates. After 24 h, cells were treated with Dox (Selleck, Houston, TX, USA) or cisplatin (Jiangsu Hansoh Pharmaceutical, Jiangsu, China) for 72 h. Then, 20 µL MTT (5 mg/mL, Sigma Aldrich, St. Louis, MO, USA; Merck, Kenilworth, NJ, USA) was added to the medium and subsequently incubated at 37 °C for 4 h. Finally, the absorbance was obtained from an enzyme labeling instrument at 570 nm (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

In vivo study

Animal experiments were performed at the Experimental Animal Center of Fujian Medical University (Fuzhou, China). The experiments were conducted according to institutional guidelines and with prior approval from the Institutional Animal Care and Use Committee (2019-0003). Mice were housed in standard autoclaved polypropylene cages with access to food and water ad libitum under a 12 h dark-light cycle (lighting time 08:00–20:00), at a constant temperature (23 ± 2 °C) and relative humidity ($50\% \pm 5\%$). The stable miR-519d-5p-overexpressed MCF-7 and control cells constructed by lentivirus (8×10^6 cells) were injected into the right flanks of 5-week-old female nude mice. When the tumor volume of each tumor-bearing nude mouse model reached ~ 100 mm³, each mouse was randomly divided into 2 groups, 6 per group that were treated with 0.9% NaCl or 2.5 mg·kg⁻¹ Dox (Hisun-Pfizer Pharmaceuticals Co., Zhejiang, China) through the tail vein once every 2 days, 6 times in total. The tumor volumes were monitored, and the growth curves of the tumors were plotted accordingly. At 2 days after the last injection, the nude mice were euthanized, and tumors were weighed. Tumor volume was measured and calculated by the following formula: [(width)² × (length)]/2.

Luciferase reporter assays

The 293T cells were plated in 96-well plates and transiently transfected with the plasmids or miRNAs, and then the luciferase was measured by a dual luciferase assay kit (Promega, Madison, WI, USA; #E1910). The activity was normalized to the firefly/Renilla ratio.

Western blotting

Protein was isolated using lysis buffer. Samples were

resolved on a 12% sodium dodecyl sulfate (SDS)-denaturing polyacrylamide gel and then transferred to a polyvinylidene fluoride (PVDF) membrane. After blocking for 1 h at 37 °C, the membrane was incubated with primary antibodies against RELA (1:1,000) (Proteintech, Rosemont, IL, USA; #no 10745-1-AP) and GAPDH (1:1,000) (Proteintech, #60004-1-Ig) at 4 °C overnight. Afterwards, the membrane was incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) for 1 h at room temperature. Then, the bands were visualized by a chemiluminescence detection system.

Chromatin immunoprecipitation assay

A chromatin immunoprecipitation (ChIP) assay kit (Millipore, Burlington, MA, USA; #17-10086) was used for the experiments. The ChIP assay was performed to collect the DNA, which was used as templates for PCR according to the manufacturer's protocol. Moreover, the miR-519d-5p promoter containing RELA-binding sites was amplified, and the PCR products were separated on 2% agarose gels and visualized with SYBR green. All primers are shown in [Table S2](#).

DNA pull-down assay

The DNA pull-down analysis was set up as described previously (23). In short, streptavidin-conjugated agarose beads, biotin-labeled DNA probes, and the whole-cell extracts of MCF-7 or SKBR3 cells were incubated and rocked for 2 h at room temperature. Then, the mixture was separated, and the proteins were dissolved and determined by immunoblotting with an anti-RELA antibody, in which nonspecific proteins were washed out, and only proteins having high affinity to biotin labeled DNA were detected on SDS- polyacrylamide gel electrophoresis (PAGE). The DNA probes are shown in [Table S3](#).

Statistical analysis

All data were expressed as the mean \pm standard deviation (SD). Student's *t*-test was used to determine significance between 2 samples. One-way analysis of variance (ANOVA) was conducted to assess the significance among multiple groups. A *P* value of <0.05 referred to significant difference, and ns meant no significant difference. **P* <0.05 ; ***P* <0.01 ; ****P* <0.001 .

Results

miR-519d-5p regulates chemosensitivity in BC in vitro

To explore the important role of miR-519d-5p in the occurrence and development of BC, we detected the differential expression in BC tissues and para tumor tissues in 17 participants. As presented in [Figure 1A](#), the levels of miR-519d-5p were significantly lower in BC tissues compared with para tumor tissues. This result was verified in cell lines, compared with normal breast cells Hs 578 Bst, BC cell lines MCF-7, SKBR3, MDA-MB231, and HCC1937 had significantly lower expression levels of miR-519d-5p ([Figure 1B](#)). Then, we investigated the effect of miR-519d-5p on the sensitivity of BC cells to chemotherapy drugs, and the MTT assays demonstrated that the miR-519d-5p mimic increased the sensitivity of breast cells to Dox compared with that of the control group ([Figure 1C,D](#)). In addition, the miR-519d-5p mimic could also increase the sensitivity of cells to cisplatin ([Figure 1E,F](#)).

miR-519d-5p regulates chemosensitivity in BC in vivo

In order to observe the sensitization effect of miR-519d-5p on Dox in vivo, we constructed a xenograft tumor nude mouse model. The results revealed that BC tumors form quickly, with a high tumor formation rate. Dox could inhibit the growth of tumors with an inhibition rate of 38.36%. Compared with the vector group, the growth rate of the tumor in the miR-519d-5p group was significantly slowed, with an inhibition rate of 35.81%, and the tumor in the miR-519d-5p group was more sensitive to Dox, with an inhibition rate of 67.01% ([Figure 2A,B,C](#)). These observations confirmed the key role of miR-519d-5p in the control of BC cell proliferation, and sensitivity to chemotherapeutic agents.

RELA is a direct target of miR-519d-5p in BC cells

In order to investigate the molecular mechanism underlying miR-519d-5p sensitizing chemotherapy, we used multiple database predictions and discovered that RELA contained miR-519d-5p-binding sequences in its 3'-UTR, and that RELA may be one of the target genes of miR-519d-5p ([Figure 3A](#)). A series of experiments were then performed to confirm this prediction. The luciferase reporter assay showed that the overexpression of miR-519d-5p significantly suppressed the activity of the RELA reporter,

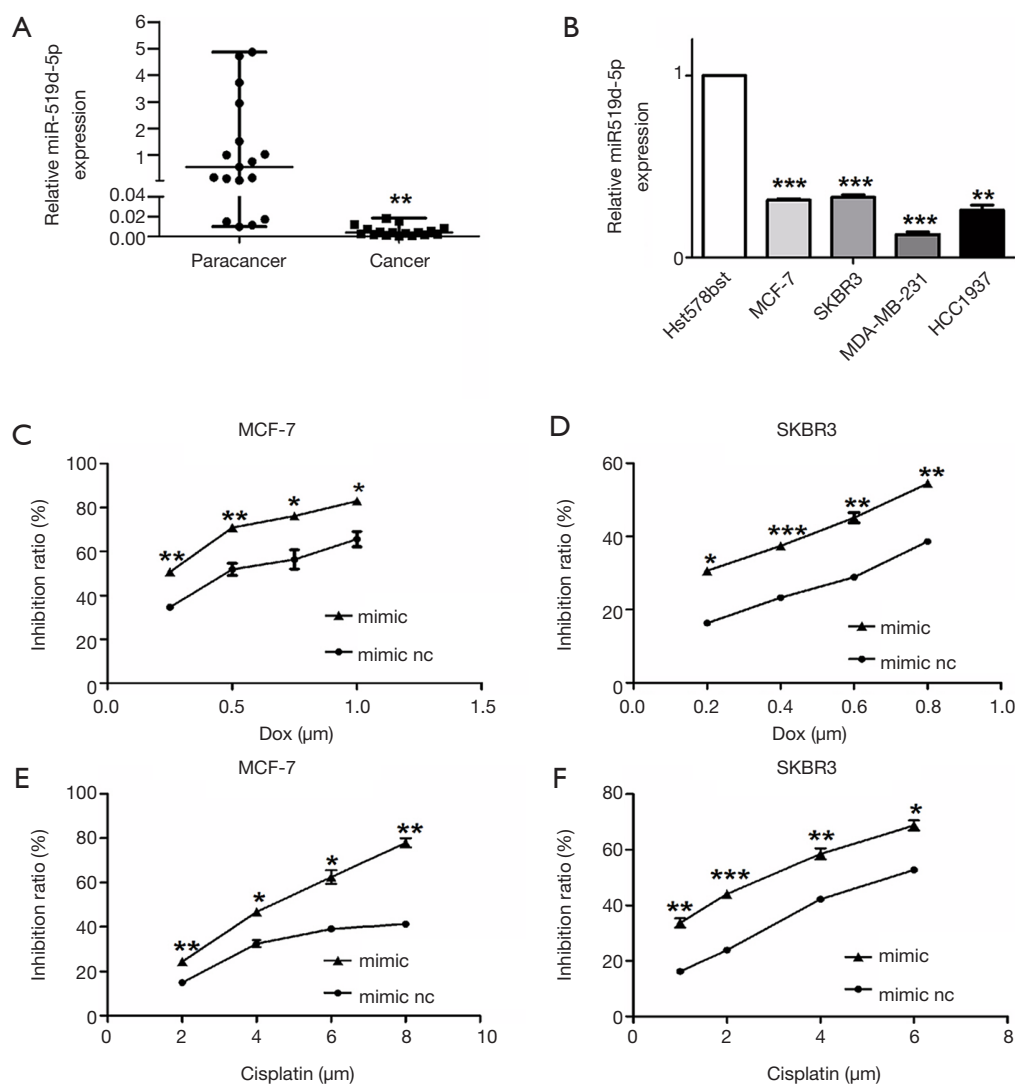


Figure 1 miR-519d-5p regulates chemosensitivity in breast cancer *in vitro*. (A) miR-519d-5p expression in 17 paired BC and para cancer tissue samples. (B) The expression of miR-519d-5p in the immortalized breast cell line Hs 578bst and BC cell lines. (C,D,E,F) MCF-7 and SKBR3 cells transfected with negative control (nc) or miR-519d-5p mimic were treated with different concentrations of Dox and cisplatin. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. BC, breast cancer; Dox, doxorubicin.

but not the mutated reporter, indicating that miR-519d-5p was complementary base-pairing with the 3'-UTR of RELA (Figure 3B). In addition, we found that the expression of RELA was inversely correlated with that of miR-519d-5p in BC cells, with RELA mRNA and protein expression levels upregulated in BC cells compared with normal breast cells. (Figure 3C,D).

To further confirm the targeted regulation of RELA by miR-519d-5p, we transfected an miR-519d-5p mimic

into MCF-7 and SKBR3 cells. The results revealed that the miR-519d-5p mimic significantly suppressed the RELA mRNA level (Figure 4A,B). Conversely, the miR-519d-5p inhibitor enhanced the RELA mRNA level (Figure 4C,D). Furthermore, the RELA protein level displayed a similar trend (Figure 4E,F). These results showed that miR-519d-5p may directly bind with the RELA 3'-UTR and inhibit RELA mRNA and protein expression levels in BC cells.

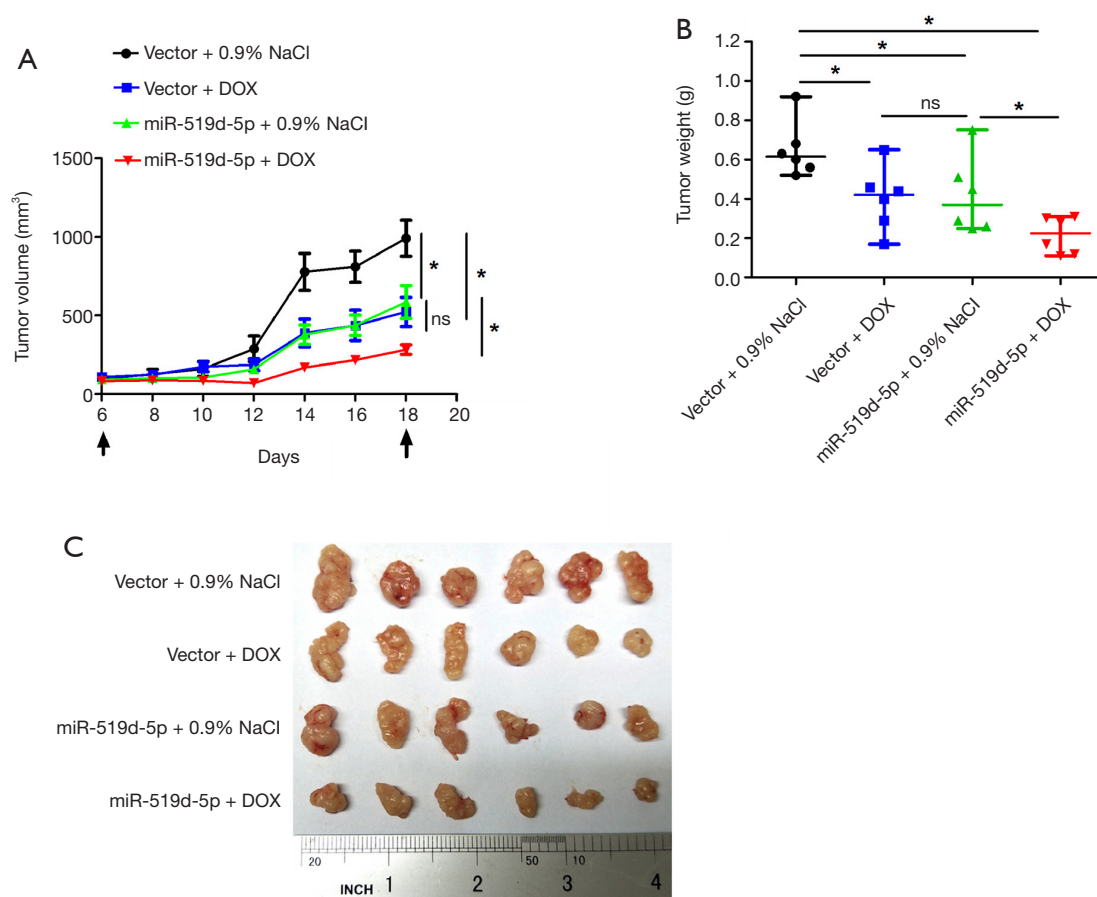


Figure 2 miR-519d-5p regulates chemosensitivity in breast cancer *in vivo*. Quantification of (A) growth curves and (B) tumor weight of xenograft tumors in mice treated with 0.9% NaCl, Dox (2.5 mg/kg Dox, i.v., every other day) 6 times after the tumors were established in the mice. (C) Representative image of tumors in nude mice after the above treatment (n=6). *P<0.05. Dox, doxorubicin.

miR-519d-5p is a transcriptional target of RELA

Since RELA is a classic transcription factor, we speculated whether it could also regulate the transcription of miR-519d-5p. We both knocked down and overexpressed RELA in MCF-7 and SKBR3 cells in order to identify the effect of RELA on the transcription level of miR-519d-5p (Figure 5A,B). The results revealed that knockdown of RELA significantly increased the expression of miR-519d-5p (Figure 5C,D), whereas overexpression of RELA significantly suppressed the expression of miR-519d-5p in MCF-7 and SKBR3 cells (Figure 5E,F).

The bioinformatics prediction tool Jasp (http://jaspar.genereg.net/) showed that there are 4 putative RELA-binding sites on the miR-519d-5p promoter region (Table S4). Then, a ChIP assay was performed in MCF-7 and SKBR3 cells to further investigate whether RELA

regulates miR-519d-5p gene expression by directly binding to the miR-519d-5p promoter. Analysis of the PCR products showed that RELA was able to bind to the promoter region of miR-519d-5p (Figure 6A,B). Furthermore, the present study assessed the potential physical recruitment of RELA to the miR-519d-5p promoter using DNA pull-down assays. As presented in Figure 6C,D, the endogenous RELA protein in MCF-7 and SKBR3 cells was efficiently recruited to the 4 binding sites. Overall, these results revealed that RELA may directly bind to the miR-519d-5p promoter and regulate miR-519d-5p transcription, and miR-519d and RELA form a negative feedback loop regulation.

Discussion

The chemoresistance of BC to clinical first-line

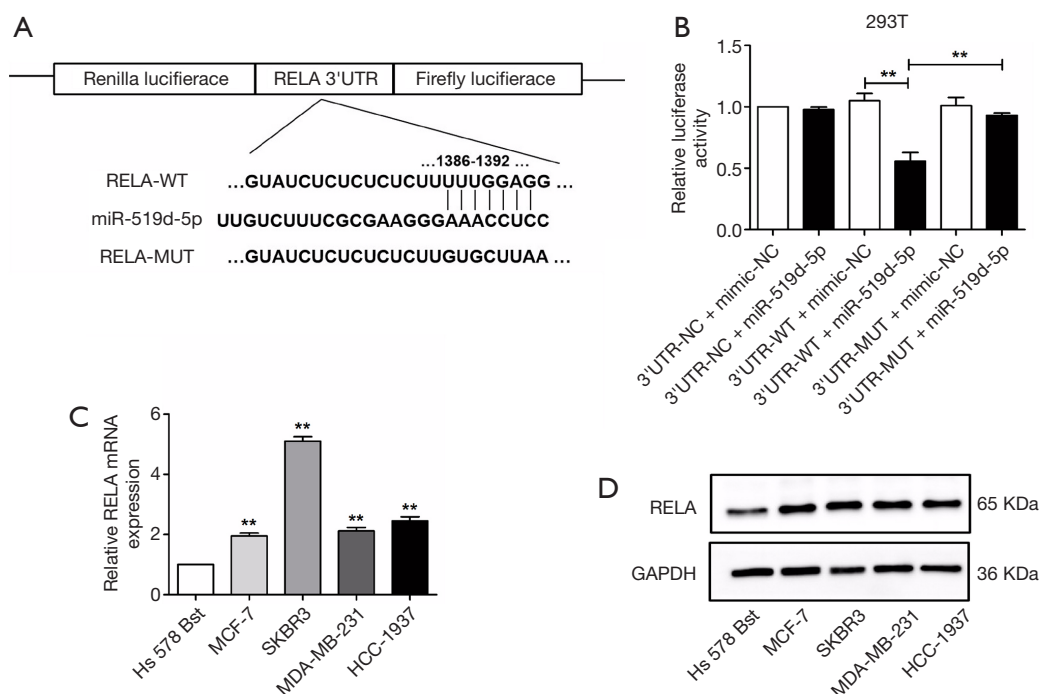


Figure 3 RELA is a direct target of miR-519d-5p in breast cancer cells. (A) Sequences of RELA 3'UTR and miR-519d-5p according to the prediction by bioinformatics databases. (B) Relative luciferase activity in 293T cells co-transfected with reporter vector control (RELA-3'UTR-NC) or reporter vector containing the wild-type binding sequence (RELA-3'UTR-WT) or mutant-type binding sequence (RELA-3'UTR-MUT) along with miR-519d-5p mimic or negative control. (C and D) Relative expression levels of RELA (C) mRNA and (D) protein in the immortalized breast cell line Hs 578bst and BC cell lines. ** $P < 0.01$. BC, breast cancer; 3'UTR-WT, 3'untranslated region-wild type; 3'UTR-MUT, 3'untranslated region-mutant type; mRNA, messenger RNA.

chemotherapeutic agents (e.g., Dox and cisplatin) has become the main cause of chemotherapy failure. Therefore, it is of clinical value to clarify the mechanisms underlying chemoresistance in BC. Accumulating evidence shows that miRNAs have a critical impact in tumorigenesis, tumor progression, as well as chemoresistance (24), and miRNA-based cancer therapies have entered clinical trials (25).

Previous reports have demonstrated that miR-519d can regulate BC tumorigenesis and metastasis (17) and mediate drug sensitivity in ovarian cancer cells and colorectal cancer cells (26). However, whether miR-519d affects the chemotherapeutic drugs sensitivity and the underlying mechanisms still need to be further clarified. Our results indicated that miR-519d-5p was expressed at lower levels in BC cells and tissues, indicating that miR-519d-5p may be associated with BC progression. Moreover, miR-519d-5p overexpression sensitized BC cells to Dox *in vitro* and *in vivo*. Long-term use of Dox not only risks the development of drug resistance in cancer cells, but can also cause serious

cardiotoxicity, bone marrow hematopoietic dysfunction, and other adverse reactions due to the accumulation of doses (27). The miR-519d-5p may be helpful for decreasing the dose and side effects of chemotherapeutic drugs and improving therapeutic effects. From a practical perspective, patients could potentially benefit from miR-519d-5p combination therapy, which may sensitize tumors to these treatments.

The functional characterization of a miRNA relies heavily on the identification of its targets and its effects on their expression. As our results revealed, the expression of miR-519d-5p showed a negative correlation with RELA, suggesting that miR-519d-5p may have a vital impact in the regulation of RELA. In the present study, bioinformatics tools, luciferase reporter assays, RT-qPCR, and western blotting were combined to reveal the regulation of RELA by miR-519d-5p. The results revealed that RELA is a vital target of miR-519d-5p in BC cells.

Despite being regulated by miRNAs, RELA also

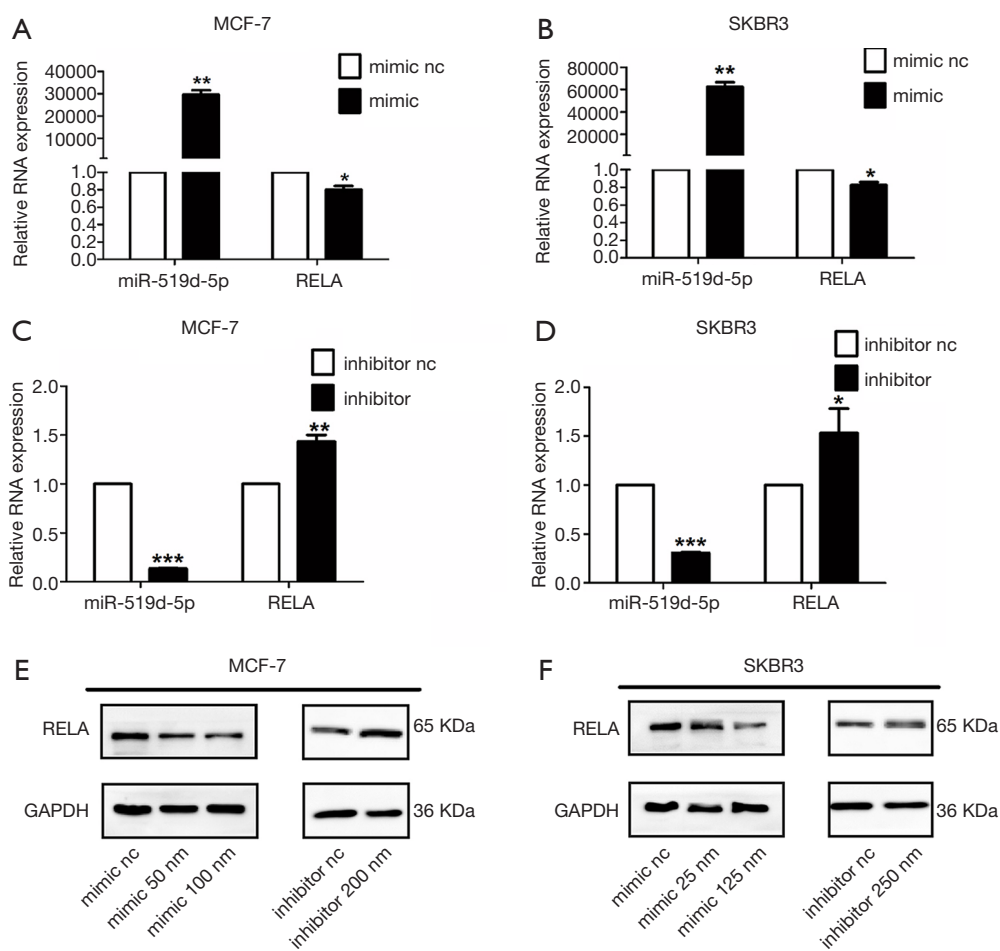


Figure 4 miR-519d-5p inhibits RELA mRNA and protein expression. (A and B) miR-519d-5p and RELA mRNA level after miR-519d-5p mimic transfection in (A) MCF-7 and (B) SKBR3 cells. (C and D) miR-519d-5p and RELA mRNA level after miR-519d-5p inhibitor transfection in (C) MCF-7 and (D) SKBR3 cells. (E and F) RELA protein level in (E) MCF-7 and (F) SKBR3 cells transfected with miR-519d-5p mimic or inhibitor. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

regulates miRNA expression by acting as a transcriptional regulator (28). The results of this study confirmed that RELA bound to the miR-519d-5p promoter and regulated the transcriptional activity of miR-519d-5p. These findings suggest that miR-519d-5p and RELA reciprocally control each other in a negative feedback loop. There is plenty of evidence that RELA is associated with chemoresistance (29,30); thus, upregulation of miR-519d-5p could be a potential strategy to target RELA.

In studying the miR-519d-5p expression in cells and tissues, predicting the molecular mechanism of potential key genes sensitizing DNA damage chemotherapeutics in BC can further supplement miRNA and tumor complex

regulatory network signaling pathways, and provide ideas and an effective basis for reversing tumor progression. Our results may provide a novel therapeutic strategy for the combinational use of miR-519d-5p and chemotherapeutic agents to overcome chemoresistance by forming a negative feedback loop with RELA.

Conclusions

In conclusion, we have established a novel therapeutic strategy for the combinational use of miR-519d-5p and chemotherapeutic agents to overcome chemotherapy resistance by forming a negative feedback loop with RELA.

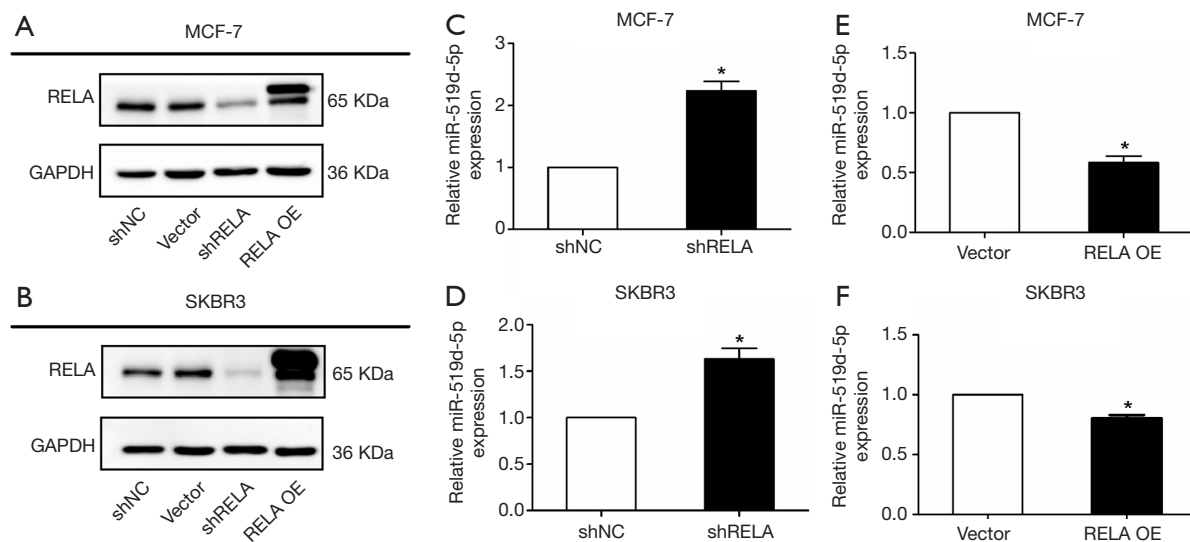


Figure 5 miR-519d-5p is a transcriptional target of RELA. (A and B) The overexpression and knockdown efficiency of RELA in (A) MCF-7 and (B) SKBR3 cells. (C and D) miR-519d-5p expression in RELA knockdown (C) MCF-7 and (D) SKBR3 cells. (E and F) miR-519d-5p expression in RELA overexpression (E) MCF-7 and (F) SKBR3 cells. * $P < 0.05$.

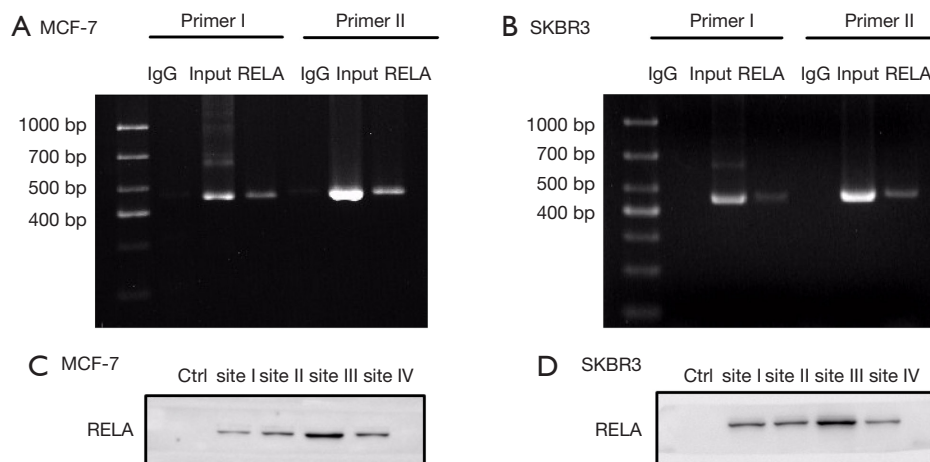


Figure 6 RELA regulates miR-519d-5p transcription by binding to its promoter. (A and B) The interactions of RELA with the miR-519d-5p promoter covering four RELA-binding sites in MCF-7 and SKBR3 cells were detected by ChIP. (C and D) Biotin-labeled miR-519d-5p promoter fragments containing site I, site II, site III, or site IV and a negative control oligonucleotide containing no binding site were used in the DNA pull-down. ChIP, chromatin immunoprecipitation assay.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at <https://dx.doi.org/10.21037/atm-21-3241>

Data Sharing Statement: Available at <https://dx.doi.org/10.21037/atm-21-3241>

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://dx.doi.org/10.21037/atm-21-3241>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All procedures performed in this study involving human participants were in accordance with the Declaration of Helsinki (as revised in 2013). The use of patient samples for research was approved by the Ethics and Scientific Committees of Henan Cancer Hospital (approval no. 2020240). Informed consent and approval were provided by all participants. Animal experiments were performed at the Experimental Animal Center of Fujian Medical University (Fuzhou, China). The experiments were conducted according to institutional guidelines and with prior approval from the Institutional Animal Care and Use Committee (approval No. 2019-0003).

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