miR-187-3p increases gemcitabine sensitivity in breast cancer cells by targeting FGF9 expression

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Abstract. Breast cancer is the most common type of malignancy in women, which remains a significant health concern worldwide. Gemcitabine is a frequently applied anticancer pharmacological agent. However, the efficacy of gemcitabine is limited by chemoresistance. In the present study, a combination of reverse transcription quantitative-PCR, cell viability, flow cytometry, luciferase reporter assay and western blot analysis were performed to elucidate the potential effects of miR-187-3p on gemcitabine sensitivity in the breast cancer cell line, MDA-MB-231. The results revealed that miR-187-3p was significantly decreased in the breast cancer tumor tissues. Moreover, the overexpression of miR-187-3p significantly inhibited cell viability and promoted apoptosis in MDA-MB-231 cells. In addition, miR-187-3p overexpression enhanced the anti-proliferative and pro-apoptotic effects of gemcitabine, indicating that miR-187-3p regulated gemcitabine sensitivity in breast cancer cells. Mechanistically, miR-187-3p negatively regulated the expression of fibroblast growth factor 9 (FGF9) by binding to its 3'-untranslated region. Overexpression of FGF9 reversed the aforementioned effects of miR-187-3p overexpression on cell viability and apoptosis in the presence of gemcitabine. In conclusion, the present study indicated that miR-187-3p increased gemcitabine sensitivity in breast cancer cells by targeting FGF9 expression.

Introduction

Breast cancer is one of the most diverse and complex types of human malignancy, which is a leading cause of mortality among women worldwide (1). In the USA, breast cancer diagnoses are particularly common, accounting for ~30% of all newly diagnosed malignancies; it was reported that breast cancer led to various malignancies, which seriously affected patients' quality of life (1-3).

Gemcitabine (2',2'-difluorodeoxycytidine, dFdC) is a pyrimidine nucleoside analog that inhibits DNA replication and transcription, leading to cell apoptosis (4). It is frequently applied as a single agent as salvage chemotherapy following several lines of treatment (5). Considering prior treatment, dose and schedule, response rates to gemcitabine were typically 14-42% (5). The combined use of gemcitabine with capecitabine, vinorelbine, platinum and other chemotherapeutic agents can enhance the anticancer effects of the overall chemotherapy (6). Since Gemcitabine has been recommended by Chinese breast cancer diagnosis and treatment guide (2017) as an effective method in treating advanced breast cancer (7). Enhancing the therapeutic effects gemcitabine would be of great significance for the treatment of this disease.

MicroRNAs (miRNAs or miRs) are a class of evolutionarily conserved non-coding RNAs that typically consist of ~22 nucleic acids (8). They normally serve as post-transcriptional regulators of gene expression by binding to the 3'untranslated regions (3'UTRs) of target mRNAs to either inhibit translation or promote degradation (8). miRNAs have been reported to participate in a number of physiological processes, including proliferation, apoptosis, cell movement and stem cell renewal (8). Accumulating evidence has revealed that a wide variety of miRNAs are involved in oncogenesis (9). Of note, miR-187-3p has been revealed to be downregulated in colorectal cancer (10), renal cell carcinoma (11), prostate cancer (12), lung cancer (13) and breast cancer (14). In addition, miR-187-3p expression has also been previously associated with patient prognosis in breast cancer (14). However, the precise function of miR-187-3p in the pathophysiology of cancer remain controversial, as a previous study has demonstrated that miR-187 overexpression promotes lymphoma progression and enhances resistance to chemotherapy in peripheral T-cell lymphoma (15).

Fibroblast growth factor 9 (FGF9) is a member of the FGF family, which includes 23 family members each serving key functions including cellular differentiation, proliferation and tumorigenesis (16). FGFs bind to and activate FGF receptors, leading to the further activation of developmental signaling pathways that are responsible for a variety of biological

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functions (17). The overexpression of FGF9 has been previously identified as a novel unfavorable prognostic indicator in lung cancer (18). Intracellular signaling pathways activated by FGF serve important roles in a wide range of malignancies, including breast cancer (17,19). Fillmore et al (20) revealed that estrogen could activate FGF9/FGFR3/T-box transcription factor 3 signaling to increase the numbers of breast cancer stem-like cells, whilst Yin et al (21) have previously reported that the miRNA-FGF9 pathway is important for pleuropulmonary blastoma development.

In the present study, it was revealed that the overexpression of miR-187-3p inhibited MDA-MB-231 cell proliferation, promoted apoptosis and reduced resistance to gemcitabine. Mechanistically, miR-187-3p overexpression resulted in the downregulation of FGF9 expression to regulate gemcitabine sensitivity in breast cancer cells, implicating miR-187-3p as a promising therapeutic target in the treatment of breast cancer.

Materials and methods

Clinical patient tissue samples. A total of 30 breast cancer tumor tissue samples and matched adjacent non-tumor tissue samples, 5 cm away from the tumors, were collected at Chifeng Municipal Hospital (Chifeng, China) from June 2015 to July 2017. All samples were collected from women aged between 27 and 65 years with an average age of 48±11 years. Patients who have received any chemo- or radio- therapies prior to use were excluded from the study. Written informed consent was provided by all participants prior to enrollment. The present study was approved by the Ethics Committee of Chifeng Municipal Hospital (approval no. 20150602CFMH; Chifeng, China). All tissue samples were immediately frozen in liquid nitrogen following surgery and stored in a -80°C refrigerator prior to use.

Cell culture and reagents. MDA-MB-231 human breast cancer cell line was purchased from the American Type Culture Collection and was subsequently cultured in DMEM (Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (HyClone; GE Healthcare Life Sciences) and 1% penicillin-streptomycin solution (Life Technologies; Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor cocktail (Merck KGaA).

Transient transfection. miR-187-3p mimic (50 nM, 5'-GCC CGACGUUGUUCUGUGCU-3') and miR-NC mimic (50 nM, 5'-UCGCUGUACAGGGCUAGC-3') were purchased from Shanghai GenePharma Co., Ltd. pcDNA3.1 (2 µg) and pcDNA-FFG9 (2 µg) were purchased from Addgene, Inc. All transfections were performed into MDA-MB-231 using Lipofectamine® 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.). After incubation for 48 h, cells were collected for the subsequent studies.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from cultured MDA-MB-231 cells and tissues using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA synthesis was performed at 37°C for 15 min and 85°C for 5 sec using a PrimeScript™ RT reagent kit (Takara Bio, Inc.) according to the manufacturer's protocols. RT-qPCR was performed in triplicate using SYBR® Premix Ex Taq™ (Takara Bio, Inc.) in a Bio-Rad CFX96 Real-Time PCR System (Bio-rad Laboratories Inc.). The thermocycling conditions were as follows: 95°C for 30 sec, followed by 35 cycles of 95°C for 5 sec and 60°C for 30 sec. Relative levels of miR-187-3p were normalized to that of U6 small nuclear RNA, whereas those of FGF9 were normalized to GAPDH. The 2-ΔΔCq method was used to quantify relative gene expression (22). The primer sequences used were as follows: Stem loop primer, 5'-CTCAACTGTGTGCTGGAGTCCGAATTCAGTGGACCGGTCT-3'; miR-187-3p forward, 5'-GCCGACTGTGCTGTCTTGGT-3' and reverse, 5'-CTCAACTGTGTGTCGTTGGA-3'; U6 forward, 5'-CTCAAC TGTTGCTGTTGGA-3' and reverse, 5'-CTCAACTGTGTGTCGTTGGA-3'; FGF9 forward, 5'-ATGGCTCCCTTAGTGAAATT-3' and reverse, 5'-CCAGGTGTACCTTAACAAAACT-3'; GAPDH forward, 5'-CAATACACCCCTCTATGACACC-3' and reverse, 5'-GACAGTGTCCGAGCTTC-3'.

Cell viability. Cell viability was assessed by performed a cell counting kit-8 assay (CCK-8; Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol. Cells (~5x10⁴/well) were seeded into 96-well plates. Following treatment with ascending concentrations of Gemcitabine (0.25, 0.5, 1, 2 and 4 nM) for 24 h at 37°C and co-transfection with miR-187-3p or miR-NC mimic and pcDNA3.1-FGF9 or pcDNA3.1 plasmid for 24 h, 10 µl CCK-8 solution was added into each well and incubated at 37°C for 2 h. Absorbance at 450 nm was subsequently measured in each well using a spectrophotometer to determine cell viability.

Apoptosis assay. An Annexin-V/Dead Cell Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to perform cell apoptosis assay according to manufacturer's protocol. Cells were harvested and washed in cold PBS, after which they were then diluted to ~1x10⁶ cells/ml using 1X Annexin-binding buffer in 100 µl per assay. Cells were subsequently treated with 5 µl Alexa Fluor® 488 annexin V and 1 µl 100 µg/ml PI per assay suspension. A total of 400 µl annexin-binding buffer was added and samples were incubated at room temperature for 15 min. Stained cells were analyzed using BD FACS Calibur™ flow cytometer (BD Biosciences) coupled with FlowJo software (version 10.2; FlowJo LLC).

Western blot analysis. Antibodies against FGF9 (cat. no. PA5-23719; 1:1,000) and β-catenin (cat. no. 71-2700; 1:1,000) were obtained from Thermo Fisher Scientific, Inc. Anti-c-Myc (cat. no. 5605; 1:1,000) and Cyclin D1 (cat. no. 2978; 1:1,000) antibodies were purchased from Cell Signaling Technology, Inc. GAPDH mouse monoclonal antibodies (cat. no. ab8245; 1:10,000) was obtained from Abcam. Anti-mouse (cat. no. CW0221S; 1:10,000) and anti-rabbit (cat. no. CW0234S; 1:10,000) secondary antibodies were purchased from Beijing ComWin Biotech Co., Ltd.

Following collection, cells were washed twice with cold PBS and lysed in cold RIPA buffer (Beyoyme Institute of Biotechnology) supplemented with protease inhibitor cocktail (Sigma-Aldrich, Merck KGaA). Samples were then incubated on ice for 30 mins. Lysates were subsequently centrifuged at
miR-187-3p expression was significantly reduced in breast cancer tumor tissues compared with non-tumor tissues (Fig. 1), indicating that miR-187-3p may serve a role in the development of breast cancer.

Overexpression of miR-187-3p inhibits cell viability and promotes apoptosis in MDA-MB-231 cells. The physiological function and underlying mechanism of miR-187-3p in breast cancer remain poorly understood. Therefore, MDA-MB-231 cells were successfully transfected with the miR-187-3p mimic to overexpress miR-187-3p compared with the miR-NC mimic (Fig. 2A). Compared with the miR-NC mimic, miR-187-3p overexpression was subsequently revealed to significantly inhibit MDA-MB-231 cell viability (Fig. 2B). Additionally, compared with the miR-NC mimic, overexpression of miR-187-3p significantly promoted MDA-MB-231 cell apoptosis (Fig. 2C and D). The results indicated that miR-187-3p overexpression reduced MDA-MB-231 cell viability and promoted apoptosis.

Overexpression of miR-187-3p enhances gemcitabine sensitivity in MDA-MB-231 cells. Liang et al (23) discovered that miR-187-3p was one of the most significantly downregulated miRNAs in multidrug-resistant MCF7 cells compared to parental MCF7 cells. Although gemcitabine is frequently applied as a therapeutic agent for advanced breast cancer, it remains associated with limited efficacy (7,24). In this present study, it was determined that, compared to the control, gemcitabine (2 nM) treatment did not significantly alter miR-187-3p expression in MDA-MB-231 cells (Fig. 3A). However, compared with the miR-NC mimic, overexpression of miR-187-3p significantly enhanced the gemcitabine-mediated (1, 2, 3 and 4 nM) reduction of cell viability in MDA-MB-231 cells (Fig. 3B) whilst significantly potentiating gemcitabine-induced MDA-MB-231 cell apoptosis (Fig. 3C and D). These results indicated that miR-187-3p overexpression removed gemcitabine resistance in breast cancer cells.

miR-187-3p regulates FGF9 expression by binding to its 3'-UTR. Following the indication that miR-187-3p enhanced the efficacy of gemcitabine, the underlying mechanism was...
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elicited. According to the TargetScan database, FGF9 was predicted to be a potential target gene of miR-187-3p, as a putative miR-187-3p binding site was observed on the 3'-UTR of FGF9 (Fig. 4A). WT FGF9 3'-UTR and Mut FGF9 3'-UTR luciferase reporter gene plasmids were constructed to verify this potential association. Dual-luciferase reporter gene assay data demonstrated that, compared with the miR-NC mimic, transfection with the miR-187-3p mimic significantly reduced relative luciferase activity in MDA-MB-231 cells co-transfected with the WT FGF9 3'-UTR plasmid (Fig. 4B).

miR-187-3p overexpression negatively regulates FGF9 expression and the associated downstream Wnt pathway. To investigate the regulatory effects of miR-187-3p on FGF9, RT-qPCR and western blot analysis were performed to measure FGF9 mRNA and protein expression in MDA-MB-231 cells following miR-187-3p overexpression. It was revealed that, compared with the miR-NC mimic, miR-187-3p overexpression significantly reduced FGF9 mRNA and protein expression (Fig. 5A-C). A previous study demonstrated that FGF9 can regulate Wnt/β-catenin signaling and that deficiencies in this pathway have resulted in reduced mesenchymal proliferation (25). In addition, FGF9 has been reported to be a target for the Wnt/β-catenin pathway in ovarian endometrioid adenocarcinomas (26). Following miR-187-3p overexpression, β-catenin, c-Myc and Cyclin D1 protein expression were revealed to be significantly reduced compared with the miR-NC mimic (Fig. 5D and E). In addition, compared with the control, gemcitabine treatment did not significantly alter FGF9 expression in MDA-MB-231 cells (Fig. 5F and G). The results indicated that miR-187-3p regulated breast cancer progression by negatively regulating FGF9 expression, which in turn inactivated the Wnt signaling pathway.

Overexpression of FGF9 reverses gemcitabine- and miR-187-3p-mediated cell viability inhibition and promotion of MDA-MB-231 cell apoptosis. To explore the functional association of miR-187-3p and FGF9 on gemcitabine sensitivity in breast cancer cells, a pcDNA3.1-FGF9 recombinant
A plasmid was constructed, which was co-transfected with the miR-187-3p mimic into MDA-MB-231 cells. Compared with the pcDNA3.1, transfection with the pcDNA3.1‑FGF9 plasmid increased FGF9 expression in MDA‑MB‑231 cells (Fig. 6A and B). Compared with the miR‑NC mimic and pcDNA3.1, overexpression of miR‑187‑3p significantly reduced the FGF9 protein expression, which was significantly reversed by co‑transfection with the FGF9 plasmid (Fig. 6C and D). Cell viability and apoptosis assays were performed to measure the effect of miR-187-3p and FGF9 overexpression on gemcitabine sensitivity in breast cancer cells. In the presence of gemcitabine, co-transfection with the FGF9 plasmid significantly reversed miR-187-3p overexpression-mediated reduction in cell viability (Fig. 6E) and promotion of apoptosis (Fig. 6F and G). Taken together, the results indicated that overexpression of miR-187-3p increased gemcitabine sensitivity in MDA-MB-231 cells by suppressing FGF9 expression.
miR-187-3p expression negatively correlates with that of FGF9 mRNA in breast tumor tissues. Following observations that miR-187-3p negatively regulates FGF9 in breast cancer cells, the potential correlation between miR-187-3p and FGF9 mRNA expression was determined in 30 breast cancer tumor and their matched adjacent non-tumor tissue samples. The results revealed that FGF9 mRNA expression was negatively correlated with miR-187-3p expression (Fig. 7), indicating that
miR-187-3p may regulate FGF9 during breast cancer pathogenesis.

Discussion

Breast cancer is the most commonly diagnosed type of cancer in women, with its incidence ranking the highest among all malignancies (27). Accumulating evidence has demonstrated that targeted therapies offer superior therapeutic efficacy compared with conventional approaches (28). Recently, miRNAs have been widely demonstrated to be associated with a variety of diseases including breast cancer (29). Gasparini et al (30) reported that the downregulation of miR-27a and miR-30e in patients with breast cancer correlated with poor patient outcomes, whereas the overexpression of miR-155 and miR-493 correlated with good patient outcomes. In addition, Kumaraswamy et al (31) revealed that miR-146a expression correlated positively with that of breast cancer gene 1, which in turn negatively regulated epidermal growth factor receptor expression in breast cancer. The miR-200 family has previously been demonstrated to serve important roles in the cell migration, metastasis, proliferation and invasion of breast cancer (32,33). miRNA expression profiles may influence responses to cancer chemotherapy (34). miRNAs regulate cancer drug resistance via a number of methods, including the expression of specific drug targets, survival and/or apoptosis signaling, cell proliferation and cell cycle progression (35). Furthermore, miR-200c was revealed to be reduced in the doxorubicin-resistant MCF7 breast cancer cell line (MCF7/ADR), where the overexpression of miR-200c increased MCF7/ADR sensitivity to epirubicin (36). In addition, the expression of miR-7, miR-326 and miR-345 were revealed to be markedly lower in the MCF7 the cislipatin-resistant cell line in comparison with the MCF7 cell line (37). The function of miR-187-3p in cancer remains controversial, as it has been previously reported to be downregulated in many diseases, including prostate cancer (38), retinal ganglion (39), type 2 diabetes (40), non-small cell lung cancer (13) and breast cancer (14). miR-187-3p has been previously associated with a number of different malignancies (13,14). Using a miRNA microarray, Liang et al (23) determined that miR-187-3p was one of the most significantly downregulated miRNAs in the chemotherapy-resistant MCF7 cells compared with parental MCF7 cells. However, the function of miR-187-3p on breast cancer drug resistance has yet to be clearly elucidated. In the present study, it was revealed that miR-187-3p expression was significantly lower in breast cancer tumor tissue compared with matched non-tumor tissues collected from patients with breast cancer. miR-187-3p overexpression significantly reduced cell viability and promoted cell apoptosis, indicating that miR-187-3p is a tumor suppressor in breast cancer. Although gemcitabine has been used for the treatment of metastatic breast cancer since 2004 (41), its therapeutic use in cancer chemotherapy has been impeded at least in part by drug resistance (42). Chaudhari et al (43) performed miRNA microarray analysis, which determined that doxorubicin treatment increased miR-187-3p expression in human cardiomyocytes. In the present study, cell viability and cell apoptosis assays revealed the effect of miR-187-3p on breast cancer gemcitabine sensitivity. Data from these analyses, it was indicated that miR-187-3p overexpression increased gemcitabine sensitivity in breast cancer cells.

Although Fillmore et al (20) have previously explored the role of FGF9 signaling in breast cancer cells, the association between miRNA expression and FGF9 in breast cancer remain unclear. According to the TargetScan database, FGF9 was predicted to be a potential target gene of miR-187-3p, which was subsequently confirmed by luciferase assays and observations that miR-187-3p negatively regulated FGF9 expression.

Target genes of miR-187-3p were involved in the regulation of signaling pathways, including phosphatidylinositol 3-kinase-AKT and hypoxia-inducible factor 1 signaling pathways in epileptic rats (44). Hendrix et al (45) revealed that FGF9 was a downstream target of the Wnt signaling pathway, which may be an indirect transcription target of β-catenin in ovarian endometrioid adenocarcinomas. Abdel-Rahma et al (46) demonstrated that 80% FGF9 mutations were associated with membranous β-catenin expression in colorectal and endometrial carcinomas. In addition, FGF9 has been reported as a target for the Wnt/β-catenin pathway in ovarian endometrioid adenocarcinomas (26). Interestingly, FGF9 interacted with Wnt/β-catenin signaling in a feed-forward loop in lung development, as determined by Yin et al (25). Wnt signaling is associated with drug resistance in cancer (47). In addition, Shi et al (48) elucidated that LGR5 suppressed docetaxel resistance in breast cancer through the inactivation of the Wnt/β-catenin signaling. However, information on the association between miR-187-3p and Wnt/β-catenin in breast cancer pathophysiology remains unclear. In the current study, overexpression of miR-187-3p in MDA-MB-231 cells significantly reduced the protein expression of β-catenin, c-Myc and Cyclin D1. In conclusion, miR-187-3p negatively regulated FGF9 expression, which inactivated Wnt/β-catenin signaling and increased breast cancer sensitivity to gemcitabine-mediated toxicity.

Taken together, the results of the present study suggested that miR-187-3p increased gemcitabine sensitivity by targeting FGF9 expression in breast cancer cells, indicating that miR-187-3p may be a therapeutic target for patients with breast cancer.
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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

YW, YQ, WL, HY and XY carried out experiments and analyzed the data. LT and JL collected the clinical samples and analyzed the data. LL designed the study, analyzed the data and prepared the manuscript. All author read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethic Committee of Chifeng Municipal Hospital. Written consent was provided by all participants prior to the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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