miR-181b promotes chemoresistance in breast cancer by regulating Bim expression

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Abstract. MicroRNAs are emerging as critical regulators of the initiation and progression of multiple types of human cancers, including breast cancer. In the present study, the expression of miR-181b in breast cancer patient serum and breast cancer cell lines was evaluated. It was demonstrated that the miR-181b level was significantly upregulated in patient serum and breast cancer cell lines compared with that in normal controls. The results of in vitro 3H thymidine incorporation and Transwell migration assay indicated that miR-181b overexpression markedly promoted the proliferation and metastasis of breast cancer cells. These data suggest that miR-181b is a tumor promoter in breast cancer. Furthermore, miR-181b expression was found to be upregulated in doxorubicin (DOX)-resistant T-47D cells (T-47D-R) compared with that in the parental T-47D cells, and upregulation of miR-181b expression decreased the anticancer effect of DOX in the T-47D cells. Mechanistic studies demonstrated that the Bim gene, an essential initiator of apoptosis, was inhibited by miR-181b overexpression. We observed that knockdown of miR-181b by its specific inhibitors significantly re-sensitized the T-47D-R cells to the cytotoxicity of DOX. Importantly, we demonstrated that miR-181b inhibitors increased the level of Bim in the T-47D-R cells, resulting in the loss of mitochondrial membrane potential (MMP) and the activation of caspases caused by DOX. In summary, the results of the present study suggest that miR-181b functions as an oncogene during breast cancer development, and the miR-181b/Bim pathway may be a novel target used to overcome the chemoresistance in breast cancer.

Introduction

Breast cancer is one of the most common malignant cancers, and is also the leading cause of cancer-related deaths among women due to the metastatic spread of the cancer to vital organs, such as the lung and liver (1,2). For patients with early breast cancer, surgery is the primary treatment which effectively improves patient long-term survival. However, it is ineffective for individuals with advanced disease, and the systemic chemotherapy is considered as an alternative option when tumor resection is not feasible (3,4). Unfortunately, chemotherapy is mostly ineffective due to the development of chemoresistance in cancer patients (5). Doxorubicin (DOX) is a widely used antitumor antibiotic for the treatment of multiple types of cancers including breast cancer. However, high-dose DOX shows cardiotoxicity as well as killing normal self-reborn cells (6,7). Therefore, efforts have been made to reduce the dose of DOX by reversing chemoresistance (8,9).

MicroRNAs (miRNAs) are a class of small non-coding RNAs, typically 19-25 nucleotides in length. They function as gene regulators by downregulating the expression of specific target genes (10,11). Approximately 50% of miRNA genes are located in tumor-associated genomic regions, and more than 30% of all human protein-coding genes may be regulated by miRNAs, including a wide range of genes involved in tumorigenesis (12,13). Therefore, it is well acknowledged that miRNAs are important for cancer development and progression, which can act as either oncogenes or tumor suppressors by regulating their respective target genes (14,15). Studies have indicated that a systematic characterization of miRNAs could enable their identification as biomarkers for the diagnosis of breast cancer, and many miRNAs may be chosen as therapeutic targets for the treatment of breast cancer (16,17). However, the role and the mechanism of miRNAs in tumorigenesis and cancer chemotherapy remain largely unknown.

In the present study, we sought to determine the role of miR-181b in the growth and migration of breast cancer cells. We demonstrated that the expression of miR-181b is upregulated in patients with breast cancer, and is involved in the development and metastasis of breast cancer cells. More importantly, we also present evidence for miR-181b upregulation as a mechanism for DOX resistance, and provide the first...
link between miR-181b and the intrinsic apoptotic pathway activated by DOX in breast cancer.

Materials and methods

Clinical samples and cell culture. Blood samples were obtained from 30 healthy controls and 30 breast cancer patients at Zhejiang Cancer Hospital (Hangzhou, China). The use of the blood for the present study was approved by the Hospital's Protection of Human Subjects Committee. MCF-10A, T-47D, MCF-7, MDA-MB-231 and MDA-MB-435 cell lines were provided by the Institute of Biochemistry and Cell Biology of the Chinese Academy of Science (Shanghai, China). The breast cancer cell lines (including T-47D, MCF-7, MDA-MB-231 and MDA-MB-435) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (both from Gibco, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate. The MCF-10A cell line was cultured in DMEM/F12 media supplemented with 5% horse serum, 10 µg/ml insulin (all from Gibco), 100 ng/ml cholera toxin, 20 ng/ml EGF and 0.5 µg/ml hydrocortisone (all from Sigma-Aldrich, USA) at 37°C in a humidified incubator with 5% CO2. To study the role of miR-181b in chemoresistance, we established a doxorubicin-resistant T-47D cell line (T-47D-R) by stepwise exposure of T-47D cells to increasing concentrations of DOX (Sigma-Aldrich). Briefly, the T-47D cells were initially cultured with 0.1 µg/ml DOX for 8 weeks, and then the DOX concentration was increased every 4 weeks by 0.02 µg/ml up to a final concentration of 0.3 µg/ml. The T-47D-R cells were exposed to DOX over a time period of 12 months. Before the following experiments were performed, the T-47D-R cells were cultured in DOX-free DMEM for 2 weeks.

RNAs and transfection. Human miR-181b mimics, 2'-omethyl modified miR-181b inhibitors, negative control oligonucleotides (NCO) and Bim siRNA were purchased from GenePharma Co. (China). The sequences of RNAs were as follows: miR-181b mimics, 5'-AACAUUCAUGCUGCCG GUGGGU-3'; miR-181b inhibitors; 5'-ACCCACCCGA CACCAUAAGAUG-3'; NCO, 5'-AUCCUAUGGGGGU UACAUUGUU-3'; and Bim siRNA, 5'-GACCGGAGAAGGU AGCAAAUU-3'. The RNAs were transfected into cells with Lipofectamine 2000 (Invitrogen, USA) at the final concentration of 50 nM according to the manufacturer's protocols.

Quantitative real-time PCR (qRT-PCR). The expression levels of miR-181b and Bim were measured by quantitative RT-PCR (qRT-PCR), using TaqMan MicroRNA assays kit and supplies (Applied Biosystems, USA) according to the manufacturer's instructions. The expression of miR-181b was normalized to U6 snRNA, and the Bim level was normalized to GAPDH. Relative quantities of miR-181b and Bim were calculated using the 2^-ΔΔCt method (18).

Luciferase reporter assay. The Bim 3'-UTR was cloned into the pMIR-REPORT™ miRNA Expression Reporter Vector (Life Technologies, USA) to generate the wild-type constructs. The mutant plasmid was created by mutating the seed regions of the miR-181b-binding sites (UGAUAGU to UGAUAGU) using the Site-Directed Mutagenesis kit (Takara, Japan) based on the wild-type constructs. Then, the Dual-Luciferase Reporter assay (Promega, Madison, WI, USA) was conducted to investigate the interaction between miR-181b and its predicted target gene Bim. Briefly, the T-47D-R cells were co-transfected with the miR-181b mimics or NCO and Bim 3'UTR or Bim 3'UTR-mutant. Forty-eight hours later, the firefly and Renilla luciferase activities were detected according to the manufacturer's protocol. The firefly luciferase activity was normalized to the Renilla luciferase activity.

Western blot analysis. The whole cells were lysed with RIPA buffer (Cell Signaling Technology, USA) containing 2 mM of phenylmethylsulfonyl fluoride. The samples were then subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with primary antibodies at 4°C overnight, and were then incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 2 h. Blots were developed using an enhanced chemiluminescence detection kit (Pierce, USA). The primary antibodies against Bim, caspase-3, caspase-9, PARP and β-actin were purchased from Cell Signaling Technology.

Cell viability and proliferation. Breast cancer cells were transfected with miR-181b mimics or miR-181b inhibitors at a final concentration of 50 µM. Forty-eight hours after transfection, the medium was replaced with fresh medium containing DOX at different concentrations. After culturing for 48 h, the cells were subjected to cell viability assay. DOX concentrations leading to 50% cell death (IC50) were calculated by the viability curve determined by the MTT assay. To determine cell proliferation, the [3H] thymidine incorporation assay was used to determine the cell proliferation during the last 6 h of incubation as previously described (19).

Cell migration in vitro. Breast cancer cells were transfected with miR-181b mimics or inhibitors for 48 h. Before being seeded, the undersurface of the upper chamber of the Transwell was coated with collagen I overnight at 4°C, and then 1x105 cells were seeded in serum-free media on the upper chambers of Transwells with a porous transparent polyethylene terephthalate membrane having a pore size of 8-micron (Corning Costar Corporation, USA), and hydroxyurea (Sigma-Aldrich) was added to stop cellular proliferation. After 24 h, cells on the undersurface of the upper units were fixed, stained and then counted under a phase-contrast microscope.

Apoptosis and mitochondrial membrane potential (MMP, ΔΨm) analysis. Apoptosis was assessed using the Annexin V-FITC apoptosis detection kit (Sigma-Aldrich) according to the manufacturer's protocol. After transfection and DOX treatment, T-47D-R cells were harvested and rinsed in cold phosphate-buffered saline (PBS), followed by resuspension in 1X Annexin binding buffer at 1x105 cells/ml. Annexin V (5 µl) and 0.1 µg of propidium iodide (PI) were then added to the cells. Samples were incubated at room temperature for 15 min in the dark and were analyzed using flow cytometry (Becton-Dickinson, USA). ΔΨm was detected using 5,5',6,6'-tetrachloro-1,1',3,3'-tetrathiyl-imidacarbo-cyanine iodide (JC-1; Molecular Probes, USA) as an indicator (20). After treatment, the T-47D-R cells were collected and resuspended with PBS containing JC-1 at...
a final concentration of 5 µM. Following a 20-min incubation period at 37˚C in the dark, MMP was determined by flow cytometric analysis.

**Statistical analysis.** Data are represented as mean ± SE of three independent experiments. The Student's t-test was conducted with SPSS 14.0 software to assess the statistical significance between treatments. p<0.05 was considered to indicate a statistically significant result.

**Results**

**miR-181b is upregulated in breast cancer cell lines and patient serum.** The expression of miR-181b was analyzed in the serum of breast cancer patients and the healthy controls using RT-qPCR. miR-181b was significantly upregulated in the cancer patient serum compared with that in the normal controls (Fig. 1A). Furthermore, we found that the expression level of miR-181b was significantly higher in all of the four breast cancer cell lines compared with the level in the normal breast cell line MCF-10A (21) (Fig. 1B). These results suggest that miR-181b may function as a tumor promoter in breast cancer.

**miR-181b promotes cell proliferation and migration in breast cancer cells.** To study the role of miR-181b in the tumor progression of breast cancer cells, miR-181b mimics or inhibitors were transfected to change the level of miR-181b in the T-47D and MCF-7 cells. Briefly, the miR-181b level was increased ~10.22-fold after the miR-181b mimics were introduced, and the miR-181b level was decreased ~4.27-fold after the miR-181b inhibitors were introduced (data not shown). Results of ³H thymidine incorporation assays demonstrated that the proliferation of both T-47D and MCF-7 cells was significantly enhanced in the miR-181b-overexpressing group compared with the control group. In contrast, the proliferation was significantly impaired in the miR-181b-knockdown cells (Fig. 2A). Furthermore, we evaluated the effect of miR-181b on cell migration using a Transwell system. We observed that the overexpression of miR-181b significantly promoted the migration in breast cancer cells, which could be obviously inhibited by knockdown of miR-181b (Fig. 2B). These results suggest that miR-181b may function as a novel oncogene in breast cancer.

**miR-181b is associated with the resistance of breast cancer cells to DOX.** To investigate whether miR-181b can modulate the sensitivity of breast cancer cells to DOX which is a potent anticancer drug, we stepwisely exposed the T-47D cells to increasing concentrations of DOX to establish a DOX-resistant T-47D cell line (T-47D-R). As shown in Fig. 3A, the expression level of miR-181b in the T-47D-R cells was significantly upregulated compared with that in the parental T-47D cells, suggesting that miR-181b promotes the chemoresistance in breast cancer. To reveal the effects of miR-181b on the efficacy of chemotherapy, T-47D cells were simultaneously treated with miR-181b and DOX. It was found that the miR-181b mimics significantly increased the IC₅₀ of DOX, which was obviously decreased by the transfection of miR-181b inhibitors compared with the controls (Fig. 3B). This indicated that the downregulation of miR-181b sensitized breast cancer cells to DOX resistance. Furthermore, knockdown of miR-181b re-sensitized the T-47D-R (DOX-resistant T-47D) cells to DOX, although the antitumor effect of DOX was not influenced by the transfection of miR-181b mimics (Fig. 3C). Taken together, these results indicated that miR-181b may play an important role in chemoresistance in breast cancer, and that miR-181b silencing reverses the resistance to DOX treatment.

**miR-181b in T-47D-R cells regulates the expression of Bim.** In order to understand the underlying mechanism for the promotion of miR-181b to DOX resistance, we used TargetScan database (http://www.targetscan.org/) and found that Bim was a putative target of miR-181b. Notably, the position 474-480 of Bim 3'-UTR was observed to be a complementary site (5’-.....UGAAUGU.....3’) for the seed region of miR-181b. Furthermore, we observed that the expression level of Bim was significantly lower in the T-47D-R cells compared with
Figure 2. miR-181b promotes cell proliferation and migration in breast cancer cells. (A) After the T-47D and MCF-7 cells were transfected with miR-181b mimics or inhibitors for 48 h, cell proliferation was measured using 3H thymidine incorporation assay. *p<0.05 vs. NCO group. (B) T-47D and MCF-7 cells were transfected with miR-181b mimics or miR-181b inhibitors for 48 h, and the migration potential was analyzed using Transwell assays. Relative cell migration was normalized to the NCO group. *p<0.05 vs. NCO group.

Figure 3. miR-181b is associated with the resistance of breast cancer cells to doxorubicin. (A) The expression of miR-181b in T-47D-R cells and their parental cells was analyzed by RT-qPCR. *p<0.05. (B) T-47D cells were transfected with 50 pmol/ml NCO, miR-181b mimics or inhibitors. After 48 h of incubation, the cells were treated with DOX for another 48 h. Cell viability was evaluated by MTT assay. The IC_{50} value was determined according to the survival curves. *p<0.05 vs. NCO group. (C) T-47D-R cells were transfected with 50 pmol/ml NCO, miR-181b mimics or inhibitors. After 48 h of incubation, the cells were treated with DOX for another 48 h. Cell viability was evaluated by MTT assay. The IC_{50} value was determined according to the survival curves. *p<0.05 vs. NCO group.
the parental T-47D cells (Fig. 4A). Therefore, we inferred that the T-47D cells became DOX resistant by downregulating Bim expression, which is a target of miR-181b. To confirm this speculation, T-47D-R cells were transfected with miR-181b mimics, miR-181b inhibitors or NCO (50 pmol/ml) followed by assay of mRNA and protein levels of Bim, respectively. *p<0.05 vs. NCO group. (C) Luciferase reporter assays in T-47D-R cells. Cells were transfected with wild-type or mutant Bim 3'-UTR-reporter constructs together with miR-181b mimics or NCO. Forty-eight hours post-transfection, the firefly luciferase activity was measured, and normalized to the Renilla luciferase using the Dual-Luciferase Reporter system.

Figure 4. Bim is a target of miR-181b in T-47D-R cells. (A) The expression of Bim at the mRNA and protein levels in T-47D and T-47D-R cells was evaluated by RT-qPCR and western blot analysis. (B) T-47D-R cells were transfected with miR-181b mimics, miR-181b inhibitors or NCO (50 pmol/ml) followed by assay of mRNA and protein levels of Bim, respectively. *p<0.05 vs. NCO group. (C) Luciferase reporter assays in T-47D-R cells. Cells were transfected with wild-type or mutant Bim 3'-UTR-reporter constructs together with miR-181b mimics or NCO. Forty-eight hours post-transfection, the firefly luciferase activity was measured, and normalized to the Renilla luciferase using the Dual-Luciferase Reporter system.

The expression of Bim was negatively regulated by miR-181b, which may play an essential role in the DOX resistance of breast cancer cells.

miR-181b inhibitors reverse DOX resistance through the miR-181b-Bim-MMP-caspase pathway. Our preceding results showed that miR-181b inhibitors reversed the DOX resistance in T-47D-R cells and that the Bim gene was the direct target of miR-181b. We, therefore, investigated the pathway and the relationship between Bim regulation and the antitumor effect of the treatment with DOX plus miR-181b inhibitors in T-47D-R cells. As Bim is the key member of the pro-apoptotic Bcl-2 family proteins (22), we found that the downregulation of miR-181b by its specific inhibitors significantly increased the apoptotic rate of the T-47D-R cells treated with DOX, and this synergistic effect of miR-181b inhibitors was abolished when Bim siRNA (after transfection, the expression of Bim was reduced
Figure 5. miR-181b inhibitors reverse DOX resistance through the miR-181b-Bim-MMP-caspase pathway. (A) T-47D-R cells were co-transfected with 50 pmol/ml Bim siRNA and/or 50 pmol/ml miR-181b inhibitors for 48 h. Then, the cells were treated with 5 µg/ml DOX for another 48 h. The apoptotic rate was analyzed using flow cytometry. (B) T-47D-R cells were treated as described above, and the mitochondrial membrane potential was analyzed by JC-1 staining. (C) T-47D-R cells were co-transfected with 50 pmol/ml Bim siRNA and/or 50 pmol/ml miR-181b inhibitors for 48 h. Then, the cells were treated with 5 µg/ml DOX for another 48 h in the absence or presence of zVAD-fmk (10 µM), and western blot analysis was performed to measure the activation of caspase-9 and -3 and its substrate PARP. (D) T-47D-R cells were treated as described above, and cell viability was measured by MTT assay. *p<0.05 vs. DOX plus miR-181b inhibitor group.
~5.12-fold, data not shown) was co-transfected (Fig. 5A). The results indicated that miR-181b regulated the DOX resistance via targeting the Bim gene. We next observed that although miR-181b inhibitors alone did not influence the MMP of the T-47D-R cells, they significantly promoted DOX to damage the mitochondria of the T-47D-R cells. Notably, the mitochondrial dysfunction caused by miR-181b inhibitors plus DOX was inhibited by Bim siRNA (Fig. 5B). Since a previous study indicated that intrinsic apoptosis is activated by mitochondrial dysfunction, mitochondrial-derived apoptotic proteins are then released from mitochondria, leading to the activation of caspase-9 which finally activates caspase-3 (23). Our results demonstrated that the apoptosis induced by miR-181b inhibitors plus DOX was caspase-dependent (Fig. 5C). Finally, using MTT assay, we indicated that both the Bim siRNA and zVAD-fmk significantly inhibited the cell death induced by DOX combined with the miR-181b inhibitors in the T-47D-R cells (Fig. 5D). Our data strongly suggest the important role of the Bim pathway in reversing the DOX resistance in breast cancer.

Discussion

Studies have demonstrated that miR-181b may act as an oncogene in multiple types of cancer. For instance, the upregulation of the miR-181 family promoted the growth, clonogenic survival, migration and invasion in hepatocellular carcinoma cells by targeting TIMP3 (24). It was also reported that expression levels of the miR-181 family were elevated in breast, colon and pancreatic cancer (25-27). Furthermore, miR-181b was found to contribute to the drug resistance of tamoxifen which is the irreproachable drug for the treatment of breast cancer (28). Although the previous studies indicated that the miR-181b level is commonly upregulated in many types of tumor, the functions and targets concerning miR-181b remain unknown. In the present study, we showed that miR-181b was significantly upregulated in the blood of breast cancer patients and in breast cancer cell lines. Moreover, we also found that the expression level of miR-181b in breast cancer cells was positively related with cell proliferation and migration in vitro, suggesting that miR-181b acts as a tumor promoter in breast cancer.

Chemoresistance is the major limitation for achieving a satisfactory chemotherapeutic effect, and much effort has been made to prove that treatment using various miRNAs may improve the antitumor effect of chemotherapeutic drugs in multiple tumor types. For instance, the anti-miR-21 oligonucleotide significantly enhanced the chemosensitivity of glioblastoma cells to DOX by inducing apoptosis (29). Enforced expression of miR-26b improved the curative effect of hepatocellular carcinoma cells to TRAIL by downregulating the anti-apoptotic gene myeloid cell leukemia-1 (Mcl-1) (30). miR-193b was proven to act as a drug sensitizer, and promoted the induction of apoptosis following cisplatin treatment in hepatocellular carcinoma (31). In this context, it was of interest to examine whether or not miR-181b is associated with chemoresistance. We found that knockdown of miR-181b re-sensitized the T-47D-R cells to DOX, significantly decreasing the IC50 value of DOX. We further found the miR-181b mimics did not further impair the antitumor effect of DOX. We explained that the expression of miR-181b in the T-47D-R cells is high enough to have a biological effect so that the transfection of miR-181b mimics became unnecessary.

Bcl-2 interacting mediator of cell death [Bim, also known as B-cell chronic lymphocytic leukemia-lymphoma-like 1 (BCL2L11)], is a member of the Bcl-2 family genes and belongs to the BH3-only subfamily of Bcl-2 family proteins (32). Bim has emerged as a crucial regulator of the mitochondrial (intrinsic) apoptotic pathway, which directly activates the pro-apoptotic function, and binds to all of the pro-apoptotic Bcl-2 family members to promote cell apoptosis (33,34). Recently, accumulating evidence shows that Bim deletion in cancers is associated with a poorer response to targeted therapy treatment (35,36). Bim may act as a biomarker to predict the survival of cancer patients (37). According to our data, miR-181b inhibitors re-sensitized T-47D-R cells to DOX by targeting Bim, depending on the upregulation of the cellular level of Bim in chemoresistant cancer cells. To further explore the mechanisms, we tested the mitochondrial membrane potential which is downstream of the Bim pathway, which controls mitochondrial (intrinsic) apoptosis (38). We found that regain of Bim mediated by miR-181b inhibitors in T-47D-R cells significantly promoted DOX to damage the mitochondria, leading to a decrease in the mitochondrial membrane potential. As a result, caspase-9 which is a biomarker of intrinsic apoptosis was activated, following the cleavage of caspase-3 (39).

In conclusion, the present study provides evidence that miR-181b is a novel oncogene and one important factor for developing the chemoresistance in breast cancer. The results suggest that the miR-181b-Bim-MMP-caspase pathway may be a potential therapeutic target for the chemotherapy of breast cancer in the future.

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References


