Abstract. It is not well established whether miR-93 is involved in drug resistance and epithelial-mesenchymal transition (EMT) in breast cancer, and its underlying mechanism remains uncertain. In the present study, the expression differences of miR-93 between paired breast cancer tissues confirmed it is involved in the progression of breast cancer. Such a difference was also observed in doxorubicin-resistant and -sensitive cells. Overexpressed miR-93 in sensitive cells revealed increases in cellular proliferation and the expression levels of drug-resistant-related genes, and a decrease in sensitivity to doxorubicin. This demonstrated the relationship between miR-93 and breast cancer drug resistance. Simultaneously, EMT was confirmed in miR-93 overexpressing sensitive cells. This indicated the triadic relationship among miR-93, EMT and drug resistance in breast cancer. We applied the Dual-luciferase Reporter assay to expose the direct interaction between miR-93 and PTEN, which suggested that miR-93 contributes to inducing EMT and drug resistance of breast cancer cells by targeting PTEN.

Introduction

Breast cancer is the leading cause of cancer-related mortality among women (1). This high incidence of mortality is, to a large extent, due to drug-resistance, which is the major obstacle to successful clinical treatment (2).

Numerous studies have demonstrated that microRNAs (miRNAs) are involved in the process of epithelial-mesenchymal transition (EMT) in various types of cancer (3-6), and various studies have revealed that the EMT may be associated with the drug resistance of cancer cells (7-10). Furthermore, by comparing the miRNA expression profiles between breast cancer cell lines in our previous study, we determined that the expression of miR-93 was increased markedly in drug-resistant MCF-7/AdrVp cells compared with the parental MCF-7 cell line (11).

Numerous studies on breast cancer have demonstrated that PTEN is involved in EMT (5,12) and in the drug resistance of cancer cells (6,13). Although a few studies have shown that miR-93 may functionally interact with PTEN, this has only been reported in cardiomyocyte apoptosis, osteosarcoma, ovarian cancer, glioma, hepatocellular carcinoma and prostate cancer (14-20). Therefore, it remains to be determined whether miR-93 is also functionally associated with PTEN in breast cancer, and whether such an association contributes to the induction of EMT and drug resistance in breast cancer cells. The present study aimed to clarify the role of miR-93 in drug resistance and EMT in breast cancer, and to investigate its target gene.

Materials and methods

Tissue samples. Sixteen pairs of tissue samples of ductal breast cancer were obtained from the West China Hospital of...
Sichuan University. All the patients underwent surgical resection to obtain the breast cancerous and corresponding adjacent non-tumorous tissues, without receiving chemotherapy or radiotherapy beforehand. All tissues were preserved in liquid nitrogen. The present study was approved by the local ethical standards of the Institutional Review Board of Sichuan University. Informed consent was obtained from all individual participants included in the present study.

Ethical approval. All procedures performed in the present study involving human participants were in accordance with the ethical standards of the Institutional and/or National Research Committee of Sichuan University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Cell lines and transfections. The MCF-7 cell line was purchased from the Shanghai Institutes for Biological Sciences (Shanghai, China) and cultured according to a protocol from the ATCC.

The doxorubicin-resistant MCF-7/ADR cell line (MCF-7/ADR) was induced by continuously culturing MCF-7 cells in medium containing progressive concentrations of doxorubicin (Sigma-Aldrich, St. Louis, MO, USA). MCF-7/ADR cells were cultured in medium with 2 µg/ml doxorubicin, and subsequently transferred into a drug-free medium for at least 2-3 weeks before use in the assays.

Using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, MCF-7 cells were transfected with 50 nM hsa-miR-93-5p mimics or hsa-miR-93-5p negative control mimics (RiboBio Co., Ltd., Guangzhou, China) in order to produce an MCF-7/ADR-miR-93 mimics group (MCF-7/ADR-miR-93 mimics) and a negative control group (MCF-7/ADR-miR-93 mimics NC), respectively. In addition, MCF-7/ADR cells were transfected with 100 nM hsa-miR-93-5p inhibitor (RiboBio Co., Ltd.) to produce an MCF-7/ADR-miR-93 inhibitor group, or an hsa-miR-93-5p inhibitor negative control (RiboBio Co., Ltd.) to produce an MCF-7/ADR-miR-93 inhibitor NC group, following the same procedure as aforementioned.

RNA isolation and qRT-PCR. Total miRNAs were extracted from cells and tissue samples using an miRcute miRNA Isolation kit (Tiangen Biotech Co., Ltd., Beijing, China). The expression of miR-93 was analyzed using the Bulge-Loop™ miRNA qRT-PCR Starter kit (RiboBio Co., Ltd.). The primers for miR-93 and endogenous control U6 were purchased from RiboBio Co., Ltd. (ssD809230675, ssD809231367, ssD809261711, ssDD0904071006, ssDD0904071007 and ssDD0904071008).

TRIzol reagent (Takara, Dalian, China) was used for total RNA extraction. The expression of different genes was analyzed using a SYBR-Green qRT-PCR kit (Takara). The primer sequences for qRT-PCR were as follows: E-cadherin, 5'-ggccgagatagaaactatttcct-3', 5'-tagggtgccagctttacagt-3' (product size, 200 bp); vimentin, 5'-gaatgagttgcctacttggccg-3', 5'-ggctggtagcacttggccg-3' (product size, 170 bp); GAPDH, 5'-ctgatccgggatggct-3', 5'-tgccagctgggagc-3' (product size, 202 bp); PTEN, 5'-ctggtgccggagttc-3', 5'-ttggctgctccgggagttc-3' (product size, 132 bp).

Flow cytometry. Cell apoptosis was detected using an Annexin V-FITC apoptosis detection kit (KeyGen Biotech Co., Ltd., Nanjing, China), according to the manufacturer’s instructions. The data were analyzed using FlowJo 9.1 software.

CCK-8 assay. Cellular proliferation and growth inhibition were assessed using Cell Counting Kit-8 (CCK-8) assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s protocol. For the detection of the proliferation of cells in the MCF-7/ADR-miR-93 mimic group, the cells were assessed at 0, 24, 48 and 72 h. In addition, to determine the effect of miR-93 on drug resistance, the cell survival ratio was assessed after cells in the MCF-7/ADR-miR-93 mimic group were cultured with 0.08, 0.4, 1 and 5 µg/ml doxorubicin (Sigma-Aldrich) for 24 h.

Dual-luciferase reporter assay. A bioinformatics analysis using the ‘miRanda’ database was performed to analyze the possibility of miR-93 binding to PTEN. Subsequently, MCF-7 cells were cotransfected with 2.5 µg pGL3 luciferase reporter plasmid (GeneCopoeia) containing either a wild-type (5'-GGAuuAAuAAAGAuGGCuuuC-3') or mutated (5'-GGAAuuAAuAAAGAuGGCuuuC-3') form of the PTEN 3'-uTR, using Lipofectamine 3000. Luciferase activity was assessed consecutively at 24 h post-transfection using a Dual-Luciferase Assay (GeneCopoeia), and normalized to the blank vector control group.

Statistical analysis. All the experiments were repeated three times independently. Data were calculated as the mean ± SD. The paired t-test was applied for statistical analysis using SPSS software (version 20.0) (SPSS, Inc., Chicago, IL, USA).

Results

Expression of miR-93 in breast cancer in vivo and in vitro. qRT-PCR was performed to identify the patterns of miR-93 expression in breast cancer tissues and a doxorubicin-resistant breast cancer cell line (MCF-7/ADR) compared with paired normal breast tissue samples and a doxorubicin-sensitive parental breast cancer cell line (MCF-7). The results revealed that 62.5% of cancer tissue samples (10 out of 16 cases) exhibited a markedly higher expression level of miR-93 compared with their corresponding paired adjacent normal tissue (Fig. 1A; p<0.05). Furthermore, the miR-93 expression level in the MCF-7/ADR cells was significantly higher than that in MCF-7 cells (Fig. 1B; p<0.01).

Proliferation and doxorubicin-resistance of MCF-7 cells transfected with miR-93 mimics. A CCK-8 assay was performed
to evaluate the proliferation and sensitivity to doxorubicin of cells in the MCF-7-miR-93 mimics group. The results revealed that the overexpression of miR-93 markedly upregulated the proliferation rate of MCF-7 cells (Fig. 2A; \( p<0.01 \)), and also significantly increased the survival ratio of cells treated with doxorubicin for 24 h (Fig. 2B; \( p<0.01 \)).

qRT-PCR was used to examine the expression levels of the multi-drug resistant-related genes MDR, MRP and BCRP in MCF-7 and MCF-7-miR-93 mimic cells. The results demonstrated that the expression levels of each of these genes were significantly higher in the MCF-7-miR-93 mimic cells than in the MCF-7 cells (Fig. 2C; \( p<0.01 \)). Furthermore, flow cytometric analysis revealed that the rate of doxorubicin-induced apoptosis was lower in the MCF-7-miR-93 mimic cells than in untransfected MCF-7 cells (Fig. 3A, B and E; \( p<0.01 \)). By contrast, in the MCF-7/ADR-miR-93 inhibitor cells, the rate

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**Figure 1.** Expressions of miR-93 in breast cancer *in vivo* and *in vitro*. (A) The expression level of miR-93 was higher in breast cancer tissues compared to the adjacent normal tissues \((n=16)\). (B) MCF-7/ADR cells showed a significantly higher expression level of miR-93 than the MCF-7 cells; \( *p<0.05, **p<0.01 \).

**Figure 2.** Effects of miR-93 on cell proliferation, cell survival and drug resistance. (A) Cell proliferation was determined by CCK-8 assay. At 0, 24, 48 and 72 h, the OD value in each group of cells was assessed by the absorbance at 450 nm. (B) Overexpression of miR-93 in MCF-7 cells increased the survival ratio. Cell viability was determined by CCK-8 assay. The MCF-7-miR-93 mimic cells and its control cells were treated with 0.08, 0.4, 1 and 5 \( \mu \)g/ml doxorubicin for 24 h. The percentage of viable cells was normalized to that of the untreated controls. (C) Overexpression of miR-93 increased the expression of multi-drug resistant marker genes (MDR, MRP and BCRP) in MCF-7 cells; \( *p<0.05, **p<0.01 \).
of doxorubicin-induced apoptosis was significantly increased compared with that in the untransfected MCF-7/ADR-miR-93 cells (Fig. 3C, D, and F; p<0.01).

**EMT in MCF-7 cells overexpressing miR-93.** To explore the possible relationship between miR-93 expression and cancer cell EMT, the changes in morphological features and the mRNA levels of EMT-related genes were examined after the transfection of MCF-7 cells with miR-93 mimics or NC. The morphological features of the cells are shown in Fig. 4A. MCF-7-miR-93 mimic-transfected cells displayed a cobbleston-like appearance and tight cell-cell junctions. By contrast, the MCF-7-miR-93 mimic NC and untransfected MCF-7 cells appeared to have spindle-cell morphology. Meanwhile, the qRT-PCR results revealed that the expression level of the epithelial marker E-cadherin was markedly decreased, while the levels of the mesenchymal markers N-cadherin, vimentin, Twist, Snail and fibronectin were markedly increased after transfection with the miR-93 mimics (Fig. 4B; p<0.01).

**Dual-luciferase reporter assay and qRT-PCR analysis of PTEN.** To detect the possible association between miR-93
Figure 4. Overexpression of miR-93 contributes to EMT progression in MCF-7 cells. (A) The morphological characteristics of the miR-93-overexpressed MCF-7 cells and its negative control. MCF-7-miR-93 mimic cells displayed a cobblestone-like appearance and tight cell-cell junction. In contrast, the MCF-7-miR-93 NC cells appeared to have spindle-cell morphology. (B) qRT-PCR analysis of EMT-related marker genes (E-cad, N-cad, vimentin, Twist, Snail and fibronectin) mRNA expression level; *p<0.05, **p<0.01.

Figure 5. miR-93 directly targets PTEN. (A) The potential binding site of miR-93 in the 3'-UTR of PTEN. (B) The luciferase activity in the PTENWT-3'-UTR was significantly lower than the PTEN-Mut-3'-UTR control when cotransfected with miR-93 mimics. (C) Overexpression of miR-93 decreased the PTEN expression as determined by qRT-PCR analysis; *p<0.05, **p<0.01.
and PTEN, a ‘miRanda’ bioinformatics analysis was initially performed. The miRNA target prediction program indicated PTEN as one of the possible target genes of miR-93. In particular, the 3′-untranslated region (UTR) of PTEN mRNA contains a binding site for miR-93 (Fig. 5A). To confirm this, a dual-luciferase reporter assay was carried out. The results revealed that, compared with the control group (MCF-7-miR-93 mimic NC), transfection with the miR-93 mimics decreased the luciferase activity of the reporter construct containing the wild-type PTEN 3′-UTR (Fig. 5B; p<0.01), whereas the miR-93 mimics induced no significant change in the activity of the reporter construct containing the mutated PTEN 3′-UTR. This indicated that miR-93 can bind directly to the PTEN 3′-UTR. Furthermore, the qRT-PCR results confirmed that the miR-93 mimics could downregulate the expression level of PTEN in MCF-7 cells compared with the MCF-7-miR-93 mimics NC group (Fig. 5C; p<0.01).

Discussion

It has been widely reported that miRNAs are involved in numerous molecular events in various types of tumors (21-23), including the acquisition of drug resistance, which is one of the most prominent clinical challenges at present. In the present study, miR-93 was selected for investigation as its function remains uncertain in many contexts, particularly in breast cancer. We aimed to clarify the role of miR-93 in the acquisition of drug resistance of breast cancer cells.

miR-93, along with miR-106b and miR-25, is a member of the miR-106b-25 cluster, which is located in its host gene, MCM7 (24). All members of the miR-106b-25 cluster, in addition to MCM7, have been reported to be involved in tumorigenesis (25-27) and drug resistance (28,29) in multiple tumors. Furthermore, our previous study on miRNA profiles revealed that miR-93 was the most upregulated miRNA of this cluster in the doxorubicin-resistant MCF-7 cells compared with the parental MCF-7 cells, which indicated that miR-93 may be the major contributor to the drug resistance of breast cancer cells in this cluster. Simultaneously, in our preliminary experiments, we also tested the differential expression trends of MCM7 in MCF-7/ADR and MCF-7 cells, which revealed a less marked difference than that of miR-93. Therefore, miR-93, rather than the whole cluster and its host gene, may play a critical role in the acquisition of drug resistance in breast cancer. To date, very few studies have focused on the role of miR-93 in breast cancer (30-36). Furthermore, these studies only reported its association with altered expression patterns and proliferation, and none have focused on its relationship with drug resistance and association with PTEN.

The results of the present study revealed a higher expression level of miR-93 in primary ductal breast cancer tissues than in corresponding tumor-adjacent normal tissues, and an increased proliferation rate in miR-93-overexpressing MCF-7 cells, which confirmed that miR-93 may be involved in the progression of breast cancer. Furthermore, a higher expression level of miR-93 was observed in MCF-7/ADR cells than in MCF-7 cells, and the survival ratio of miR-93-overexpressing MCF-7 cells following exposure to doxorubicin was increased markedly compared with that of the miR-93 mimics NC group. Furthermore, the expression levels of multi-drug resistance-related genes were significantly upregulated in the miR-93-overexpressing MCF-7 cells compared with NC-transfected cells, concomitant with the increase in doxorubicin resistance. In contrast, the miR-93 inhibitor treatment led to a reversal of doxorubicin resistance in the MCF-7/ADR cells. Thus, we suggest that miR-93 may contribute to the doxorubicin resistance of breast cancer cells.

EMT has been observed in the majority of tumors (37,38), and emerging evidence also indicates that it may be involved in drug resistance in certain types of cancer cells (7,39), including breast cancer (40,41). In the present study, the observed morphological changes and the upregulation of EMT-related genes in miR-93-overexpressing MCF-7 cells indicated that miR-93 participates in EMT in breast cancer cells. Moreover, the significant upregulation of drug resistance-related genes in the miR-93-overexpressing MCF-7 cells was observed concomitantly with EMT, which infers that EMT may be involved in the miR-93-induced drug resistance of breast cancer cells.

Following bioinformatics analysis and the retrieval of related literature (18), we speculated that PTEN may be a direct target gene of miR-93, and the results of a dual-luciferase reporter assay and the downregulation of PTEN in miR-93-overexpressing MCF-7 cells confirmed this direct interaction in breast cancer cells. The PI3K/Akt signaling pathway is considered to be one of the mechanisms underlying EMT in cancer cells, and PTEN has been shown to be a regulatory factor upstream of this pathway (42). Combined with our results, we hypothesize that miR-93 affects EMT through its interaction with PTEN, subsequently inducing drug resistance in breast cancer cells.

In conclusion, miR-93 may play an important role in EMT and drug resistance of breast cancer cells by targeting PTEN. The present study provides novel insights into the biological function of miR-93 in breast cancer drug resistance. MiR-93 may be considered a potential biomarker of prognosis, and a promising therapeutic target for the reversal of drug resistance in patients with breast cancer.

References


