Overexpression of microRNA-24 increases the sensitivity to paclitaxel in drug-resistant breast carcinoma cell lines via targeting ABCB9

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Abstract. Paclitaxel has been widely used in the treatment of breast cancer. However, the development of drug resistance often increases the failure of chemotherapy. Growing evidence has reported the significant role of microRNAs (miRs) in drug resistance. The present study identified that miR-24 was significantly downregulated in paclitaxel-resistant (PR) breast cancer patients and in MCF-7/PR human breast carcinoma cells, and that overexpression of miR-24 could increase the effect of paclitaxel on drug-resistant breast carcinoma cells. Furthermore, miR-24 could directly bind to the 3’-untranslated region of ATP binding cassette B9 to downregulate its expression, thereby reducing drug transportation and improving the anti-tumor effect of paclitaxel on breast cancer cells. In vivo experiments also demonstrated that overexpression of miR-24 could increase the sensitivity of drug-resistant MCF-7 cells to paclitaxel. In conclusion, the present results suggested a novel function for miR-24 in reducing paclitaxel resistance in breast cancer, which may be of important clinical significance.

Introduction

Breast cancer is one of the most common cancers in women, and is the leading cause of cancer-induced mortality (1). With the development of therapeutic methods, including surgical resection, radiotherapy and particularly chemotherapy, the outcomes in breast cancer patients have improved in the last decades (2,3). Multiple anti-cancer drugs have been developed for chemotherapy in cancer therapy. As a natural compound isolated from the bark of the \textit{Taxus baccata} plant, paclitaxel could bind to the β-tubulin subunit of microtubules, thereby stabilizing it and forcing dividing cancer cells to arrest in the M phase of the cell cycle, thus promoting cell death (4). Paclitaxel has been successfully used in clinical treatment for >2 decades, which significantly improved the outcomes of breast cancer patients (4).

However, acquired drug resistance during treatment occurs in numerous patients and contributes to 90% of the chemotherapy failures (5). Thus, to explore how cancer cells can eliminate the damage effect of chemotherapeutic drugs such as paclitaxel and improve drug sensitivity could be important for the treatment of cancers. A crucial cause of drug resistance may be the increased expression of efflux pumps (6). Certain transmembrane proteins could transport drugs out of cancer cells. It has been reported that human transporter associated with antigen processing-like, also known as ATP binding cassette (ABC) B9, and other members of the ABC transporter family could mainly contribute to paclitaxel resistance (7,8). In addition, increased metabolism of chemotherapeutic agents by cytochrome p450, various β-tubulin mutations and other mechanisms could together contribute to the resistance to paclitaxel and other chemotherapeutic agents (9,10). Therefore, reducing drug resistance may improve the treatment of breast and other cancers.

MicroRNAs (miRs) are a class of small non-coding RNAs that can control the expression of multiple genes via binding to their 3’-untranslated region (UTR) and consequently inducing post-transcriptional repression or degradation of the messenger RNAs (mRNAs) (11). Recent studies have reported
that microRNAs are important in drug resistance (12,13). For example, miR-29 could modulate collagen type I alpha 1 expression to improve cisplatin-induced cytotoxicity in ovarian cancer cells (14), and miR-30a increased doxorubicin resistance in osteosarcoma cells by regulating AMP-activated protein kinase α1 (15). In addition, paclitaxel resistance could also be regulated by miRs (16).

miR-24 has been widely reported to be involved in multiple cancers and to mediate tumor growth and apoptosis (17,18). However, the role of miR-24 in cancer is complex and remains unclear. In the present study, it was observed that miR-24 was significantly decreased in paclitaxel-resistant (PR) MCF-7 human breast cancer cells. To further investigate the role of miR-24 in drug resistance of breast cancer, MCF-7/PR cells were established, and the effect of miR-24 on the sensitivity to paclitaxel of MCF-7/PR cells was studied both in vitro and in vivo.

Materials and methods

Cell culture and establishment of PR cell lines. MCF-7 and SKBR3 human breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle medium (containing 25 mM glucose and 10% fetal bovine serum) (GE Healthcare Life Sciences HyClone Laboratories, Logan, UT, USA) at 37°C and 5% CO2. To establish PR cell lines, the cells were incubated with 1 µM paclitaxel (Sigma-Aldrich; St. Louis, MO, USA) at 37°C for 24 hours. Then, Hoechst 33342 staining was performed as previously described (19). For western blot analysis, following different treatments, MCF-7 and MCF-7/PR cells were lysed, and western blotting was performed as previously described (19). All western blot analyses were performed in triplicates. The blots were analyzed by Quantity One v4.62 software (Bio-Rad Laboratories, Hercules, CA, USA). Primary antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), including anti-B-cell lymphoma (Bcl)-2 (cat. no. sc-7383; dilution 1:500), anti-Bcl-2-associated X protein (Bax) (cat. no. sc-526; dilution 1:1,000), anti-cleaved caspase-3 (cat. no. sc-7872; dilution 1:1,000), and anti-Bcl-2 (cat. no. sc-7383; dilution 1:1,000). Secondary antibodies were all purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA), including anti-rabbit IgG (cat. no. sc-2005; dilution 1:1,000), and anti-mouse IgG (cat. no. sc-2004; dilution 1:1,000).

Luciferase assay. The miR-24 response element (wild type or mutated) in the 3'UTR of ABCB9 was transfected into the pMIR-reporter plasmid (Ambion, Austin, TX, USA), which contained the luciferase reporter gene. MCF-7 cells were co-transfected with luciferase reporter constructs containing wild type or mutated type ABCB9 3'UTR and miR-24 mimic in the presence/absence of miR-24 inhibitor. A luciferase assay kit (Promega Corporation, Madison, WI, USA) was used to analyze the luciferase activities according to the manufacturer's protocol.

Study population. A total of 20 human breast cancer tissues were selected from breast cancer patients that exhibited poor chemo-response after undergoing surgery at The Affiliated Cancer Hospital of Nanjing Medical University (Nanjing, China) between July 2010 and June 2014. Another group of patients (n=20) with a good chemo-response was recruited as control. Tumor tissues were washed and stored in liquid nitrogen prior to the experiments. In combination with the pathological diagnosis, >90% necrosis was considered drug-sensitive, while tumor necrosis <90% was considered...
overexpression of miR-24 improves the sensitivity toward paclitaxel of breast cancer cells. (A) The cytotoxicity of paclitaxel on MCF-7 (P0) and MCF-7/PR (P60) cells was determined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay (n=6). (B) Upon induction of paclitaxel resistance in MCF-7 cells for different days, the expression of miR-24 was determined by qPCR (P<0.05, **P<0.001 compared with P0 cells, n=6). (C) Using a microRNA mimic to overexpress miR-24 in MCF-7/PR cells, the expression of miR-24 was detected by qPCR in normal MCF-7 and MCF-7/PR cells (**P<0.001 compared with MCF-7/PR cells, n=6). (D) The miR-24 mimic was added to MCF-7 or MCF-7/PR cells, and then the IC50 of paclitaxel in each cell model was determined (n=6). (E) The miR-24 mimic was added to SKBR3 or SKBR3/PR cells, and then the IC50 of paclitaxel to each cell model was determined (n=6). PR, paclitaxel-resistant; miR, microRNA; IC50, half maximal inhibitory concentration; qPCR, quantitative polymerase chain reaction; P, passage.

**Results**

**Overexpression of miR-24 increases the sensitivity to paclitaxel of breast cancer cell lines.** After the MCF-7 breast cancer cells were treated for 60 days with 1 µM paclitaxel, the IC50 of paclitaxel in MCF-7/PR cells increased by 16-fold compared with that in normal MCF-7 cells (Fig. 1A). In this PR cell line, the expression of miR-24 decreased in a time-dependent manner (Fig. 1B). Thus, to further investigate the effect of miR-24 on the cytotoxicity of paclitaxel, a microRNA mimic was used to overexpress miR-24 in MCF-7 (Fig. 1C) and other breast cancer cell lines (data not shown). It was observed that overexpression of miR-24 did not affect the IC50 of paclitaxel in normal MCF-7 cells (IC50 changed from 2.627 to 3.429 µM, Fig. 1D). On the contrary, using a microRNA mimic to increase the miR-24 expression level in MCF-7/PR cells could obviously improve the sensitivity toward paclitaxel of this drug-resistant cell model (IC50 changed from 8.944 to 48.760 µM, Fig. 1D). Similar results were also observed in SKBR3 breast cancer cells (Fig. 1E and F).

**Animal models.** A total of 40 female 8-week-old nude BALB/c mice (20-22 g) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China), and written informed consent was obtained from all participants.

**Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining.** Tumor tissues were fixed in 4% paraformaldehyde for paraffin sections. Then, a TUNEL kit (Roche Diagnostics, Indianapolis, IN, USA) was used to stain the apoptotic cells according to the manufacturer's protocol. The apoptotic ratio was calculated as the number of TUNEL-positive cells divided by the total number of cells.

**Statistical analysis.** Data were expressed as the mean ± standard deviation. All statistical analysis was performed using PASW Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA). Statistical comparisons between groups were analyzed using the t test and one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.
Overexpression of miR-24 increases paclitaxel-induced apoptosis and inhibition of migration in MCF-7/PR cells via regulation of ABCB9 expression. As miR-24 could increase the cytotoxic effect of paclitaxel on breast cancer cells, the present study further investigated the effect of miR-24 on paclitaxel-induced cell apoptosis and inhibition of migration. Using Hoechst 33342 staining, paclitaxel-induced apoptosis was determined. The apoptotic cells displayed highly bright and shrunk nuclei (Fig. 2A). The apoptotic rate in 10 µM paclitaxel-treated MCF-7 cells reached 40%, which was 4-fold higher than that in control cells (Fig. 2B). However, this cytotoxic effect was not obvious in MCF-7/PR cells (Fig. 2B).

Using a miR-24 mimic, the cytotoxicity of 10 µM paclitaxel on MCF-7/PR cells was partially recovered (Fig. 2B). Furthermore, it was noticed that 10 µM paclitaxel could reduce the expression of the anti-apoptotic protein Bcl-2, increase the expression of the apoptotic marker Bax and activate caspase-3 in normal MCF-7 cells, while these effects were suppressed in MCF-7/PR cells (Fig. 2C). However, increased miR-24 expression level restored the pro-apoptotic effect of paclitaxel on drug-resistant cells (Fig. 2C). In addition, the effect of miR-24 on the paclitaxel-regulated migration of MCF-7/PR cells was also detected. Scratch test revealed that the inhibitory effect of 10 µM paclitaxel on the migration of MCF-7/PR cells was not as obvious as that observed in normal MCF-7 cells (Fig. 2C). However, in accordance with the results of the apoptotic experiments, overexpression of miR-24 improved the inhibitory effect of paclitaxel on the migration of MCF-7/PR cells (Fig. 2D).

The above results demonstrated that upregulating miR-24 expression did not affect normal MCF-7 cells survival (Fig. 1D). Thus, it was speculated that miR-24 may increase the sensitivity to paclitaxel of MCF-7/PR cells through regulating efflux pumps or reducing the metabolism of the compound. Using online microRNA target prediction tools [including TargetScanHuman 6.2 (http://www.targetscan.org/vert_71/), miRBase (http://www.mirbase.org/) and starBase (http://starbase.sysu.edu.cn/)], it was predicted that the 3′-UTR region of ABCB9 was a target of miR-24.

Figure 2. Overexpression of miR-24 increases paclitaxel-induced apoptosis and inhibition of migration in MCF-7/PR cells via regulation of ABCB9 expression. (A) Hoechst 33342 staining was used to mark apoptotic cells, and images were recorded by fluorescence microscopy (scale bar=50 µm). (B) The apoptotic-positive cells were counted and divided by the total cell number to calculate the apoptotic ratio. In total, 15 random images from six parallel wells were selected (**P<0.01 compared with control MCF-7 cells, ***P<0.001 compared with paclitaxel-treated MCF-7/PR cells, n=6). (C) The expression of apoptotic markers in different groups was determined by western blotting. β-actin was used as internal reference. All blots were repeated ≥3 times. (D) The migration of cells was investigated by scratch test (scale bar=100 µm). (E) Prediction of the miR-24 binding site on ABCB9 and ABCB9 mutation site. (F) Luciferase assay was performed to detect whether miR-24 could directly binding to the 3′-UTR region of ABCB9 (**P<0.01, ***P<0.001, n=6). (G) The messenger RNA expression of ABCB9 was determined by quantitative polymerase chain reaction (*P<0.01, n=6). (H) The protein expression of ABCB9 was determined by western blotting (**P<0.01, ***P<0.001, n=3). PR, paclitaxel-resistant; miR, microRNA; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; ABC, ATP-binding cassette; mu, mutant; UTR, untranslated region.
ABCB9 has possible binding positions for miR-24 (Fig. 2E). To confirm this concept, a miR-24 mimic and a ABCB9 3’-UTR wild type or mutated luciferase reporter were co-transfected into MCF-7/PR cells. It was observed that the miR-24 mimic decreased the luciferase activity of ABCB9 3’-UTR wild type-transfected cells, but not that of cells transfected with the mutated version (Fig. 2F). In addition, using a designed miR-24 inhibitor, ABCB9 3’-UTR wild type yielded similar results.

Figure 3. Expression of miR-24 and ABCB9 in breast cancer patients. (A and B) The expression of (A) miR-24 and (B) ABCB9 was detected by quantitative polymerase chain reaction in drug-resistant and drug-sensitive breast cancer patients (n=20). (C) The expression of ABCB9 was detected by western blotting in drug-resistant and drug-sensitive breast cancer patients (six samples were analyzed in each group). (D) Correlation analysis was performed between the expression level of miR-24 and the ABCB9 gene in breast cancer patients. miR, microRNA; ABC, ATP-binding cassette.

Figure 4. Effect of paclitaxel on miR-24-overexpressing MCF-7/PR cells in implanted nude mice. (A) Tumor growth in nude mice was determined every 2 days. *P<0.05, **P<0.01 in the MCF-7/PR-miR-24 group treated with paclitaxel compared with the control MCF-7 group; *P<0.05, **P<0.01 in the MCF-7/PR-miR-24 group treated with paclitaxel compared with the MCF-7/PR group treated with paclitaxel, n=8. (B) TUNEL assay was performed to observe the apoptotic cells. Apoptotic nuclei exhibited brown staining, while all nuclei were stained by hematoxylin with blue dye. Scale bar=100 μm. (C) The apoptotic ratio was calculated as the number of TUNEL-positive cells divided by the total cell number. *P<0.05, **P<0.01, n=8. PR, paclitaxel-resistant; miR, microRNA; P, passage; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.
compared with the mutated version (Fig. 2F). The expression of ABCB9 was next determined at both the mRNA and protein level by qPCR and western blot analysis, respectively. It was observed that the expression of ABCB9 increased significantly in MCF-7/PR cells in comparison with that in normal MCF-7 cells, but miR-24 mimic reduced ABCB9 expression at both the mRNA and protein level (Fig. 2F-H). Meanwhile, miR-24 inhibitor could abolish this effect.

**Expression of miR-24 and ABCB9 in drug-resistant breast cancer patients.** As miR-24 and ABCB9 are important in paclitaxel resistance of breast cancer cells, the expression level of miR-24 and ABCB9 in PR breast cancer patients was examined. Fig. 3A indicates that the miR-24 level was significantly decreased in tumor tissues from breast cancer patients with poor response to paclitaxel compared with that observed in tumor tissues from patients with good drug sensitivity. On the contrary, the expression of ABCB9 in the drug-resistant group was significantly higher than that in patients with good drug sensitivity (Fig. 3B). This result was also verified by western blotting (Fig. 3C). Furthermore, correlation analysis revealed that the expression of miR-24 was negatively correlated with the expression of ABCB9 (Fig. 3D), which supported the evidence that miR-24 downregulated the expression of ABCB9, thereby increasing drug sensitivity.

**Overexpression of miR-24 increases the sensitivity of paclitaxel to MCF-7/PR cells in vivo.** The effect of miR-24 on the sensitivity toward paclitaxel of MCF-7/PR cells in vivo was next investigated using a nude mouse model. Using a miR-24 mimic, a high expression level of miR-24 could be sustained for >2 weeks (data not shown). It was observed that 10 mg/kg paclitaxel could significantly reduce tumor growth in MCF-7-implanted nude mice (Fig. 4A). However, paclitaxel at this dose was not able to affect the growth of tumors derived from MCF-7/PR cells (Fig. 4A). On the contrary, when MCF-7/PR cells were pre-transfected with a miR-24 mimic, the anti-tumor effect of 10 mg/kg paclitaxel was partially recovered (Fig. 4A). Apoptosis in tumor tissues was detected by TUNEL assay. Following 2 weeks of treatment, 10 mg/kg paclitaxel could markedly induce tumor cell apoptosis (Fig. 4B and C). In accordance with the results of the experiment performed in vitro, paclitaxel induced less apoptosis in MCF-7/PR cell-implanted tumor tissue than that in the tissues of normal MCF-7 cell-implanted nude mice (Fig. 4B and C). Furthermore, pre-transfection with the miR-24 mimic could recover the pro-apoptotic effect of paclitaxel in vivo.

**Discussion**

Drug resistance in breast cancer has been regarded as one of the primary obstacles contributing to chemotherapy failure (22). Thus, it is necessary to explore novel therapeutic methods to reduce drug resistance for the treatment of breast cancer. In the present study, it was observed that the expression of miR-24 was significantly reduced in tumor tissues from PR breast cancer patients and PR breast cancer cell lines. Further in vitro experiments confirmed that upregulating miR-24 expression could partially recover the sensitivity to paclitaxel of breast cancer cells. Therefore, miR-24 may be a useful target to reduce drug resistance and a possible marker to guide clinical medication.

miR-24 has been reported to be an oncogene in oral carcinoma (23), prostate cancer (24) and lung cancer (25). In addition, miR-24 could promote cell proliferation in lung cancer (25) and hepatocellular carcinoma (26). However, miR-24 could inhibit cell proliferation in gastric cancer (27,28), and acts as a tumor suppressor in colon cancer (29). In breast cancer, the role of miR-24 also remains controversial. The expression of miR-24 in serum from early breast cancer patients was decreased in a previous study (30), but another study using paraffin-embedded tissues observed that the expression of miR-24 was upregulated (31). In addition, Du et al. reported that the expression of miR-24 was higher in breast cancer samples than that in benign breast tissues, and speculated that miR-24 may enhance tumor invasion (32). Therefore, as an important microRNA associated with cancer development, the role of miR-24 in breast and other cancers should be further investigated.

Using microRNA target prediction tools, the present authors identified that ABCB9 could be one of the target genes of miR-24. ABCB9 is a member of the ABC sub-family B, which has been reported to be involved in drug intracellular trafficking, thereby affecting chemotherapy-related multidrug resistance (MDR) (33,34). Thus, it was speculated that miR-24 may affect the expression of ABCB9 to regulate the sensitivity toward paclitaxel of breast cancer cells. It was identified that miR-24 could directly bind to the 3′-UTR of ABCB9, thereby inhibiting the translation of ABCB9. These results were in accordance with those from previous studies (35,36). Overall, the above results indicated that downregulation of ABCB9 may contribute to the effect of miR-24 on the sensitivity toward paclitaxel of breast cancer cells. Furthermore, the present study confirmed that ABCB9 was also upregulated in chemotherapy-resistant breast cancer patients, which was negatively correlated with the level of miR-24, suggesting that miR-24 was associated with drug resistance in breast cancer via regulating ABCB9 expression. Unlike other members of the ABC transporter protein family, the regulatory effect of ABCB9 on drug resistance has not been frequently reported (34,36). The present study provided novel evidence regarding the role of ABCB9 in chemotherapy-related MDR. Furthermore, the role of miR-24 in PR breast cancer cells was evaluated in vivo using a nude mouse model. It was observed that miR-24 increased the sensitivity to paclitaxel of MCF-7/PR-implanted nude mice. Although multiple mechanisms may be involved, including p450-related drug metabolism and various β-tubulin mutations, which require further investigation in miR-24-associated drug resistance, the present results suggested that miR-24 could be important in drug resistance of breast cancer cells, and could improve the sensitivity toward paclitaxel both in vitro and in vivo.

In conclusion, the present study demonstrated for the first time that miR-24 improves the sensitivity toward paclitaxel of breast cancer cells, suggested an important novel function for miR-24, and provided a possible microRNA target and a useful marker in the clinical treatment of breast cancer.

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