MiR-489 regulates chemoresistance in breast cancer via epithelial mesenchymal transition pathway

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ABSTRACT

To investigate the role of microRNAs in the development of chemoresistance and related epithelial–mesenchymal transition (EMT), we examined the effect of miR-489 in adriamycin (ADM)-resistant human breast cancer cells (MCF-7/ADM). MiR-489 was significantly suppressed in MCF-7/ADM cells compared with chemosensitive parental control MCF-7/WT cells. Forced-expression of miR-489 reversed chemoresistance. Furthermore, Smad3 was identified as the target of miR-489 and is highly expressed in MCF-7/ADM cells. Forced expression of miR-489 both inhibited Smad3 expression and Smad3 related EMT properties. Finally, the interactions between Smad3, miR-489 and EMT were confirmed in chemoresistant tumor xenografts and clinical samples, indicating their potential implication for treatment of chemoresistance.

Keywords:
miRNAs
Chemoresistance
Breast cancer
Epithelial mesenchymal transition

1. Introduction

Breast cancer is the leading cause of cancer death among females. Chemotherapy is frequently used for breast cancer treatment, but the major impediment to success is the development of chemoresistance [1–3]. Various factors may cause chemoresistance, and recent studies have indicated that epithelial–mesenchymal transition (EMT) is intensively involved. EMT is an important process that plays key roles in embryonic development, as well as in cancer and other diseases [4–7]. During the acquisition of EMT characteristics, epithelial cells gain mesenchymal features by losing polarity and cell–cell contacts, together with dramatic remodeling of the cytoskeleton [8], a process that endows the epithelial cells with high motility. Cancer epithelial cells have been found to hijack the EMT pathways to develop chemoresistance [9,10]. Cancer cells are able to manipulate the expression of genes that activate the EMT process, such as decreasing the expression of E-cadherin (E-cad), as well as increasing the expression of vimentin (VIM) and N-cadherin. The EMT process provides cancer cells with enhanced abilities for migration and invasion [11,12]. The increase of cell motility by the EMT process thus enables cancers to be resistant to anoikis, as well to escape from immune surveillance and chemotherapeutic drugs, finally developing chemoresistance and inducing metastasis [13,14]. Therefore, the development of EMT inhibitors may provide novel strategies for the prevention, diagnosis, and treatment of cancers.

MicroRNAs (miRNAs) constitute another important factor in the development of chemoresistance in cancer cells [15,16]. miRNAs are endogenously-expressed non-coding RNAs that target the 3′ untranslated regions (3′UTRs) of mRNAs for cleavage or the inhibition of translation [17]. Recent studies have shown that miRNAs interact intensively with EMT to develop chemoresistance. Bryant et al. [18] identified 13 miRNAs that participate in the development of chemoresistance to anti-epidermal growth factor receptor (EGFR) therapy in non-small-cell lung cancer cells; these miRNAs are also involved in EMT signaling in cancer cells. Among those miRNAs, miR-200c (or the miR-200 family) is one of the most extensively studied miRNAs associated with both chemoresistance and EMT in various kinds of cancer cells [19]. However, to date the list of miRNAs linked both to EMT and chemoresistance is small and the underlying mechanisms remain...
unclear. In this study, we reported that miR-489 mediated EMT-related chemoresistance in breast cancer cells. MiR-489 was down-regulated in adriamycin (ADM)-resistant human breast cancer cells (MCF-7/ADM) compared with parental control cells (MCF-7/WT). Re-expression of miR-489 reversed the chemoresistance and EMT features of these cells by targeting Smad3. In xenograft tumors and clinical samples, Smad3, miR-489, and EMT biomarkers were correlated with chemoresistance. Our novel findings provide a potential target to overcome chemoresistance by miRNA via the EMT pathway.

2. Materials and methods

2.1. Ethics statement

All animal experiments were performed in accordance with the laboratory animal guidelines and with approval from the Animal Experimentations Ethics Committee, Jiangnan University. The study using clinical samples was approved by Institutional Review Board at the affiliated hospital, Jiangnan University.

2.2. Cell culture and reagents

MCF-7/WT (human breast cancer) cells were purchased from the ATCC. Adriamycin (ADM)-resistant (MCF-7/ADM) cells were derived by treating MCF-7/WT cells with stepwise increasing concentrations of ADM over 8 months. All cells were maintained in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) with 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were cultured in triplicate, and data are presented as mean ± standard error of the mean (S.E.M.).

2.3. Clinical samples collection and in situ hybridization (ISH) of miRNA

All procedures were conducted with the approval of the Ethics Committees of the University and the Hospital. Tissue samples were collected from breast cancer patients in The Affiliated Hospital of Jiangnan University (Wuxi, China). Samples were embedded in TissueTek OCT (Sakura, Japan) and stored at −80 °C.

In situ hybridization (ISH) was performed using the locked nucleic acid (LNA)-modified oligonucleotide probe (Biolink, China). Fixed cultures were incubated with 0.1 mM PBS, permeabilized with 0.4% Triton X-100, and treated with 1 µg/ml proteinase K (Beyotime, China). The cells were washed with 4% (v/v) parafomaldehyde for 5 min to inhibit the effects of the proteinase. To reduce non-specific signals, cells were washed on 1× SSC for 15 min at 37 °C, and once with NTE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, 20 µg/ml RNase A) for 30 min at 37 °C to digest the single-stranded RNA, then rinsed once with 1× SSC and 0.5× SSC (15 min each) at 37 °C, and finally washed twice with Buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5) for 10 min. All ISH images of miR-489 were captured by a confocal laser scanning microscope (Leica TCS SP8, Germany).

2.4. Transfection of miRNA

MCF-7/ADM cells were resuspended in Opti-MEM I medium (Invitrogen, USA) with 400 nM agomiR-489 (mimic) or agomiR-control (negative control). After incubation at 37 °C under 5% CO2 for 6 h, RPMI 1640 supplemented with 10% FBS was added 3-fold and cells were cultivated as described above. Then culture was continued for 48 h for assay.

2.5. Reverse transcription and quantitative real-time PCR

Total RNA was prepared using Trizol (Beyotime, China) according to the manufacturer’s instructions. A CDNA library was generated using a Reverse Transcriptase M-MLV (TakaRa, Japan). The cDNA was used for the amplification of genes of E-cadherin, vimentin, and β-actin. The reverse transcription primer pairs for E-cadherin (E-cad) were forward 5'-gcctgtagattaacggcagca-3', reverse 5'-accacccctaaggcactct-3', vimentin (Vim) were forward 5'-ggctcagatccgaaagacgac-3', reverse 5'-gttcatccacgccgaaattctc-3', β-actin were forward 5'-tgccacacgggagcata-3', reverse 5'-ttcacgtggtttgag-3'. Reverse transcription data were analyzed with ImageJ software (NIH, USA), and β-actin was used as an endogenous control. For mature miRNA quantification, cDNA was synthesized using Stem-loop RT primers and a Hairpin-it microRNA qPCR Quantitation Kit (GenePharma, China). U6 small nuclear RNA (snRNA) was used as an internal control. The relative expression level of miR-489 in each sample was calculated using the comparative expression level 2^(-ΔΔCt) method [20] and normalized to the expression of U6 snRNA. All qRT-PCRs were performed in triplicate, and data are presented as mean ± standard error of the mean (S.E.M.).

2.6. Western blot analysis

Whole-cell lysates of treated cells were prepared and separated on 10% SDS–PAGE. Membranes were immunoblotted against Smad3. The antigen–antibody complexes were visualized using an enhance chemiluminescence detection system. The expression of Smad3 was quantified by ImageJ software for gray value, and normalized to β-actin.

2.7. Immunofluorescence staining

Cells were fixed with 4% (m/v) parafomaldehyde, permeabilized with 0.1% TritonX-100, and blocked with 5% BSA. Then the cells were stained with primary antibody and subsequently with Alexa Fluor 488 conjugated donkey anti-mouse secondary antibody (Beyotime, China), and Alexa Fluor 546 secondary antibody (Invitrogen, USA).

2.8. Migration assays

For motility assays, 10^5 cells were added to the top chamber of uncoated PET membranes in 24-well Corning plates. Medium supplemented with 10% FBS was added to the lower chamber of each well as a chemoattractant. Cells were incubated for 48 h, and those that did not migrate through the filter (on the upper surface) were removed with a cotton swab. Cells that had migrated to the lower surface of the filter were fixed with 4% (m/v) parafomaldehyde and stained with crystal violet. Migrated cells were counted under a microscope. Images were captured by a video camera (Nikon Coolpix 54, Japan) mounted on the microscope (Leica CME, Germany).
2.9. Xenograft tumors

Twenty female nude mice (4–6 weeks old) were purchased from model animal research center of Nanjing University (Nanjing, China), housed under aseptic conditions, and cared for in accordance with the guidelines of the Laboratory Animal Unit of Jiangnan University. Tumor xenografts were established by subcutaneous injecting MCF-7/WT or MCF-7/ADM cells into the back flanks of mice ($5 \times 10^6$ cells per mouse). Tumor sizes were measured every 3 days until reached $\sim 200 \text{ mm}^3$.

2.10. Statistical analysis

Results are presented as mean ± S.E.M. Statistical differences were determined by Student's $t$-test. We performed statistics analyses using Graphpad Prism 5.0 software. All statistical tests were two-tailed, and a value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. MiR-489 is involved in chemoresistance in breast cancer cells

We established the MCF-7/ADM cell line as described in our previous study [21,22]. MCF-7/ADM cells displayed a 630-fold greater resistance to ADM than the parental line MCF-7/WT. In previous studies, we used high-throughput small-RNA sequencing to compare MCF-7/WT and MCF-7/ADM cells (unpublished data) and identified miR-489 was one of the most markedly changed miRNAs. We then used qRT-PCR (Fig. 1A) and in situ hybridization (ISH) (Fig. 1B) to validate the expression of miR-489; this showed that miR-489 was significantly down-regulated in MCF-7/ADM cells compared to MCF-7/WT cells. With transfection of agomir-489, which mimics endogenous miRNA, qRT-PCR showed that MCF-7/ADM cells transfected with miR-489 agomir expressed approximately 600-fold more miR-489 compared to those transfected with negative controls (Fig. 1C). MTT assay showed that after agomir-489 transfection, MCF-7/ADM cells became more sensitive to ADM treatment (Fig. 1D), indicating the potential of miR-489 to reverse drug resistance.

3.2. Smad3 is the target of miR-489

Smad3 was predicted as a potential target by TargetScan software (www.targetscan.org) and the luciferase reporter assay was used to verify the binding. The 3’UTR region of Smad3 mRNA was cloned into luciferase reporter vector (pLUC). HEK293T cells were then transfected with miR-489 together with the luciferase reporter vector and the luciferase activity was assessed. The results demonstrated that miR-489 suppressed the expression of luciferase and thus targeted the 3’UTR of Smad3 (Fig. 2A).

We then examined the interaction between Smad3 and miR-489 in MCF-7/ADM cells. Western blot results showed that Smad3 was up-regulated in MCF-7/ADM cells compared to MCF-7/WT cells (Fig. 2B), while forced expression of miR-489 in MCF-7/ADM cells significantly decreased Smad3 expression (Fig. 2C and D).

3.3. MiR-489 regulates EMT in breast cancer cells

Because Smad3 plays an important role in EMT [23,24], and we found that MCF-7/ADM cells displayed mesenchymal-like markers, such as the high expression of vimentin, the absence of E-cadherin, and high migratory ability (Fig. 3A and B), suggesting EMT-like features. We then investigated whether the down-regulation of miR-489 was involved in the gaining of EMT properties. For this
purpose, MCF-7/ADM cells were transfected with agomir-489, and the results showed that restoring the activity of miR-489 decreased the expression of vimentin and increased the expression of E-cadherin at the RNA and protein levels (Fig. 3C and D). As a result, miR-489 significantly inhibited the migration of MCF-7/ADM cells (Fig. 3E).
3.4. MiR-489 is associated with EMT features and chemotherapeutic response in human breast tumor xenografts in athymic nude mice and in clinical samples

After validating the gene/miRNA expression changes in MCF-7/WT and MCF-7/ADM cells, we assessed gene/miRNA expression in nude mice bearing MCF-7/WT and MCF-7/ADM xenograft tumors. Immunohistochemistry showed low E-cadherin and abundant vimentin (Fig. 4A) and Smad3 (Fig. 4B) in MCF-7/ADM xenograft tumors compared with MCF-7/WT tumors. In situ hybridization showed that miR-489 expression was lower in MCF-7/ADM (Fig. 4B) than in MCF-7/WT xenograft tumors. These results correspond to the gene/miRNA expression in cells.

To further verify our results, we assessed the expression and correlation of miR-489, Smad3, E-cadherin, and vimentin in clinical samples of breast cancers. Tissues from patients receiving anthracycline–taxane-based neoadjuvant chemotherapy were collected. Tumor masses of which the volume did not significantly decrease after neoadjuvant therapy (i.e. from non-responders) were considered to be chemoresistant. Post-chemotherapy samples were compared to identify the gene/miRNA expression changes in chemoresistant and chemosensitive cases. Results showed that miR-489 was down-regulated, while Smad3 was up-regulated in non-responders compared to responders (Fig. 5A). At the same time, we found that low miR-489 expression and high-Smad3 expression were highly correlated with the EMT features of low E-cadherin (Fig. 5B) and high vimentin expression in the samples from non-responders.

4. Discussion

ADM (adriamycin)-based chemotherapy is one of the standard protocols for treating breast cancers, but ADM resistance occurs often and results in failure. It is becoming increasingly evident that miRNAs are critical modulators of chemoresistance and EMT in many types of cancers. A few reports have revealed roles of miRNAs in ADM resistance, in which EMT was also implicated. For example, Chen et al. [25] reported that an EMT modulator, miR-200c, is down-regulated in ADM-resistant MCF-7 cells by interrupting the normal expression of p-glycoprotein (p-gp), which is a well-known membrane protein that pumps out chemotherapeutic drugs [26]. More recently, it was shown that miR-34a regulates the chemosensitivity of breast cancer cells to ADM by targeting Notch1 [27], which is a key activator in the EMT pathway [28]. However, none of these studies clearly addressed the co-regulation of EMT and ADM-resistance by miRNAs. Furthermore, because both EMT and miRNAs are essential for the development of chemoresistance, it is important to find more miRNAs involved in both chemoresistance and EMT in cancer cells.

We found that miR-489 was down-regulated in MCF-7/ADM compared with MCF-7/WT cells, and that restoring its activity decreased chemoresistance, suggesting a role of miR-489 in chemoresistance. MiR-489 has already been reported to be down-regulated in squamous cell carcinoma. A recent study by Kikkawa et al. [29] described the tumor-suppressive effects of miR-489 in squamous cell carcinoma, where they showed that miR-489 inhibited cell growth in all head and neck cancer cell-
In a search for potential targets of miR-489 that might be involved in the resistance to ADM and the EMT-like phenotype in MCF-7/ADM cells, we identified Smad3 as a candidate gene. MiR-489 directly targeted Smad3, in that down-regulation of miR-489 expression led to high expression of Smad3 in MCF-7/ADM cells. Smad3 has been shown in a number of studies to be involved in EMT progression [30]. Smad3 was found to be critical for TGF-β-induced EMT and can mediate the invasion of mammary epithelium [31]. In response to TGF-β, Smad3 is essential to facilitate the function of Snail1 by forming a complex with it, the complex then plays as a transcriptional repressor in EMT signaling and directly targets the E-cadherin expression [32]. It was already extensively found interrupting the Snail1 pathway effectively affected the EMT pathway [33], so activation of Smad3 by miR-489 must increase EMT, and then inhibit chemoresistance. Indeed, in our model of MCF-7/ADM cells, which displayed some mesenchymal features and high expression of Smad3, we found that disrupting the high activity of Smad3 by restoring the miR-489 activity not only reduced the chemoresistance, but also decreased the EMT features, including (1) an enhanced expression of E-cadherin which is a proved target of Smad3 [32], the increase in E-cadherin fastened the cell–cell contact of epithelial MCF-7/ADM cells and decreased their migration; (2) a losing of vimentin which is a major cytoskeletal component of mesenchymal cells, diminished vimentin changed mesenchymal cell shape and motility, as well as decreased the migration [34]. Therefore, our data suggest the miR-489–Smad3 signaling pathway mediates chemoresistance to ADM through EMT.

To further confirm our results, we constructed a xenograft model of nude mice to study the EMT features, miR-489, and Smad3 expression in human breast tumor xenografts. EMT markers and Smad3 expression were found together with low miR-489 in MCF-7/ADM xenograft tumors, which were more easily formed than those by MCF-7/WT, as we showed previously. Therefore, these data not only showed the connection between EMT features, miR-489, and Smad3, but also revealed that miR-489 and Smad3 may participate in tumorigenesis, a critical step for metastasis.

To extend our findings into chemoresistance to other type of drugs used clinically, we examined the expression of miR-489, Smad3, and several EMT markers in breast cancer samples from patients who had undergone anthracycline–taxane-based neoadjuvant chemotherapy. Our result also showed a strong correlation of miR-489–Smad3 with chemoresistance and EMT, suggesting the general importance of miR-489 in chemoresistance.

In summary, we showed that loss of miR-489/gain of Smad3 is a potential modulator of both chemoresistance and EMT-like properties in breast cancers. Therefore, re-expression of miR-489 or inhibition of Smad3 might be potential therapeutic approaches for the treatment of chemoresistant breast cancers.

**Acknowledgements**

We thank Prof B Jiang, J Xu, KY Lu in the State Key laboratory of Food Science and Technology, Jiangnan University for insightful comments and technical assistance. This work was supported by the Program for New Century Excellent Talents in University of The Ministry of Education of China NCET-12-0880 (to X.M.); Fundamental Research Funds for the Central Universities JUS-
RP51311A (to X.M. & J.J.); China National Natural Science Foundation grants 81100185 (to X.M.), 81273437 (to J.J.), 31200126 and 31371317 (to D.X.H.); NSFC-RGC joint grant 81361168001 (to J.J.).

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