MicroRNA-224 modulates chemosensitivity of breast cancer cells to docetaxel by apoptosis inhibitor 5

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Summary

Purpose: The view that microRNA-224 (miR-224) may lead to tumorigenesis has been accepted in many studies. However, its role remains unclear in modulating the chemosensitivity of breast cancer cells to docetaxel (DOC). Thus, the aim of this study was to estimate what's the role of miR-224 in the chemosensitivity of breast cancer cells to DOC.

Methods: The role of miR-224 in breast cancer cells was analyzed using CCK-8 assay, real-time PCR, flow cytometry assay and Western blot. Dual-luciferase reporter assay and API-5-siRNA technology were performed to analyze the association between miR-224 and Apoptosis inhibitor 5 (API-5).

Results: Overexpression of miR-224 could significantly decrease the chemosensitivity of MCF-7 breast cancer cells to DOC. The luciferase activity of MCF-7/DOC cells containing wild-type 3'UTR of API-5 could be inhibited by miR-224 mimics. Similarly, the chemoresistance of MCF-7 cells to DOC induced by miR-224 mimics could be partially reversed by API-5-siRNA.

Conclusion: An inverse association between miR-224 and API-5 in breast cancer cells was revealed. Dysregulation of miR-224 plays a vital role in the acquired DOC resistance of breast cancer and at least partially via targeting API-5.

Key words: API-5, breast cancer, chemosensitivity, docetaxel, microRNA-224

Introduction

The fact that breast cancer has almost been a leading cause of death in women in nearly all countries is undeniable [1]. Also, a considerable percentage of patients with breast carcinoma have poor response to chemotherapy, thereinto, multi-drug resistance of tumor cells towards anticancer drugs is the major reason for chemotherapy failure. Docetaxel (DOC) is a common chemotherapeutic drugs; but unfortunately, as with many other anti-cancer drug, chemoresistance induced by DOC results in treatment failure in breast cancer patients. Current findings have revealed that the acquisition of chemoresistance may be closely related with abnormal regulation in miR levels. MiRs are small non-coding RNAs and serve as master regulators in many important biological processes. It was indicated that miR-222 and miR-29a played an important role in breast cancer cells...
via PTEN [2], while in colorectal carcinoma, down-regulation of miR-224 was suggested to have close association with the sensitivity of tumor cells to the antifolate methotrexate [3]. However, little is known about the role of miR-224 in breast cancer. Current studies have shown that miR-224 increased the proliferation of epithelial ovarian cancer cell lines through KLLN [4] and that the overexpression of miR-224 played crucial roles in metastasis of cancer cells by suppressing the PKP1 [5]. Additionally, Lee et al reported that miR-224 regulated API-5 expression via binding to 3' UTR of API-5 in hepatocellular carcinoma [6]. Lu et al confirmed that miR-224 and miR-145 affected their target proteins STAT-1 and API-5 in systemic lupus erythematosus (SLE) [7]. API-5 is a 55 kDa protein with anti-apoptotic signaling in cancer cells.

Our study aimed to show that miR-224 expression modulated chemoresponse of breast cancer to DOC by targeting API-5 and that API-5 signaling pathway might play an indispensable role in the acquisition of chemotherapeutic agents resistance of breast cancer cells.

**Methods**

**Cell culture**

The human breast cancer cell line MCF-7 was obtained from Harvard University, while the docetaxel-resistant MCF-7 cells (MCF-7/DOC) was purchased from AiBiological Research Co. Ltd (Shanghai, China). Both of them were cultured in DMEM with high glucose (Gibco, Grand Island, NY, USA) and 10% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified atmosphere containing 5% CO₂.

**Quantitative real-time polymerase chain reaction (qRT-PCR) assay**

Total RNA was extracted from MCF-7 and MCF-7/DOC cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Then, total RNA was reverse-transcribed to cDNA using the PrimeScript RT Master mix (Takara, Kumamoto Prefecture, Kyushu, Japan). The SYBR Green PCR Master Mix (Applied Biosystems, CA) and an ABI 7300 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA) were used in qRT-PCR. The miR-224 sequence-specific reverse transcription-PCR primers were purchased from GenePharma Co. Ltd, Shanghai, China and the internal control RNU6B was used following operating instructions. The other primers for API-5 and β-actin in real-time PCR reaction were purchased from GenePharma Co., Ltd as well. All cycling parameters that needed to be detected were followed as protocol described. Each sample above was analyzed in triplicate. Ultimate detection results were analyzed by comparing CYCLE THRESHOLD (Ct) values.

**MicroRNA target gene identification and microRNA microarray**

TargetScan and PicTar were used to identify the microRNA target genes. FlashTag RNA Labeling Kit (GENISPHERE) was used to label the RNA and all the operation was done according to Affymetrix manufacturer’s protocol. The last results were treated by miR QC tool software (Affymetrix).

**MicroRNA-224 transfection**

MiR-224 mimics (5'-CAAGUCACUAGGGUUC-GGUU-3', 5'- CAGAACCACUAGACCUUGU-3'), miR-224 inhibitor (5'-AAGGAACACUAGUUGACUUG-3') and mimics control (5'- UUCCGAACGUGUCACGUU-3', 5'-ACGGACCCGUUCCGAGATA TT-3'), inhibitor control (5'-AGGUGACCAUUGCGGAATT-3') were synthesized by GenePharma Co., Ltd (Shanghai, China). MiR-224 was overexpressed in MCF-7 sensitive cells using miR-224 mimics, while in MCF-7/DOC cells, miR-224 was knocked down using miR-224 inhibitor. These cells were plated into 6-well plates at a density of 2.0×10⁵ cells/well 24 h before transfection. Then, miR-224 mimics or inhibitor were used according to the manufacturer’s protocol to transfect the cells by using Lipofectamine 2000 (Invitrogen). After 48 h, the transected cells were harvested for further analysis. All the experiments were repeated three times.

**Cell survival analysis**

MCF-7 sensitive (MCF-7/S) cells and MCF-7/DOC cells were transfected with miR-224 mimics or inhibitor, respectively. Then, the cells with transfection were seeded into 96-well plates and treated with DOC at different concentrations for 36 h. CCK-8 assay was performed to analyze the cell viability rate. Each group had three replicates. 10 μL of CCK-8 (Dojindo, Kumamoto Prefecture, Kyushu, Japan) per well was added and incubated for 4 h at 37°C. Then, 100 μL DMEM was added to each well and the absorbance per well was measured at 450 nM on a microplate reader.

**Apoptosis assay**

MCF-7/S and MCF-7/DOC cells transfected with miR-224 mimics or inhibitor were incubated with 40 μM and 0.5 μM DOC for 24 h, respectively. Then, a total of 1.0×10⁵ cells with transfection were collected, washed twice with ice-cold phosphate buffered solution (PBS) and transferred into a tube containing 5 μL Annexin V and 1 μL propidium iodide (PI). Next, the cells were incubated for 15 min at room temperature in the dark and then were analyzed using flow cytometry.

**Western blot**

Protein expression was examined by Western Blot following a standard procedure. Cells were collected and then concentrated to obtain proteins. OneDrop OD-1000+ Spectrophotometer (Shanghai, China) was used to estimate the protein concentrations. Then SDS-PAGE buffer (Beyotime) was used to mix the proteins and the mixture was subsequently boiled for 5 min. Then, the proteins

JBUON 2021; 26(2): 451
that had been boiled were transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA), blocked by non-fat milk, and incubated with antibodies against API-5 (Dako, Japan). The protein bands were visualized using ECL detection reagent (Millipore, Billerica, MA, USA). β-actin was used as internal loading control. Three experiments without interference were performed.

Luciferase reporter assay for targeting API-5-3' untranslated region

To validate whether the API-5 gene was an exact target of miR-224, the 3'-untranslated region (UTR) segment of API-5 gene was first amplified by PCR. Then, HEK293 cells were transfected with firefly luciferase UTR-report vector, control luciferase pRL-TK vector and precursor miR-224 for the API-5-3'UTR construct according to EndoFectin Lenti protocol (GeneCopoeia). Then, HEK293 cells that have transfected were seeded into 6-well plates with 2.7×10⁵ cells per well. 48 h after transfection, cells were collected and firefly luciferases were assayed using dual-luciferase reporter assay system according to GeneCopoeia Luc-Pair miR Luciferase Kit protocol (GeneCopoeia).

Statistics

SPSS 20.0 statistical package was used for analyses. Correlation between groups was analyzed by Spearman’s rank test. Differences between groups were analyzed using Kruskal-Wallis test and Kaplan-Meier method and compared with log-rank test. All the tests were two-tailed and p<0.05 was considered statistically significant.

Results

Inverse association of miR-224 and API-5 in MCF-7 and MCF-7/DOC

The half maximal inhibitory concentration (IC₅₀) of DOC in sensitive MCF-7 was 65.1593±5.73470 nM, while in resistant MCF-7/DOC it was 479.3140±39.27063 nM (p<0.05, Figure 1A). MiR-224 was significantly upregulated in MCF-7/DOC compared with its parental MCF-7 by miR microarray analysis. The above results were confirmed by the qRT-PCR (p<0.05, Figure 1B). Then, for further validation, API-5 was chosen to investigate its expression level in MCF-7/S and resistant MCF-7/DOC by Western blot analysis and qRT-PCR. In MCF-7/DOC, the expression of API-5 mRNA was decreased compared to MCF-7 (p<0.05, Figure 1C). API-5 protein expression was downregulated significantly in MCF-7/DOC compared with that in MCF-7 (Figure 1D).

MiR-224 regulates the expression of API-5

API-5 was known to be targeted by miR-224, which was significantly decreased in MCF-7/DOC.

Figure 1. Expression of miR-224 and API-5 in MCF-7 and MCF-7/DOC breast cancer cells. A: IC₅₀ of DOC in MCF-7 and MCF-7/DOC breast cancer cells was 65.1593±5.73470 nM and 479.3140±39.27063 nM, respectively (p<0.05). B,C: Expression of miR-224 and API-5 in MCF-7 and MCF-7/DOC cells by RT-PCR assay, respectively. D: Expression of API-5 protein by western blot assay. All the experiments were performed in triplicate.
cells but increased in MCF-7 cells. However, whether overexpression of miR-224 induced downregulation of API-5 was associated with DOC resistance remains unclear. In order to investigate whether the expression of API-5 was downregulated by miR-224, miR-224 inhibitor and mimics were respectively transfected into MCF-7/DOC and MCF-7, and the expression of miR-224 was determined by qRT-PCR 48 h after transfection. The relative level of miR-224 expression in MCF-7 cells transfected with miR-224 mimics was increased 7.6-fold (p<0.05, Figure 2A) when compared with control MCF-7. The relative level of miR-224 expression in miR-224 inhibitor-transfected MCF-7/DOC cells was decreased to 53% (p<0.05, Figure 2B) when compared with control MCF-7/DOC cells. Likewise, after transfection for 48 h, the mRNA expression levels of API-5 were determined by quantitative RT-PCR assay. The relative level of mRNA API-5 expression in MCF-7 cells transfected with miR-224 mimics were decreased significantly compared with that in control MCF-7 (p<0.05, Figure 2C). Conversely, the mRNA expression of API-5 in MCF-7/DOC cells transfected with miR-224 inhibitor was increased significantly compared with that in MCF-7/DOC cells transfected with control oligonucleotide (p<0.05, Figure 2D). Furthermore, western blot was used to detect the expression of API-5 protein. As shown in Figure 3A, the API-5 protein level in MCF-7 transfected with miR-224 mimics (MCF-7/mimics) cells was decreased significantly compared with that in control cells. And the result in MCF-7/DOC cells transfected miR-224 inhibitor (MCF-7/DOC/inhibitor) cells was just the opposite, i.e. the level of API-5 protein expression in MCF-7/DOC/inhibitor cells was increased significantly compared with that in MCF-7/DOC/control cells (Figure 3B). These results suggested that API-5 was transcriptionally regulated by miR-224 in MCF-7 breast cancer cells.

Influence of miR-224 on the sensitivity of MCF-7 breast cancer cells to DOC

To test the association between the expression of miR-224 and the sensitivity of breast cancer cells to DOC, the IC\textsubscript{50} value of DOC in MCF-7/mim-

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![Figure 2](image_url)

**Figure 2.** MiR-224 regulates the expression of API-5. A: Expression of miR-224 in MCF-7 breast cancer cells transfected with control mimic or miR-224 mimic (p<0.05). B: Expression of miR-224 in MCF-7/DOC breast cancer cells transfected with control inhibitor or miR-224 inhibitor (p<0.05). C,D: Real-Time PCR was used to detect API-5 mRNA expression in MCF-7 and MCF-7/DOC breast cancer cells transfected with miR-224 mimic and inhibitor, respectively (p<0.05). All the experiments were performed in triplicate.
Figure 3. A: The expression of API-5 protein in MCF-7 breast cancer cells transfected with miR-224 mimic and miR-224 negative control by Western Blot assay. The Figure shows that miR-224 was inversely correlated with the expression of API-5. B: The expression of API-5 protein in MCF-7/DOC cancer cells transfected with miR-224 inhibitor and negative control. The Figure shows that upregulated miR-224 downregulated the API-5 expression and decreased the sensitivity of MCF-7 cells to DOC.

Figure 4. Effect of miR-224 expression on the sensitivity of MCF-7 and MCF-7/DOC breast cancer cells to DOC. A: The IC\textsubscript{50} value of DOC was determined by CCK-8 assay after MCF-7 breast cancer cells were transfected with miR-224 mimics or control mimic for 24 h (p<0.05). B: The IC\textsubscript{50} value of DOC was determined by CCK-8 assays after MCF-7/DOC breast cancer cells were transfected with miR-224 inhibitor or control (p<0.05). All experiments were performed in triplicate.

Figure 5. The apoptotic rate of breast cancer cells induced by DOC in MCF-7 and MCF-7/DOC cells transfected with miR-224 mimic, inhibitor and their corresponding negative controls by flow cytometry assay, respectively. A: MCF-7 breast cancer cells were treated with DOC with a final concentration of 40\,\mu\text{M} at 24 h post-transfection, and after 36 h of incubation the cells were collected for analysis by flow cytometry assay. The Figure shows that upregulation of miR-224 expression decreased the sensitivity of MCF-7 cells to DOC. B: DOC was added into MCF-7/DOC cancer cells with a final concentration of 0.5 \,\mu\text{M}, and after 36 h of incubation the cells were collected for analysis by flow cytometry assay. The Figure shows that downregulation of miR-224 increased the sensitivity of MCF-7/DOC cells to DOC.
ics or MCF-7/DOC/inhibitor were determined by CCK-8 assay. The IC\textsubscript{50} value of DOC in MCF-7/mimics and control cells was 3.9267±0.27737 μg/mL and 1.3080±0.17610 μg/mL, respectively (p<0.05, Figure 4A). The results clearly suggested that up-regulation of miR-224 expression could decrease the sensitivity of MCF-7 cells to DOC. Additionally, the IC\textsubscript{50} value of DOC in MCF-7/DOC/inhibitor cells and control cells was 0.1773±0.05372 μg/mL and 0.5803±0.01950 μg/mL, respectively (p<0.05, Figure 4B). These results were an indication that down-regulation of miR-224 could increase the sensitivity of MCF-7/DOC cells to DOC. Then, all the above data proved that the miR-224 expression could significantly decrease the sensitivity of breast cancer cells to DOC.

Involvement of miR-224 in DOC-induced apoptosis

Flow cytometry was used to elucidate the cellular apoptosis. In MCF-7/mimics, the apoptotic rate was lower than that in MCF-7 control cells (p<0.05, Figure 5A). On the contrary, the results showed more apoptotic rate in MCF-7/DOC/inhibitor cells compared to inhibitor controls (p<0.05, Figure 5B). All these data revealed that in MCF-7 breast cancer cells, miR-224 decreased DOC sensitivity through inhibiting DOC-induced apoptosis.

MiR-224 targets API-5 gene in MCF-7 breast cancer cells

Two putative miR-224-binding sites in the API-5 3’-untranslated region (UTR) (position 1093, 1833) were found according to the TargetScan software (Figure 6A, 6B). To validate that API-5 was indeed a gene target of miR-224, the two putative sites that miR-224 may be binded with were

![Figure 6](image1.png)

**Figure 6.** MiR-224 directly targets API-5. A,B: The two complementary sites of miR-224 in API-5 3’UTR as predicted through bioinformatics analysis: has-miR-224 1093, 1833.

![Figure 7](image2.png)

**Figure 7.** API-5 is a direct target by miR-224. Dual luciferase activity assay in HEK293 cells with co-transfection of API-5 3’UTR-wild or 3’UTR-mutant and miR-224 mimic or miR-224 control as indicated.

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Table 1. Firefly luciferase (Fluc) and RENILLA LUCIFERASE (R luc) expression of HmiT021206-MT01
mir-224 in breast cancer

Mutated (named mutant 1 and mutant 2) and the segment of API-5-3'-UTR containing the miR-224 complementary sites was cloned into 3'-UTR of a dual luciferase reporter system and the resulting reporter vector together with transfection controls and miR-224 mimics, miR-224 inhibitor were then transfected into HEK293 cells. Figure 7 shows that the interaction between miR-224 and 3'-UTR of API-5 remarkably suppressed reporter gene activity. The inhibition ratio of HmiR0406-MR03 to target HmiT021206-MT01, mutant target CCS-HmiT021206-MT01-1 and CCS-HmiT021206-MT01-2 was 52.6%, 20.2% and 27.8%, respectively (Table 1 and 2). All the results revealed that miR-224 interacted the 3'UTR of the API-5 gene (HmiT021206-MT01 is the clone of API-5-3', CCS-HmiT021206-MT01-1 and CCS-HmiT021206-MT01-2 represents mutant 1 and mutant 2, respectively. HmiR0406-MR03 and CmiR0001 are the miR precursor clone and miR control clone, HmiR-AN0343-AM03 and CmiR-AN0001-AM03 are the inhibitor clone and control.

### Discussion

Chemotherapy is one of the vital treatment methods in cancer, and that in breast cancer is no exception. However, a large number of patients were observed to be refractory to chemotherapy and showed resistance to antineoplastic agents. Drug-resistance could minimize the effectiveness of chemotherapy and it is a complex phenomenon with multiple mechanisms [8,9]. Whereby, the most well-known mechanism of drug resistance is via increased efflux of drug leading to upregulation of ATP binding transporters, such as breast cancer resistance protein [10].

MiRs have been confirmed to play important roles in carcinogenesis and their expression has been found to be dysregulated in various human tumor types. MiR-224 has also been studied by many researchers, but so far it is still controversial whether small non-coding RNAs contribute to drug resistance. For instance, Huang et al have reported that the overexpression of miR-224 in-

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% of maximal activity for Control = 100%
Fluc/Rluc (Sample) Average Fluc/Rluc (control)
duced the resistant phenotype in colon carcinoma cells and miR-224 expression was associated with methotrexate resistance [11]. In the current study, we have shown that the miR-224 expression was significantly higher in MCF-7/DOC cells compared with that in MCF-7 cells by qRT-PCR assay. Then, to further investigate the link between miR-224 expression and the sensitivity of breast cancer cells to DOC, miR-224 mimics was transfected into MCF-7 cells and CCK-8 assay was used to determine the IC_{50} value of miR-224-transfected MCF-7 cancer cells. The above results showed clearly that overexpression of miR-224 obviously decreased the sensitivity of MCF-7 cancer cells to DOC and miR-224 mimics could significantly decrease apoptotic rate of DOC-induced MCF-7 cells and sensitivity of MCF-7 breast cancer cells to DOC, while miR-224 inhibitor increased apoptosis of DOC-induced MCF-7/DOC cells by flow cytometry assay. Currently, many studies reported that miR-224 was a regulator of cell progression, invasion and metastasis in tumor cells and targeted so many predicted genes such as CDS2 and HSPC159 [3]. Among the above targets regulated by miR-224, API-5 was confirmed to be targeted by miR-224 through TargetScan and PicTar. The concept that API-5 could be a target controlled by miR-224 has been accepted by many people. For example, Wang et al reported that miR-224 interacted with 3'-UTR of API-5 and the interaction could inhibit reporter activity [6]. Koc et al showed that the expression of API-5 was increased in metastatic cervical cancer tissue while it was low in normal tissue. They also found that API-5 was markedly upregulated in patients with B-cell lymphoma. API-5 has been identified, based on its antiapoptotic action, as an oncogene and ectopic expression of API-5 could enhance the tumor cell survival [12]. Kim et al [13] and Rigou et al [14] have reported that API-5 was markedly expressed in nasopharyngeal human leukemia and cervical cancer cells and abundant expression of API-5 was closely associated with many other cancers. Apoptosis was an innovative idea which helped understanding the cancer treatment. The discovery of apoptosis inhibitors were so important because they induced chemotherapeutic drugs resistance [15,16]. API-5 was one such apoptosis inhibitor. But the correlation between miR-224 and API-5 in mediating the response of tumor cells to chemotherapeutic drugs is still not well known. In our study, API-5 expression was upregulated in MCF-7 cancer cells compared with that in MCF-7/DOC cells. Additionally, miR-224 mimics could downregulate the API-5 mRNA expression and protein in MCF-7 cells. On the contrary, miR-224 inhibitor upregulated the expression of API-5 mRNA and protein in MCF-7/DOC cells. Then, luciferase reporter gene assay revealed that miR-224 modulated the API-5 expression via directly binding to the 3'UTR of API-5 in breast cancer cells. All these results revealed that miR-224 could modulate the sensitivity of breast cancer cells to DOC partly by regulating the expression of API-5 protein. However, the exact role of API-5 signaling pathway in miR-224-mediated drug resistance still needs further research.

**Conclusion**

This study demonstrated that miR-224 expression was inversely correlated with the expression of API-5 in the breast cancer drug-resistant cells. Furthermore, upregulated miR-224 in the MCF-7 breast cancer cells downregulated the API-5 expression and decreased the sensitivity of MCF-7 cells to DOC. These findings indicated that miR-224 was a predictor of tumor response to DOC-based chemotherapy, at least in part by targeting API-5. Additionally, miR-224 could provide a potential strategy in treating breast cancer. Of course, this regulatory pathway, which was revealed in this study, was only the tip of the iceberg for the complicated regulated network. Future research will be focused on exploring the exact mechanism of drug resistance regulated by miR-224 and relevant genes.

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**Conflict of interests**

The authors declare no conflict of interests.

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