Original Article
DNA methylation of miR-93: an important event in acquiring drug resistance in breast cancer cells

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Received August 6, 2017; Accepted March 7, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Aim: The aim of this study was to identify the function of miR-93 in gaining drug resistance to breast cancer cells, examining possible regulatory mechanisms. Methods: miR-93 overexpressed MCF-7 cells and miR-93 downregulated MCF-7/ADR cells were built, respectively. CCK-8 and flow cytometry were used to evaluate the effects of different miR-93 expression levels in these cells. Quantitative DNA methylation analyses on drug sensitive and resistant cells was conducted. Results: In drug resistant breast cancer cells, high expression of miR-93 and drug resistance-related genes was observed, simultaneously. Downregulation of miR-93 increased apoptosis ratios. Higher proliferation ratios and lower apoptosis ratios were observed when overexpressing miR-93 in drug sensitive cells. Significantly lower methylation levels of miR-93 were detected in drug resistant cells and 7 specific CpG sites were found. Conclusion: Highly-expressed miR-93 contributes to drug resistance in breast cancer cells and expression levels of miR-93 are regulated by DNA demethylation.

Keywords: miR-93, breast cancer, doxorubicin-resistance, DNA methylation

Introduction

Chemoresistance is one of the biggest challenges in breast cancer treatment [1]. Although numerous studies have reported possible mechanisms for chemoresistance [2-4], mechanisms of drug resistance in breast cancer cells still require further research.

Recently, accumulating evidence has demonstrated that dysregulation of miRNAs is associated with chemoresistance of multiple tumors [3, 5]. Kutanzki et al. reported that miR-21 participates in drug resistance of breast cancer cells to gemcitabine by targeting PTEN/Akt signaling pathways [6]. miR-644a has been reported regulate expression of CTBP1 and P53 in mediating breast cancer drug resistance [7]. On the other hand, many studies have pointed out that miR-93 shows significant expression level changes in multiple tumors, such as endometrial carcinoma, gastric carcinoma, and non-small lung cancer [8-10]. A typical member of the miR-106b-25 cluster, the function of miR-93 is widely related to metastasis, invasion, and proliferation [11-18]. Particularly, Liu et al. reported that the repression of miR-93 enhanced the sensitivity of bladder carcinoma and promoted chemo-induced cell apoptosis [19]. This role of miR-93 in drug resistance has also been observed in ovarian cancer and gliomas [20-22]. However, the functional roles and underlying mechanisms in drug resistance of breast cancer remain uncertain. A previous study found that miR-93 showed dramatically different expression levels between sensitive and drug resistant breast cancer cells, according to miRNA microarray analysis [23]. In addition, this study checked the gene sequence on its corresponding DNA, finding
that it contains many CpG sites. Thus, it was conjectured that high expression of miR-93 contributes to drug resistance in breast cancer. High expression levels may be induced by DNA methylation.

**Materials and methods**

**Cell lines**

MCF-7 cell line was purchased from Shanghai Institutes for Biological Sciences (Shanghai, China). It was cultured in DMEM medium supplemented with 10% FBS.

MCF-7/ADR (doxorubicin-resistant) cell line was derived from MCF-7 cell lines by continuously culturing in medium containing progressive concentrations of doxorubicin (Sigma-Aldrich). MCF-7/ADR was cultured in DMEM medium supplemented with 10% FBS and 2 ug/mL doxorubicin. It was then transferred into a drug-free medium for >2-3 weeks before experimentation.

MCF-7 cells were transfected with a final concentration of 50 nM of hsa-miR-93-5p mimics and hsa-miR-93-5p mimics with negative control (Ribiobio) to obtain miR-93 overexpressed cells (MCF-7-miR-93 mimics) and corresponding negative control cells (MCF-7-miR-93 mimics NC). Lipofectamine 3000 was used, (Invitrogen) according to manufacturer instructions. MCF-7/ADR cells were transfected with a final concentration of 100 nM hsa-miR-93-5p inhibitor (Ribiobio) to obtain miR-93 downregulated cells (MCF-7/ADR-miR-93 inhibitor) and its corresponding negative hsa-miR-93-5p inhibitor control (MCF-7/ADR-miR-93 inhibitor NC), respectively.

The current demethylation experiment was performed by treating MCF-7 cells with 5 μM 5-aza-2'-deoxycytidine (5-aza-dC; Sigma-Aldrich) for 72 hours.

**RNA isolation and qRT-PCR**

Total RNA was extracted from cells using an miRcute miRNA isolation kit (Tiangen). Next, miR-93 expression was analyzed using the Bulge-LoopTM miRNA qRT-PCR Starter Kit (Ribiobio). Primers for miR-93 and endogenous control U6 were purchased from Ribiobio (ssD809230675, ssD809231367, ssD80926-1711, ssDD0904071006, ssDD0904071007, ssDD0904071008).

Total RNA extraction was performed according to the protocol of TRizol Reagent (Takara, Dalian, CN). SYBR Green qRT-PCR (Takara) was used for detecting expression of different genes. Primer sequences for qRT-PCR were as follows: MDR: 5’-gctctgtgtatgcctagaagctg-3’, 5’-tcttcacctcaggctcagt-3’ (product Size, 202 bp); MRP: 5’-aggtttgacagttttgtgtc-3’, 5’-ctctgtggatacaggc-3’ (product Size, 181 bp); BCRP: 5’-cactttatggctcaggaga-3’, 5’-ctctgtttgaagc-3’ (product Size, 206 bp); GAPDH: 5’-ccttggatctgtaagactc-3’, 5’-gtagagtcgaggt-gatgt-3’ (product Size, 132 bp).

**CCK-8 assay**

CCK-8 assay (Dojindo Laboratories, Kumamoto, Japan) was performed, according to manufacturer protocol. Cellular survival ratios were tested after MCF-7 cells were transfected with miR-93 mimics. They were then transferred into gradient concentrations of doxorubicin (Sigma-Aldrich) for 24 hours, respectively.

**EdU proliferation assay**

Cellular proliferation was tested by EdU proliferation assay (Ribiobio), according to manufacturer protocol. DAPI was used to label the nuclei. Percentages of EdU-positive cells were calculated after analysis using fluorescent microscopy.

**Flow cytometry analysis**

Annexin V-FITC Apoptosis Detection Kit (KeyGen BioTECH) was used for cellular apoptosis. Data were analyzed with FlowJo9.1 software.

**Quantitative DNA methylation analyses**

NCBI (http://www.ncbi.nlm.nih.gov/), UCSC (http://genome.ucsc.edu/), Methprime (http://www.urogene.org/methprime), and CpG Island Finder (http://dbcat.cgm.ntu.edu.tw/) were used to perform bioinformatics analysis. Genomic DNA of MCF-7 cells and MCF-7/ADR cells was extracted by the Tissue DNA Kit (Qiagen), according to manufacturer protocol. A total of 200 ng of genomic DNA from both cell lines was bisulfite-treated with the Methylamp DNA Modification kit (Epigentek). The quality of
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the bisulfite conversion was controlled using PCR products. Sequenom MassARRAY platform (Capitalbio, Beijing, China) was used to perform quantitative methylation analysis of miR-93 host gene MCM7. PCR primers were as follows: MCM7-2: 5'-aggagagagagtaatgaattatatgttgaggaggag-3', 3'-cagtaatacactactatagggagaagggtttctatctcttcctccaaacaacc-5'. MCM7-3: 5'-aggagagagaggagtttttttggaggtgaggaattt-3', 3'-cagtaatacactactatagggagctaatcccccttataaaaaaaataaa-5'. MCM7-4: 5'-aggagagagaggagaatttttggagagttgaaagtt-3', 3'-cagtaatacactactatagggagctaatcccccttataaaaaaaataaa-5'. MCM7-5: 5'-aggagagagagtttaggagagtttttggagtgttttttg-3', 3'-cagtaatacactactatagggagctaatcccccttataaaaaaaataaa-5'. Spectra methylation ratios were generated with Epityper software version 1.0 (Sequenom).

Statistical analysis

Experiments were repeated three times, independently. Paired t-test was applied for statistical analysis. SPSS software (version 21.0) was adopted for statistical analysis. Measurement data are expressed as mean ± standard deviation and comparisons between groups were examined by variance analysis or t-test. $P < 0.05$ indicates statistical significance.

Results

Expression of miR-93 in MCF-7 and MCF-7/ADR

Next, qRT-PCR was carried out to identify expression levels of miR-93 in MCF-7 and MCF-7/ADR, respectively. Results showed that miR-93 expression levels in MCF-7/ADR were 2.7 fold higher than those in MCF-7 cells (Figure 1, $P < 0.01$).

Effects of miR-93 on cell proliferation and apoptosis

EdU proliferation assay demonstrated that miR-93 overexpressed MCF-7 cells showed significantly higher proliferation ratios (Figure 2, $P < 0.01$). On the other hand, under doxorubicin culturing, cells showed increased survival ratios, according to CCK-8 assay (Figure 3A, $P < 0.05$). Flow cytometry analysis was used to test doxorubicin-induced apoptosis ratios in both MCF-7-miR-93 mimics and MCF-7/ADR-miR-93 inhibitors. Results of MCF-7-miR-93 mimics showed a decreasing pattern, while it was increased in MCF-7/ADR-miR-93 inhibitors (Figure 3B-E, $P < 0.01$).

Expression of drug resistance-related genes

In this study, qRT-PCR was performed to identify expression of drug resistance-related genes, including multi-drug resistance (MDR), multi-drug resistance associated protein (MRP), and breast cancer resistance protein (BCRP). miR-93 upregulation resulted in corresponding increased expression of all three genes in MCF-7 cell lines: 23.5-fold increase of MDR, 47.6-fold of MRP, and 40.3-fold of BCRP, respectively (Figure 4, $P < 0.01$).

Bioinformatics analysis

Analyzing the promoter of MCM7, the host gene of miR-93, a total of 9 possible CpG islands were found (Figure 5A). The densest CpG island was chosen for investigation (Figure 5B): Fragment MCM7-2: base pairs between -1499 to -1177 (contains 23 CpG sites), Fragment MCM7-3: base pairs between -1202 to -789 (contains 46 CpG sites), Fragment MCM7-4: base pairs between -796 to -338 (contains 38 CpG sites), and Fragment MCM7-5: base pairs...
Quantitative DNA methylation analysis

Results are shown in Figure 6. Global methylation levels of miR-93 host gene MCM7 were significantly lower in MCF-7/ADR cells than in MCF-7 cells, in all four fragments (Figure 7A, P < 0.05). Furthermore, the percentages of hypomethylated CpG sites of MCM7-2, 3, 4, and 5 were 41.6%, 50%, 37.5%, and 66.7%, respectively. Among these CpG sites, sites 16, 18, and 46 of MCM7-3 and sites 7, 8, 9, and 35 of MCM7-5 showed particular hypomethylation status (Figure 7B-E, P < 0.05).

Demethylation experiment

For this experiment, qRT-PCR was used to test expression levels of miR-93 in MCF-7 after
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Results showed that the expression level of mi-93 was 3.3-fold upregulated (Figure 8, \( P < 0.01 \)).

**Discussion**

The current study found that expression levels of miR-93 are extensively upregulated in drug resistant breast cancer cells (MCF-7/ADR), confirming previous results by miRNA microarray analysis [23]. In 2011, Tissa et al. studied the differential expression of microRNAs in MCF-7 and tamoxifen-resistant LBY2 breast cancer cells [24]. They found that expression levels of miR-93 were markedly upregulated in tamoxifen-resistant cell lines. Combined with present results on doxorubicin-
resistant cell lines, upregulation of miR-93 may be a common molecular event during which breast cancer cells gain the character of drug resistance. Furthermore, in 2014, Zhou et al. studied expression changes of the miR-106b-25 cluster, with miR-93 included. They also found that high expression of miR-93 was associated with drug-induced motility and invasion in breast cancer [25]. Thus, it was conjectured that miR-93 is a key regulator in gaining drug resistance in breast cancer cells.

In this study, EdU proliferation assays, CCK-8 assays, and flow cytometry analysis were used to examine the effects of miR-93 on breast cancer cell lines. Upregulation of miR-93 may be a common molecular event during which breast cancer cells gain the character of drug resistance. Furthermore, in 2014, Zhou et al. studied expression changes of the miR-106b-25 cluster, with miR-93 included. They also found that high expression of miR-93 was associated with drug-induced motility and invasion in breast cancer [25]. Thus, it was conjectured that miR-93 is a key regulator in gaining drug resistance in breast cancer cells.

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DNA methylation of miR-93 and acquisition of drug-resistance in breast cancer

In recent years, many reports have suggested that DNA methylation is involved in multiple cancers [26-28], including breast cancer [29-31], by regulating expression of oncogenes and anti-oncogenes. Moreover, miR-93 has been found to be one of the five hypomethylated and upregulated miRNAs in hepatocellular carcinoma [32], providing a clue about the upstream regulatory mechanisms of miR-93.

In reviewing references, it was noticed that miR-93 is co-transcribed with its host gene MCM7 in gastric, prostate, and hepatocellular cancer [33-36]. Analyzing the promoter of MCM7, the densest CpG island was chosen for testing in this study. It was found that the global methylation status trended toward demethylation in drug resistant cancer cells of the chosen fragment. This result can explain the reason for miR-93 upregulation, according to DNA methylation theory [37]. Moreover, this study noticed some specific CpG sites: 16, 18, and 46 of MCM7-3 and sites 7, 8, 9, and 35 of MCM7-5 (Figures 6B, 6D and 7C, 7E). According to statistical analysis, it was assumed that these sites may play vital roles in demethylation of MCM7 promoter. It has been hypothesized by researchers that methylation or demethylation may occur on some specific CpG sites at first, then spread to the other sites of CpG island. Reviews by Marchal et al. and Miotto et al. brought out the theory that methylation or demethylation may not occur on all CpG sites, but on some of the key sites, regulating the function of the gene [38]. Concerning the above, high expression of miR-93 during drug resistance in breast cancer cell proliferation and apoptosis under doxorubicin. Present results showed that, in miR-93 overexpressed sensitive breast cancer cells, proliferation was markedly increased, while apoptosis was markedly decreased. These miR-93-induced drug resistance trends can also be noticed in urinary bladder cancer cells, ovarian cancer cells, and glioma cells [19-22]. To further confirm results, this study detected drug resistance-related gene expression, finding that MDR, MRP, and BCRP were significantly upregulated. Therefore, results suggest that miR-93 contributes to drug resistance of MCF-7 cells and high expression of miR-93 can be considered an important factor leading to drug resistance in breast cancer.

![Figure 6. Methylation levels of CpG sites in miR-93 host gene MCM7 promoter fragment. (A) fragment MCM7-2, (B) fragment MCM7-3, (C) fragment MCM7-4, (D) and fragment MCM7-5.](image-url)
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Figure 7. Sequenom MassARRAY analysis of miR-93 host gene MCM7 promoter region methylation levels. (A) The global methylation of miR-93 host gene MCM7 promoter in MCF-7/ADR and MCF-7 cells; (B-E) Quantitative methylation analysis of miR-93 host gene MCM7 promoter in MCF-7/ADR and MCF-7 cells (A-D); *P < 0.05, **P < 0.01.

Figure 8. qRT-PCR analysis of miR-93 expression in demethylation agent treated MCF-7 cells. The expression level of miR-93 was significantly upregulated when MCF-7 cells were treated with inhibitors of DNA methylation (5-aza-dC); **P < 0.01.

resistance is induced by DNA methylation/demethylation regulation. However, the current study performed demethylation experiments as a reverse validation. Results confirmed the present hypothesis.

In conclusion, the current study proves that the gaining of drug resistance of breast cancer cells is accompanied with high expression of miR-93. This may lead to increased expression of drug resistance-related genes. Moreover, high expression of miR-93 can be induced by DNA demethylation. Furthermore, this study
identified some key CpG sites of this procedure. Present findings suggest that hypermethylation agents may be a novel therapeutic strategy for reversal of drug resistance.

Acknowledgements

This paper is funded by the National Natural Science Foundation of China (project approval number: 31871214).

Disclosure of conflict of interest

None.

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