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Original Research

MicroRNA 494 increases chemosensitivity to doxorubicin in gastric cancer cells by targeting phosphodiesterases 4D

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Abstract: Acquired drug resistance is one of the main limitations in pharmacological therapy of malignancies including gastric cancer. MicroRNAs (miRNAs) are a class of small noncoding RNAs that suppress their targets by binding to the 3'UTR region of genes. In this study, we explored investigate the target gene of miR-494 and its roles in chemoresistance of gastric cancer. We found that miR-494 was significantly down-regulated in gastric cancer cells lines compared to the normal gastric epithelial cell line. Exogenous overexpression of miR-494 increased the chemosensitivity of gastric cancer cells to doxorubicin. Moreover, miR-494 expression was reduced in a doxorubicin-resistant gastric cancer cells (AGS/dox) compared with the parental cells. MTT assay showed that AGS/dox cells exhibited an elevated viability compared with the parental cells. Enforced expression of miR-494 inhibited AGS/dox cell viability and colony formation ability. In addition, we demonstrated that elevated expression of miR-494 inhibited the mRNA and protein expression of phosphodiesterases 4D (PDE4D) in gastric cancer cell. Luciferase assay showed that miR-494 directly targeted the 3'UTR region of PDE4D. Furthermore, restoration of PDE4D recovered the chemoresistance in miR-494-overexpressed gastric cancer cells. Taken together, this study demonstrated that miR-494 enhanced doxorubicin sensitivity via regulation of PDE4D expression, suggesting a novel therapeutic strategy for anti-chemoresistance in gastric cancer.

Key words: Gastric cancer; MicroRNA; miR-494; Chemosensitivity; Phosphodiesterases 4D.

Introduction

Gastric cancer is the most frequent gastrointestinal tumor in East Asia, Eastern Europe, and parts of Central and South America. Moreover, gastric cancer is the second leading cause of cancer-related deaths (1). During the past decades, significant advances have been made in diagnostic technique, surgical method and novel chemotherapy regimens. However, the long-term survival rate for gastric cancer is still quite low (2). Therefore, it is urgent to elucidate the pathogenesis of gastric cancer and further to develop more effective therapeutic strategy.

MicroRNAs (miRNAs) are a class of 18 to 24 nucleotides and non-coding RNAs that bind to the 3'-untranslated region (3'-UTR) of the target mRNAs so as to regulate protein expression at the posttranscriptional level (3). Amounting studies have indicated that miRNAs play critical roles in a wide range of pathological and physiological processes, such as proliferation, differentiation, apoptosis, tumorigenesis, and drug resistance (4,5). Remarkably, aberrant expression of miRNAs has been demonstrated to be involved in the development of diverse neoplasia and in some cases correlate with clinical-pathological features of tumors, which represents potential prognostic markers and novel therapeutic targets in cancer (6). For instance, Wang et al showed that miR-30 may act as an oncomiR in gastric cancer cells through regulation of p53-mediated mitochondrial apoptotic pathway (7). It was suggested that miR-221 and miR-222 simultaneously targeted PTEN to regulate gastric carcinoma cell proliferation and radioresistance (8). miR-494 is located on chromosome 14q32.31 (9). Recent studies have found the aberrant expression of miR-494 in several carcinomas, such as lung cancer, gastrointestinal cancer and liver cancer (10-12). However, the potential role of miR-494 in gastric cancer progression has not been fully clarified. In the present study, we explored the potential roles of miR-494 -modulated doxorubicin sensitivity as well as the underlying mechanism in gastric cancer cells.

Materials and Methods

Cell culture

The normal gastric epithelial cell line GES-1 and gastric cancer cell lines (AGS and SGC-7901) were purchased from Shanghai Institute of Cell Biology (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, California, USA) supplemented with 10% fetal bovine serum, 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated at 37°C in a humidified environment containing 5% CO_2. AGS cells were cultured in the culture media with a gradient concentration of doxorubicin (Sigma-

Aldrich, USA) and the doxorubicin-resistant AGS cell line (AGS/dox) was constructed by selecting the viable cells in culture media.

Cell transfection

Gastric cancer cells were seeded into 6-well plate and transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, USA) when cells were grown to 70–80% confluence. miR-494 mimic and control were purchased from GenePharma Co. Ltd. (Shanghai, China). Plasmid DNA was transfected into cell using the Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacture's construction followed by the downstream assays.

Cell viability assay

Cell viability was detected using 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay. To be brief, cells were seeded into 96-well plates at a density of 5000 cells per well and then were transfected with negative control or miR-494 mimic followed by treatment with doxorubicin (1μg/ml). Subsequently, 20 ml of 5 mg/mL MTT (Sigma, Shanghai, China) was added into each well and incubated for 4 h in a humidified incubator. After discarding the medium, 200 μL of DMSO (dimethyl sulfoxide) was added to each well and the optical density (OD) was evaluated by measuring the absorbance at a wavelength of 490 nm.

Colony forming assay

AGS and SGC-7901 cells were plated at $1x10^6$ cells/plate in a 100-mm culture dish one day before transfection. Then, cells were transfected with negative control or miR-494 mimic followed by treatment with doxorubicin (1μg/ml). The surviving colonies were stained with crystal violet and visible colonies were counted using an automatic colony-counting system (Media Cybernetics, Inc., Bethesda, MD, USA).

Real time PCR

Total RNAs were extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. RNAs were reverse-transcribed into cDNA using a TaqMan Gold RT–PCR Kit (Applied Biosystems, Foster City, CA, USA). The real-time PCR results were normalized to small nucleolar RNA (RNU6), which was selected as an internal control. The relative expression levels were calculated using the 2-ΔΔCt method.

Luciferase reporter assay

pMIR-PDE4D-3' UTR reporter was constructed by cloning wild type PDE4D 3'UTR or mutated 3'UTR (5' UCUCUUCUGUUGUGCCCGCCAAC) into the pMIR-REPORT vector (Life Technologies). The reporters and control mimic or miR-494 mimic were co-transfected into cells using Lipofectamine 2000 transfection reagent (Invitrogen, USA). The luciferase activity was measured by the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI, USA) according to the manufacturer's instruction.

Western blot

Gastric cancer cells were subjected to lysis in a RIPA buffer (Thermo Fisher, Waltham, MA, USA). Lysate was then centrifuged at 12,000 rpm for 10 min and protein concentration was determined by Bradford assay. Protein samples were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Invitrogen, USA). The membranes were blocked with 5% non-fat dry milk for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibody against PDE4D and GAPDH (Santa Cruz, USA) at 4 ℃ overnight followed by incubation with horseradish peroxidase conjugated anti-rabbit secondary antibody (Santa Cruz, USA). Finally, chemiluminescence was generated by using Pierce Western blotting Substrate (Thermo Fisher Scientific).

Statistical analysis

Experimental data were expressed as mean ± SD. Student's t-test and one-way analysis of variance (ANOVA) were used to calculate statistical significance using SPSS 13.0 statistics software (SPSS Inc, USA). $P < 0.05$ was considered statistically significant.

Results

miR-494 regulates chemosensitivity of gastric cancer cells

In order to explore the role of miR-494 in gastric cancer, we detected the expression level of miR-494 in human gastric cancer cells. As a result, we found that miR-494 was significantly down-regulated in gastric cancer cell lines, AGS and SGC-7901, compared to the normal gastric epithelial cell line, GES-1 (Fig. 1A). In addition, we evaluated the sensitivity of gastric cancer to doxorubicin with or without exogenous overexpression of miR-494. Consequently, AGS and SGC-7901 cells with transfection of miR-494 mimic were more sensitive to doxorubicin (Fig. 1B and C). These results suggested that overexpression of miR-494 increased the chemosensitivity of gastric cancer cells to doxorubicin.

miR-494 is down-regulated in doxorubicin-resistant gastric cancer cells

In order to explore the role of miR-494 in doxorubicin chemoresistance, we constructed a doxorubicin-

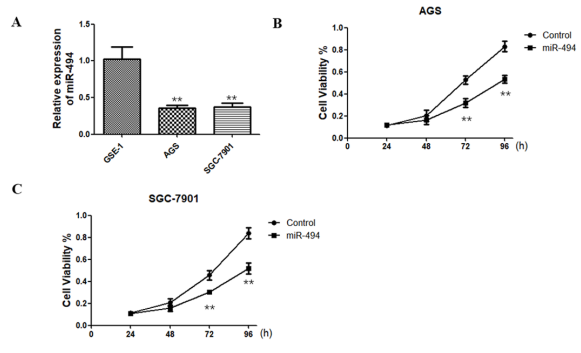

Figure 1. miR-494 regulates chemosensitivity of gastric cancer cells. (A) The expression levels of miR-494 were analyzed by real time PCR in gastric cancer cell lines, AGS and SGC-7901, and a normal gastric epithelial cell line, GES-1. Next, AGS (B) and SGC-7901 (C) cells were transfected with miR-494 mimic or control mimic, and cell viability was determined by MTT assay. Data was presented as means ± SD. Columns, mean of three independent experiments; bars, SD; * $P < 0.05$; ** $P < 0.01$.

resistant AGS cell line (AGS/dox). When cultured in normal condition without doxorubicin, there was no difference in viability between the parental and AGS/dox cells (Fig. 2A). However, MTT assay showed that AGS/dox cells exhibited an elevated viability compared with the parental cells (Fig. 2B). In addition, real time PCR was performed to detect the expression of miR-494, and we found that miR-494 was down-regulated in AGS/dox cells compared with the parental cells (Fig. 2C).

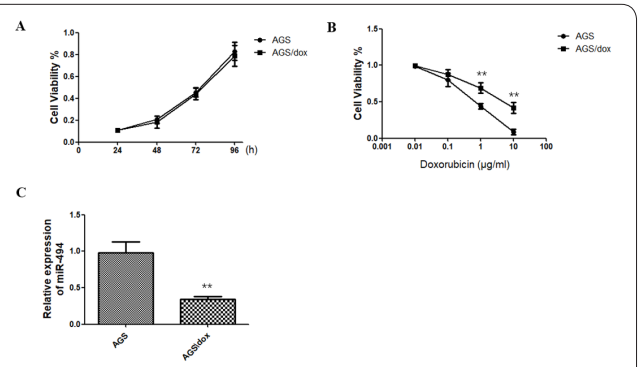

Figure 2. miR-494 is down-regulated in doxorubicin-resistant gastric cancer cells. (A) Doxorubicin-resistant AGS cells (AGS/dox) cells and the parental AGS cells were cultured in a normal condition without doxorubicin, cell viability was determined by MTT assay. AGS/dox cells and AGS cells were cultured in a condition with a gradient concentration of doxorubicin, then cell viability was determined by MTT assay (B). Real time PCR was performed to detect the expression of miR-494 in AGS and AGS/dox cells (C). Data was presented as means ± SD. Columns, mean of three independent experiments; bars, SD; * $P < 0.05$; ** $P < 0.01$.

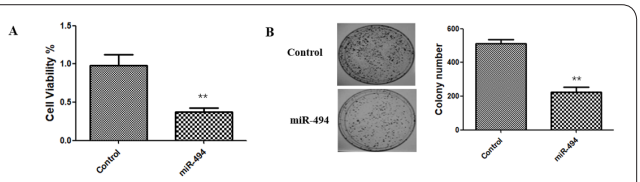

Figure 3. miR-494 modulates chemosensitivity of gastric cancer cells to doxorubicin. AGS/dox cells were transfected with control mimic or miR-494 mimic for 48 h and then cells were treated with doxorubicin for 48 h. Afterwards, cell viability was measured by MTT assay (A) and colony formation ability was determined by colony formation assay (B). Data was presented as means ± SD. Columns, mean of three independent experiments; bars, SD; * $P < 0.05$; ** $P < 0.01$.

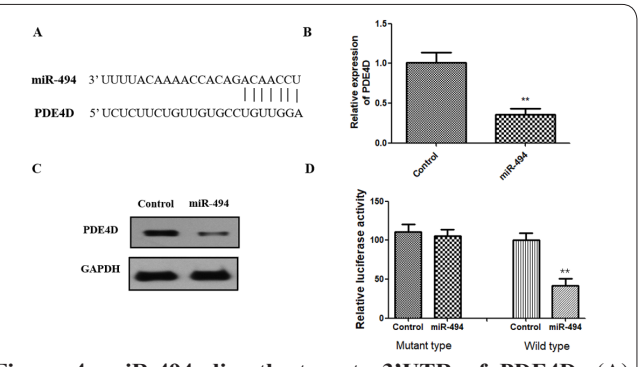

Figure 4. miR-494 directly targets 3'UTR of PDE4D. (A) PDE4D was predicted to be a potential target of miR-494. AGS cells were transfected with miR-494 mimic or control mimic. Real time PCR (B) and western blot (C) analysis was performed to detect the mRNA and protein levels of PDE4D in AGS cells transfected with miR-494 mimic. (D) Luciferase assay showed that miR-494 bond to 3'UTR of PDE4D. Data was presented as means ± SD. Columns, mean of three independent experiments; bars, SD; *, $P < 0.05$; **, $P < 0.01$.

miR-494 modulates chemosensitivity of gastric cancer cells to doxorubicin

To explore the role of miR-494 in drug resistance, we used miR-494 mimics to increase the expression of miR-494 in AGS/dox cells. As a result, MTT assay that enforced expression of miR-494 inhibited viability of AGS/dox cells to doxorubicin compared with their parental cells (Fig. 3A). Furthermore, colony formation assay revealed that up-regulation of miR-494 also reduced colony number of AGS/dox cells in response to doxorubicin (Fig. 3B). These results demonstrated that miR-494 may increase the chemosensitivity of gastric cancer cells.

miR-494 directly targets 3'UTR of PDE4D

To identify the potential miRNA that targets PDE4D, we searched the TargetScan database, and predicted that miR-494 could bind to the 3'-UTR of PDE4D (Fig. 4A). The, we examined whether PDE4D was a direct target of miR-494 in gastric cancer cells. AGS cells were transfected with miR-494 mimic or control mimic, respectively. Real time PCR and western blot analysis revealed that the mRNA and protein levels of PDE4D were significantly suppressed after overexpression of miR-494 in AGS cells (Fig. 4B and C). In addition, luciferase assay was performed to test whether miR-494 could directly target 3'UTR of PDE4D mRNA. Consequently, results showed that enforced expression of miR-494 obviously decreased the luciferase activity of vector containing wild-type PDE4D-3'UTR rather than mutated PDE4D-3' UTR (Fig. 4D). Taken together, these results showed that PDE4D was a direct target of miR-494 in gastric cancer cells.

Effects of restoration of PDE4D on the doxorubicin sensitivity of gastric cancer cells

The aforementioned results suggested that PDE4D served as a target of miR-494, we thus hypothesized that PDE4D was involved in the miR-494-modulated chemosensitivity in gastric cancer cells. To validate this hypothesis, we transfected miR-494 mimic or PDE4D-encoding vectors alone or in combination into AGS/dox cells (Fig. 5A and B). As expected, we found that enforced expression of miR-494 inhibited viability of AGS/dox cells to doxorubicin whereas restoring PDE4D recovered the drug resistance in miR-494-overexpressed

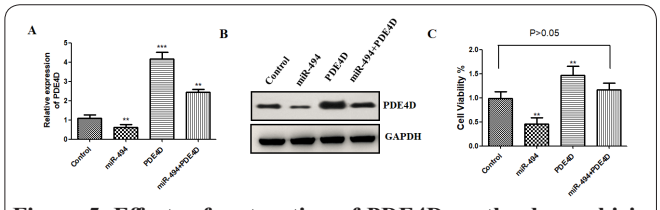

Figure 5. Effects of restoration of PDE4D on the doxorubicin sensitivity of gastric cancer cells. AGS/dox cells were transfected with miR-494 mimic alone or in combination with PDE4D vector. Real time PCR (A) and western blot (B) analysis were performed to detect the expression of PDE4D. In addition, cell viability (C) was measured by MTT assay. Data was presented as means ± SD. Columns, mean of three independent experiments; bars, SD; ** $P < 0.01$; *** $P < 0.01$.

AGS/dox cells (Fig. 5C). Collectively, these results demonstrated that miR-494 regulated the chemosensitivity of gastric cancer cells through direct targeting PDE4D.

Discussion

A large amount of studies reveal that miRNAs play important roles in the pathogenesis and progression of gastric cancer (13). For example, shin et al found that miR-135a expression was down-regulated and suppressed lymph node metastasis by targeting ROCK1 in gastric cancer (14). Jin et al showed that the expression of miR-21 was upregulated in the paclitaxel resistant gastric cancer cells and over-expression of miR-21 decreased antiproliferative effects and apoptosis induced by paclitaxel by regulating P-glycoprotein in gastric cancer cells (15). These findings demonstrate that miRNAs play a diver role gastric cancer progression.

MiR-494 has been reported to serve as a tumor-suppressive miRNA in several human malignancies. For instance, miR-494 could inhibit cell proliferation in oral cancer cells by regulating HOXA10(16). Liu et al found that miR-494 suppressed the proliferation, invasion and chemoresistance of pancreatic cancer by targeting c-Myc and SIRT1(17). Particularly, miR-494 has been found to function as an anti-oncogene in gastric carcinoma (18). However, the detailed role and mechanism of miR-494 in gastric cancer chemoresistance has not been elucidated. In the present study, we observed a significant reduction of miR-494 in gastric cancer cell lines, AGS and SGC-7901, compared to the normal gastric epithelial cell line, GES-1. Exogenous overexpression of miR-494 increased the sensitivity of gastric cancer cells to doxorubicin. In addition, AGS/dox cells exhibited an elevated viability, but overexpression of miR-494 sensitized AGS/dox cells to doxorubicin.

The phosphodiesterases (PDEs) are metallohydrolases that hydrolyze cyclic guanosine monophosphate (cGMP) or cyclic adenosine monophosphate (cAMP) or into 5′-GMP or 5′-AMP. Accumulating studies have shown that PDEs play a critical role in a wide range of physiological and pathological processes including skeletal development, tumor initiation and progression, alzheimer's disease, and osteoarthritis [19,20]. PDE4D, belongs to the PDE4 subfamily, has been shown to act as a proliferative factor in prostate cancer (21). Kolosionek et al found that upregulated PDE4D may contribute to the metastasis and invasion of A549 cells (22). Recently, Mishra et al demonstrated that inhibition of PDE4D may represent a novel therapeutic target for acquired tamoxifen resistance in estrogen receptor (ER)-positive breast cancer (23). In the present study, we performed bioinformatic analysis and found that PDE4D was a potential target of miR-494. Additionally, we confirmed that miR-494 directly targeted PDE4D in gastric cancer cells. Furthermore, restoration of PDE4D recovered the drug resistance in miR-494-overexpressed AGS/dox cells.

In summary, our study demonstrated that miR-494 sensitized gastric cancer cell to doxorubicin through direct targeting PDE4D. These results suggest that miR-494 may be developed into a novel therapeutic target for anti-chemoresistance in gastric cancer cells.

Acknowledgements

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