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ORIGINAL ARTICLE

MiR-16-1 Targeted Silences Far Upstream Element Binding Protein 1 to Advance the Chemosensitivity to Adriamycin in Gastric Cancer

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Abstract Chemotherapy can prevent metastasis and recurrence of gastric cancer (GC), and is a well supplement for operation. But, chemotherapy resistance has severely restricted the application of chemotherapy. This study aimed to investigate the regulatory roles and molecular mechanism of miR-16-1 to the chemosensitivity to adriamycin in GC. In this study, the expression of miR-16-1 and FUBP1 was down-regulated and up-regulated respectively in adriamycin-resistant GC tissues and cell lines, and represented a negative relationship between them. MiR-16-1 could silence FUBP1 directly and specifically, FUBP1 was a target gene of miR-16-1. Silence of FUBP1 inhibited the half maximal inhibitory concentration (IC50) of SGC7901/AR cell line to adriamycin, chemosensitivity enhanced significantly. Moreover, FUBP1 silence in SGC7901/AR cell line also inhibited proliferation and invasion, and advanced cell apoptosis. To sum up, the expression of miR-16-1 was positively related with the chemosensitivity of GC to adriamycin, and miR-16-1 could targeted silence FUBP1 to advance the chemosensitivity to adriamycin in GC, which might be a novel potential therapeutic target for GC.

Keywords Gastric cancer · microRNA-16-1 · Far upstream element binding protein 1 · Chemosensitivity · Adriamycin

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Introduction

Gastric cancer (GC) is the most common solid tumor originated from digestive system [1]. Because advanced GC has high frequent metastasis and relapse, its 5 years survival rate was 30% ~ 50%, and its prognosis is poor. Surgical resection is the preferred and key therapy strategy. Postoperative chemotherapy can prevent metastasis and recurrence of GC efficaciously, and is a well supplement for operation [2]. But, chemotherapy resistance has severely restricted the clinic application of chemotherapy. Thus, it is necessary to find a novel therapeutic target to improve the poor prognosis of GC.

In the past decade, non coding RNAs (ncRNAs) are the hotspot of medical oncology. MicroRNAs is a class of short ncRNAs, can silence their target genes to part in almost all biological processes, including cell proliferation, apoptosis, invasioness, autophagy and so on [3, 4]. Aberrant expression level and functional roles of microRNAs were confirmed to be involved in most of human diseases, especially in tumors by acting as oncogenes or tumor suppressor genes [5–7]. MiR-16-1 gene was cloned by independent groups which mapping to 13q14.2. MiR-16-1 has been shown to be deleted in more than half of B cell chronic lymphocytic leukemias (CLL) [8]. MiR-16-1 had been found high-expression in some malignant cancers, such as non-small cell lung carcinoma, colorectal cancer, nasopharyngeal carcinoma, glioma and osteosarcoma, and acted as a tumor suppressor to modulate critical biological characteristics [9–13]. Recent studies reported that miR-16-1 also exerted tumor suppressor function in GC [14, 15]. Nevertheless, little is known about its correlation with chemosensitivity of GC, and the regulatory effects and mechanism of miR-16-1 to chemosensitivity of GC is still unknown.

In this study, this hypothesis will be validated whether miR-16-1 could targeted silence FUBP1 to advance the

chemosensitivity to adriamycin in GC, which may provide a novel potential therapeutic target and improve the clinical therapeutic effects in GC.

Material and Methods

Clinical Specimens

46 GC specimens treated with adriamycin were gathered from Affiliated Second Hospital of Dalian Medical University through gastroscope from Oct 2012 to Apr 2015. According to the therapeutic effects, those patients were divided into sensitive group (n=19) and resistant group (n=27). This study was approved by the Ethics Committees of Dalian Medical University, and permissions of surgical patients were achieved before operation. The tissue samples were stored in liquid nitrogen.

Cell Culture

Human gastric cancer SGC7901 cells were stored in our laboratory and cultured in DMEM medium with 10% fetal bovine serum (Gibco, USA) with 5% CO_2 at 37 °C. SGC7901/AR cell line (resistant to adriamycin) was gifted from Deportment of surgical oncology in Affiliated First Hospital of China Medical University and cultured with 1 μg/mL adriamycin (Sigma, USA) to maintain the sensitivity phenotype [16].

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, USA), and the expression of miR-16-1 and FUBP1 expression was examined with One Step SYBR RT-PCR Kit (TaKaRa, Japan). The primers of FUBP1 were 5′- ACACCCGA AAGGATAGCAC -3′ (sense) and 5′- TTGCCTTG ACCTCTACCTC -3′ (antisense). The relative expression of miR-16-1 and FUBP1 was quantified with relative quantitative method when normalized to the expression of endogenous control gene U6 and GAPDH.

Vector Construction and Transfection

MiR-16-1 agonist (agomir-16-1) and agomir-NC were achieved from GenePharma Company (Shanghai, China). FUBP1 silence vector pS-FUBP1 and negative control vector (pS-NC) were constructed by Genescript Company (Nanjing, China). The microRNAs and vectors were transfected respectively with Lipofectamine 3000 (Invitrogen, USA) according to manufacturer's instructions. G418 (Invitrogen, CA, USA) was used to establish stable cell lines, and qRT-PCR was applied to validate the silenced efficiencies.

Luciferase Reporter Assay

The wild-type luciferase reporter vector pmiR-FUBP1-wt and mutant-type vector pmiR-FUBP1-mut were constructed by the Genescript company (Nanjing, China) which included wild-type binding site and mutant binding site in 3′ untranslated region (3'UTR) of FUBP1. The microRNAs and luciferase reporter vector were cotransfected into HEK 293 T cell line, and relative luciferase activity was examined with dual-luciferase reporter assay system (Promega, USA) according to manufacturer's instructions.

Western Blot

Protein was extracted by lysing with RIPA buffer (Beyotime, Shangahi, China) and the concentration was determined using the BCA assay (Beyotime Biotechnology,China) according to manufacturer's instructions. Total 30 μg of protein were subjected to 10% SDS-PAGE and transferred to a 0.22 μm PVDF membrane. After incubated with specific antibodies on a shaker overnight at 4 °C, signals were visualized using the infrared labeled antibody and scanned with the Dual Color Infra-red Laser Imaging System (Gene, HK, China) by normalized to the inference gene of GAPDH.

Cell Proliferation Assay and Chemosensitivity Assay

The cells (2×10^4/well) were seeded in 96-well plate. 96-well plate was added with 10 μl Cell Counting Kit-8 (Beyotime, Jiangsu, China) per well, and incubated for 2 h at 37 °C. Absorbance at 450 nm was recorded using the SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cells in 96-well plates (3000 cells/well) were treated with adriamycin at various concentrations (0.1 μg/mL, 1 μg/mL, 5 μg/mL, 10 μg/mL, 20 μg/mL) 24 h later [16]. The cell viability was detected 48 h later. The dose-response curve was drew to count the half maximal inhibitory concentration (IC50) using a Probit regression model.

Apoptosis Detection

Annexin V-FITC apoptosis detection kit (KeyGEN, China) was used to examine the apoptosis rate was examined according to the manufacturer's instructions. The apoptosis data was analyzed by CELLQuest 3.0 software (BD, Franklin Lakes, NJ, USA). Cells in the right lower quadrant were regarded as apoptosis.

Invasion Assay

Cell invasion assay in vitro was detected by Transwell chamber (Costar, Corning, NY, USA) with polycarbonic membrane (6.5mmin diameter, 8 μm pore size) and Matrigel (BD, Franklin Lakes, NJ, USA) according to manufacturer's instructions. The lower surface were stained with haematoxylin & eosine and counted from randomly chosen fields.

Statistical Analysis

All data were showed as mean ± SD of five independent experiments and analyzed with SPSS 21.0 software (IBM, Somers, NY, USA). The difference comparison between them was analyzed by pared Student's t-test and one-way ANOVA. The correlation between the expression of miR-16-1 and FUBP1 expression was analyzed by Spearman's rank test. $P < 0.05$ means significant difference.

Results

Low-Expression of miR-16-1 was Related Positively with Chemosensitivity to Adriamycin of GC

Firstly, the expression level of miR-16-1 in GC patients treated with adriamycin was detected, and results showed the miR-16-1 expression in resistant group patients was much lower than that in sensitive group (Fig. 1a, $P<0.05$).

Secondly, the IC50 of SGC7901 and SGC7901/AR cell lines to adriamycin were 1.27 ± 0.19 μg/mL and 5.26 ± 0.37 μg/mL (Fig. 1b, $P<0.05$). Compared with SGC790 cell line, the sensitivity of SGC7901/AR cell line was much lower. And, the following qRT-PCR assay found the expression of miR-16-1 in SGC7901/AR cell line was down-regulated compared to SGC7901 cell line (Fig. 1c).

Finally, transfection with agomir-16-1 could inhibit the IC50 of adriamycin and advance the chemosensitivity of SGC7901/AR cell line to adriamycin. Together, these founding proved that low-expression of miR-16-1 was related positively with chemosensitivity to adriamycin of GC.

FUBP1 Gene was a Target Gene of miR-16-1 in GC

Firstly, the potential target genes of miR-16-1 were predicted by bioinformatics analysis, and a conserved binding site of miR-16-1 was found in the 3'UTR of FUBP1 at 807–814 bp (Fig. 2a).

Secondly, the expression of FUBP1 in adriamycin-resistant GC tissues and cell line was much higher than that in sensitive tissues and cell line (Fig. 2b, c, $P<0.05$). And, the expression of them showed a significant inverse correlation ($rs = -0.812$, $P < 0.01$).

Thirdly, luciferase reporter assay was used to verify the direct interaction between miR-16-1 and FUBP1. Results showed that miR-16-1 could combine the wild-type binding site, but not to mutant binding site, to inhibit the relative luciferase activity (Fig. 2d, $P<0.05$). And, the agomir-NC had no similar regulatory effects.

Finally, western blot assay indicate that miR-16-1 overexpression could significantly depress the FUBP1 protein expression in SGC7901/AR cell line (Fig. 2e, $P<0.05$). To sum up, miR-16-1 could specifically silence the expression of FUBP1, FUBP1 was a target gene of miR-16-1.

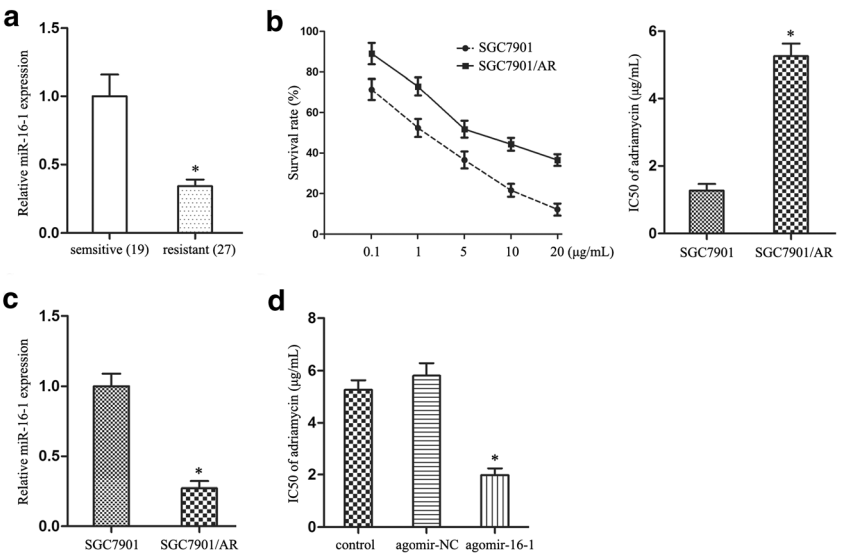

Fig. 1 Low-expression of miR-16-1 was related positively with chemosensitivity to adriamycin of GC. **a** The expression of miR-16-1 in GC patients treated with adriamycin. **b** The dose-response curve and IC50 of SGC7901 and SGC7901/AR cell lines to adriamycin. **c** The expression of miR-16-1 in SGC7901 and SGC7901/AR cell lines. **d** The IC50 of SGC7901/AR cell line with agomir-16-1 transfection. * $P < 0.05$

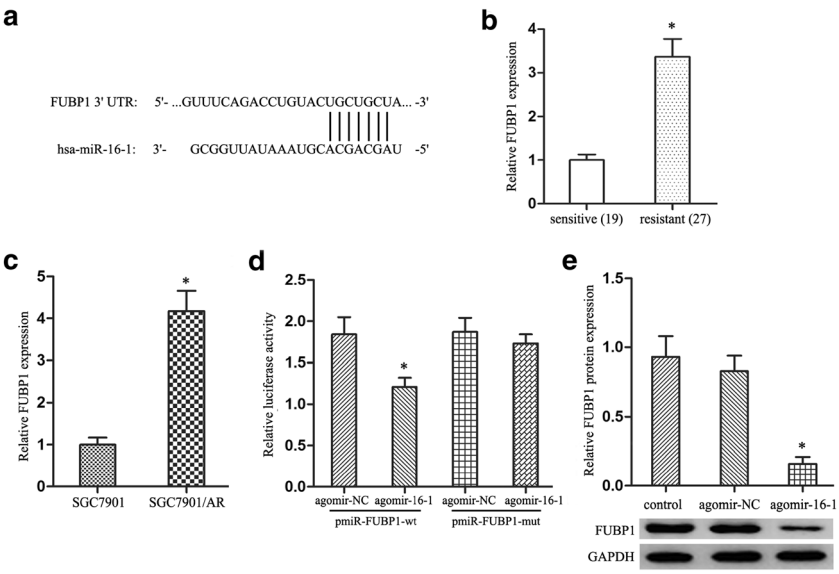

Fig. 2 FUBP1 was a target gene of miR-16-1 in GC. **a** The conserved binding site of miR-16-1 in the 3′ UTR of FUBP1. **b** The expression of FUBP1 in GC patients treated with adriamycin. **c** The expression of FUBP1 in SGC7901 and SGC7901/AR cell lines. **d** The relative luciferase activity of HEK 293 T cell line. Firefly luciferase activity was normalized to Renilla luciferase. **e** The expression of FUBP1 protein in SGC7901/AR cell line.* $P < 0.05$

Silence of FUBP1 Advanced Chemosensitivity of SGC7901/AR Cell Line to Adriamycin

Fig. 3a showed the expression of FUBP1 in SGC7901/AR cells was silenced by pS-FUBP1 transfection ($P < 0.05$).

The IC50 of adriamycin was depressed by FUBP1 silence from 5.26 ± 0.37 μg/mL to 1.79 ± 0.21 μg/mL in SGC7901/AR cell line (Fig. 3b, $P<0.05$), which demonstrated that FUBP1 silence advanced chemosensitivity of SGC7901/AR cell line to adriamycin.

Furthermore, SGC7901/AR cell line was treated with 0.5 μg/ml adriamycin, and the impact of FUBP1 silence to cell biological behaviors was examined. In comparison to the NC groups, FUBP1 silence inhibited the cell viability and invasioness of SGC7901/AR cell line, and promoted apoptosis (Fig. 3c-e, $P<0.05$).

Discussion

MicroRNAs are well known to part in almost all vital biological characteristics, including chemosensitivity, which restrict the clinic application of chemotherapy and result in poor prognosis. For example, Lu C et al. reported that miR-129 was down-regulated in cisplatin-resistant GC tissues/cells, and over-expression of miR-129 decreased cisplatin-resistance in cisplatin-resistant GC cells through inhibiting the P-gp expression [17]. And, miR-1284 inhibited the chemoresistance to

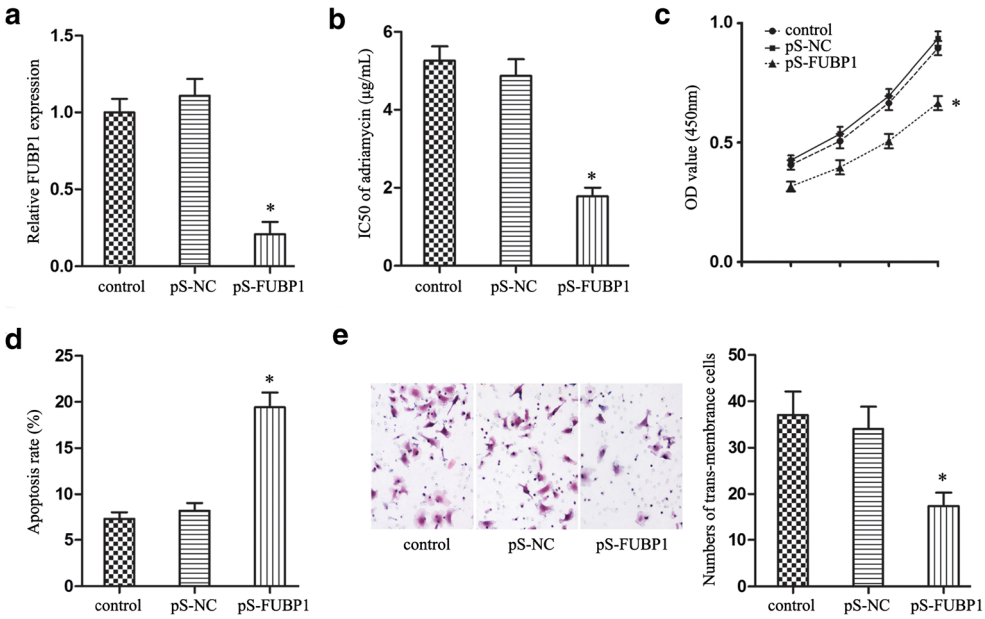

Fig. 3 Silence of FUBP1 advanced chemosensitivity of SGC7901/AR cell line to adriamycin. **a** The expression of FUBP1 in SGC7901/AR cell line with pS-FUBP1 transfection. **b** The IC50 of SGC7901/AR cell to adriamycin. **c** The cell viability of SGC7901/AR cell line. **d** The cell apotosis of SGC7901/AR cell line. **e** The invasioness of SGC7901/AR cell line. * $P < 0.05$

vincristine of SGC7901/VCR cells, blocked at the G0/G1 phase, advanced cell apoptosis, and depression migration and invasiveness [18].

In this study, high-expression of miR-16-1 in adriamycin-resistant GC tissues and cell line was found, and mir-16-1 over-expression could advanced the chemosensitivity of SGC7901/AR cell line to adriamycin. These founding proved that low-expression of miR-16-1 was related positively with chemosensitivity of GC to adriamycin, but the regulatory mechanism was still unknown.

Given that microRNAs play their roles by silencing their target genes, the hypothesis was set up that miR-16-1 can regulate some target genes to modify the regulative network in GC. So, the potential target genes of miR-16-1 were predicted by bioinformatics analysis, and a conserved binding site of miR-16-1 was found in the 3′ UTR of FUBP1.

FUPB1 gene is a member of single-stranded DNA binding protein family, and expressed generally in human tissues. FUPB1 controlled the expression of tumor-related gene c-myc in transcript level, and participate in the regulation of biological behaviors [19, 20]. It also can specifically combine with some RNAs, and play a regulative role in cell growth and neuronal differentiation [18]. Recently, the aberrant expression of FUBP1 gene had been reported in some malignant tumors, including hepatocellular carcinoma, lung cancer and nasopharyngeal carcinoma, and display potential oncogene roles [21–23].

To discuss the roles of FUBP1 in GC chemoresistance, the expression of FUBP1 was silenced by pS-FUBP1 transfection. Then, the results showed FUBP1 silence decreased the IC50 of adriamycin, advanced chemosensitivity of SGC7901/AR cell line. Moerover, after treatment with 0.5 μg/ml adriamycin, FUBP1 silence inhibited the cell viability and invasioness of SGC7901/AR cell line, and promoted apoptosis. Hong Y et al. found that FUBP1 silence could enhance the chemosensitivity of glioma U251 cells to DDP [24]. In summary, silence of FUBP1 advanced significantly chemosensitivity of GC drug-resistant cells to adriamycin, which might be a novel potential therapeutic target for GC.

Recent literatures reported FUBP1 gene bound to the far upstream element (FUSE) of some target genes, such as c-Myc, to modulate their expression, and play its oncogene role in some malignant tumors [25, 26]. FUBP1 might play its oncogene role in GC through regulating its target genes expression, but the target genes and regulative mechanism in GC cells is still unknown, which need more intensive investigations.

In conclusion, the expression level of miR-16-1 was positively related with the chemosensitivity of GC to adriamycin, and miR-16-1 could targeted silence FUBP1 to advance the chemosensitivity to adriamycin in GC, which might provide a novel potential therapeutic target and improve the clinical therapeutic effects in GC.

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Author Contributions DZ and LS participated in the study design and drafted the manuscript. DZ and YZ carried out the in vitro studies and performed the statistical analysis. DZ conceived of the study and helped to draft the manuscript. All authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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