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RESEARCH

MicroRNA-200c Regulates the Sensitivity of Chemotherapy of Gastric Cancer SGC7901/DDP Cells by Directly Targeting RhoE

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Abstract Gastric cancer remains a worldwide burden as the second leading cause of cancer-related death. Drug resistance of chemotherapy looms as a major clinical obstacle to successful treatment. Recent evidence indicated that miRNA-200c can restore the sensitivity of NSCLC cells to cisplatin and cetuximab. The expression of miRNA-200c and RhoE were investigated in gastric cancer tissues and cells (SGC7901 and SGC7901/DDP) by qRT-PCR. A luciferase reporter assay was done to understand the potential correlation between miRNA-200c and RhoE. Pre-miR-200c was transfected in SGC7901/DDP cells to confirm whether miRNA-200c could regulate RhoE expression. RhoE was knocked down to explore the role of RhoE on sensitivity of chemotherapy in gastric cancer by MTT. Western blot analysis was performed to further explore the mechanism of RhoE in regulating drug resistance. The results showed that miRNA-200c was significantly lower in cancerous tissues than those in the paired normal tissues, whereas the expression of RhoE was just the opposite. The significant difference of miRNA-200c and RhoE were observed between SGC7901 cells and SGC7901/DDP cells. miRNA-200c has target sites in the 3'-UTR of RhoE mRNA by luciferase reporter assay. Transfection of pre-miR-200c reduces RhoE expression. Meanwhile, the knockdown of RhoE enhanced the sensitivity of SGC7901/DDP cells and changed expression of some genes. These suggested that miRNA-200c regulated the sensitivity of chemotherapy to cisplatin (DDP) in gastric cancer by possibly targeting RhoE.

Keywords MicroRNA-200c · Gastric cancer · RhoE · Drug resistance

Introduction

Gastric cancer still remains a worldwide burden as the second leading cause of cancer-related death. The incidence is higher in Eastern countries (such as Japan, Korea and China) compared to the west [1, 2]. Since the disease is often diagnosed at advanced stages, the available therapeutic methods in most patients are limited. Meanwhile, chemotherapy is an important option, but drug resistance looms as a major clinical obstacle to successful treatment in gastric cancer [3–5]. Therefore, the prognosis with a reported 5-year survival rate still remains poor.

MicroRNAs (miRNAs) are 19–25 nucleotides of noncoding RNA molecules that mediate posttranscriptional gene expression through the 3'-UTR of their target mRNAs [6, 7]. miRNAs play a crucial role in many complex biological processes, including cancer development, proliferation, differentiation and apoptosis. miRNA-200c is a member of the miRNA-200 family that play an important role in the epithelial mesenchymal transition (EMT) [8]. Recent evidence indicated that miRNA-200c inhibits lung adenocarcinoma cell invasion and metastasis by targeting Flt1/VEGFR1 [9]. Yu et al. reported that miRNA-200c is an independent prognostic factor in pancreatic cancer and its upregulation inhibits pancreatic cancer invasion but increases cell proliferation [10]. In addition, miRNA-200c was found to be restore the sensitivity of NSCLC cells to cisplatin and cetuximab

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[11]. Chen et al. showed that miRNA-200c may act as a promising therapeutic target for improvement of responsiveness to chemotherapy in breast cancer [12]. However, it remains unknown whether miRNA-200c can modulate the sensitivity of chemotherapy in gastric cancer, and the target gene of miRNA-200c in regulate drug resistance remains unacquainted.

RhoE (Rnd3), Rnd1 and Rnd2 constitute a separate subgroup of Rho GTPase family. Unlike the other Rho family proteins, RhoE can bind to GTP but lacks intrinsic GTPase activity [13, 14]. Increasing evidence showed that RhoE can regulate a series of cellular functions, including cell cycle progression, cell transformation, cell migration and apoptosis [15]. Li et al. demonstrated that RhoE may promote the multidrug resistance phenotype of gastric cancer cells by decreasing the expression of Bax at posttranscriptional level, thus inhibiting vincristine-induced apoptosis [16].

We have found that RhoE may serve as a target gene of miRNA-200c through targetscan and pictar bioinformatics software. In this study, we intended to observe that miRNA-200c regulates the sensitivity of chemotherapy to cisplatin (DDP) in gastric cancer by possibly targeting RhoE.

Materials and Methods

Human Tissue Samples

This study included 27 patients with gastric cancer at the Fourth Hospital of Hebei Medical University from 2010 to 2011. The fresh cancer tissues and the paired normal tissues (5 cm away from tumor) were obtained from surgical resection specimens collected by the department of surgery. The study was approved by the Ethics Review Board at the Fourth Hospital of Hebei Medical University. The informed consent was obtained from each subject. All tissue specimens were immediately cut and snap frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Histology of each tumor tissues and paired non-cancerous tissue were independently evaluated by experienced pathologists.

Cell Culture

The human gastric cancer SGC7901 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai,China). Its drug-resistant SGC7901/DDP cells were purchased from the Keygen Biotech development Co., LTD (Nanjing, China). The cell lines were cultured in RPIM-1640 (Gibco, USA) medium with 10 % fetal bovine serum (Hangzhou sijiqing, China), and maintained in a humidified incubator at 37 °C with atmosphere of 5 % CO_2.

Luciferase Reporter Assay

The DNA encoding 3'UTR of RhoE was PCR-amplified from human genomic DNA and was cloned downstream of firefly luciferase reporter gene in pGL3-control plasmid (Promega). Gastric cancer SGC7901 cells in 96-well plates were transfected with lipofectamine 2000 with the RhoE 3'-UTR reporter or empty vector, and pre-miR-NC or pre-miR-200c according to the manufacturer's instructions.

Real-Time Quantitative PCR

Total RNA including miRNA was extracted from the tissues and cell lines using the miRNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Total RNA was subsequently reverse transcribed to cDNA. The U6 small nuclear RNA was used as internal controls for miRNA-200c. The GAPDH was used as internal controls for RhoE. Primer sequences used were listed in Table 1.

Cell Viability Assay

The SGC7901/DDP cells were transfected with RhoE siRNA and its negative control. The transfected cells were seeded in 96-well plates for 24 h and then treated with different concentrations of DDP (QiLu Pharmaceutical Co.,LTD Shandong, China), 5-FU(KingYork Group Co.,LTD Tianjin, China), paclitaxel (Yangtze River Pharmaceutical Group Jiangsu, China) and ADR(Hisun Pharmaceutical Co.,LTD Zhejiang, China). After 48 h, the cell viability was evaluated by the MTT assay according to the manual. The absorbance at 490 nm was measured on an ELISA reader.

Table 1 Primer sequences for RT and real-time PCR

Gene	Primer	Sequence
miR-200c	RT	GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATACG ACTCCATCA
	PCR-F	GCCGATTTAATACTGCCGGGT
U6	RT	GTCGTATCCAGTGCAGGGTCCG AGGTATTCGCACTGGATAC GACAAAATATGGAACTGC
	PCR-F	GGGTGCTCGCTTCGGCAGC
Common	PCR-R	CAGTGCAGGGTCCGAGGT
RhoE	PCR-F	TTACCCTGATTCGGATGCTGT
	PCR-R	CTAACATCTGTCCGCAGATCA
GAPDH	PCR-F	AAGGTGAAGGTCGGAGTCAAC
	PCR-R	GGGGTCATTGATGGCAACAATA

Western Blot Analysis

The transfected cells were lysed in RIPA buffer. Protein concentration was determined by Protein Assay. 50 μg of protein per lane was separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (PVDF). The membrane was incubated with the RhoE, ROCK1, PTEN, p-AKT, Bcl-2, Bax or β-actin antibody (Santa Cruz Biotechnology), and then detected by the Odyssey two-color infrared imaging system.

Statistical Analysis

Data were expressed as the mean ± standard deviation. The results of assay were analyzed by Student's-test and One-way ANOVA. The findings were considered statistically significant at a value of $P<0.05$. All statistical tests were performed by Statistical Analysis System V8 (SAS Institute Inc. USA).

Results

Expression of miRNA-200c and RhoE in Gastric Cancer Tissues

The expression of miRNA-200c and RhoE in 27 cancerous (T) and matched non-cancerous (N) tissues were detected by qRT-PCR. The levels of expression were presented in Fig. 1a–b. Through the analysis of data, the result showed that miRNA-200c was significantly lower in cancerous tissues than those in the paired normal tissues ($P=0.0009$). Conversely, the RhoE was examined overexpressed in gastric cancer tissues compared with the corresponding normal tissues ($P=0.0303$).

Expression of miRNA-200c and RhoE in Gastric Cancer SGC7901 and SGC7901/DDP Cells

To investigate the potential role of miRNA-200c on drug resistance in gastric cancer, the expression of miRNA-200c

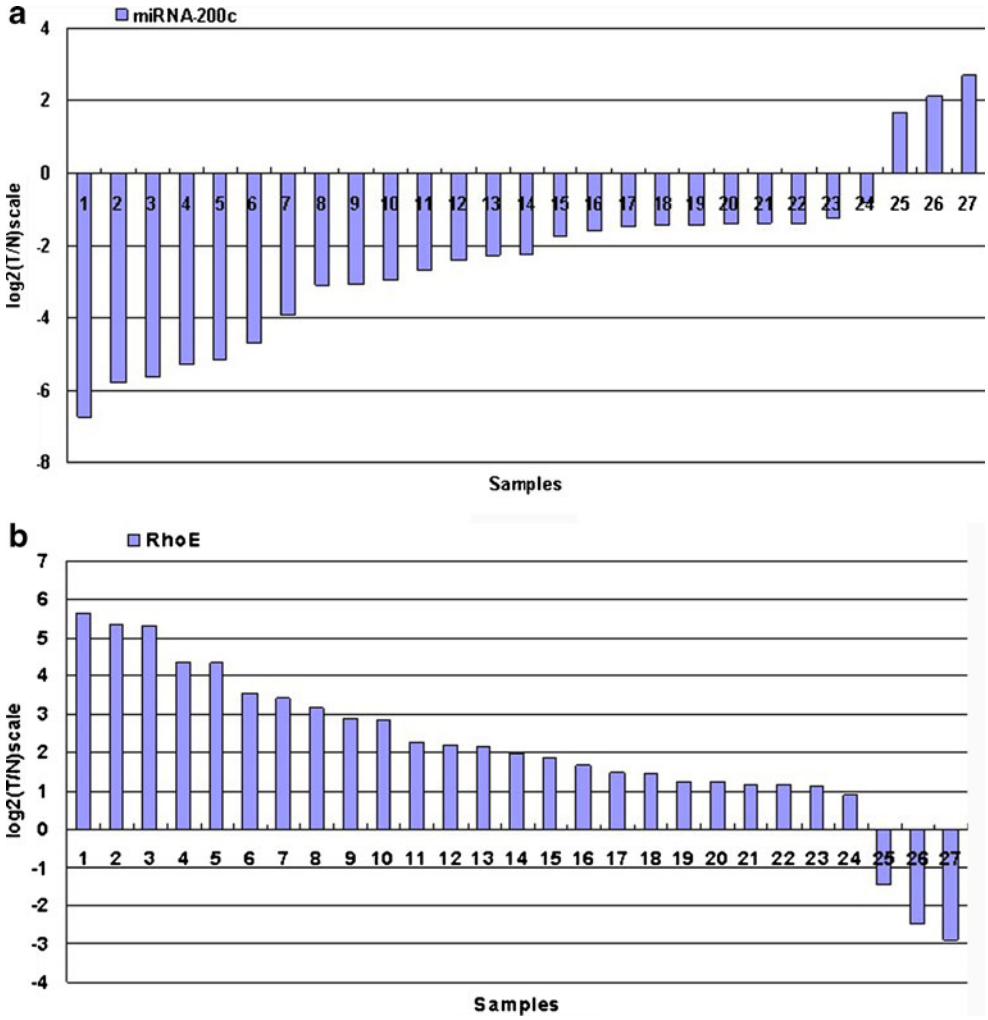

Fig. 1 Expression of miRNA-200c and RhoE in gastric cancer tissues. The expression of miRNA-200c (**a**) and RhoE (**b**) in 27 cancerous (T) and matched non-cancerous (N) tissues were detected by qRT-PCR

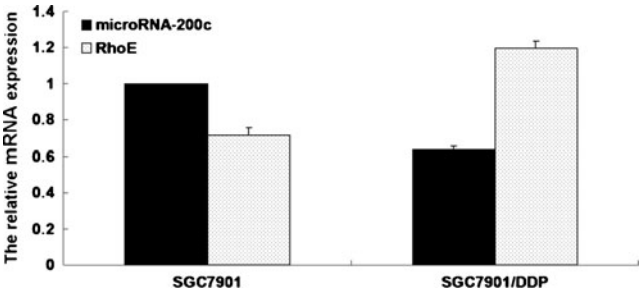

Fig. 2 Expression of miRNA-200c and RhoE in gastric cancer SGC7901 and SGC7901/DDP cells. To investigate the potential role of miRNA-200c on drug resistance in gastric cancer, the expression of miRNA-200c in SGC7901 and SGC7901/DDP cells was evaluated by qRT-PCR

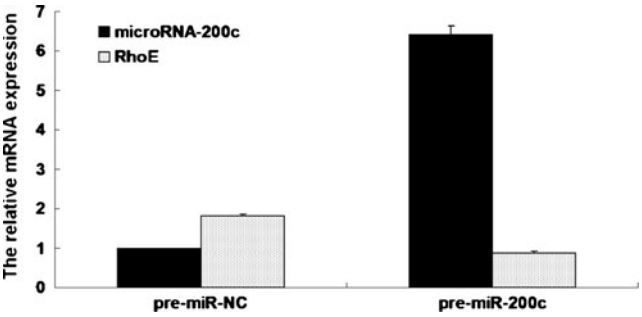

Fig. 4 Transfection of pre-miR-200c reduces RhoE expression. To investigate whether miRNA-200c could regulate RhoE expression, pre-miR-200c and its negative control were transfected respectively in SGC7901/DDP cells

in SGC7901 and SGC7901/DDP cells was evaluated by qRT-PCR. The significant difference was observed between SGC7901 cells and its drug-resistant SGC7901/DDP cells. The data were reported in Fig. 2. Meanwhile, the expression of RhoE was increased in SGC7901/DDP cells compared with in SGC7901 cells. There may be potential correlation between miRNA-200c and RhoE.

miRNA-200c Directly Targets the 3'-UTR of RhoE

To understand the potential correlation between miRNA-200c and RhoE, a luciferase reporter assay was done in SGC7901/DDP cells. The results were presented in Fig. 3. The luminescence intensity was significantly decreased in RhoE 3'-UTR reporter + pre-miR-200c transfected cells than empty vector + pre-miR-NC, empty vector + pre-miR-200c and RhoE 3'-UTR reporter + pre-miR-NC transfected cells. It suggested that miRNA-200c has target sites in the 3'-UTR of RhoE mRNA.

Transfection of pre-miR-200c Reduces RhoE Expression

To investigate whether miRNA-200c could regulate RhoE expression, pre-miR-200c and its negative control were transfected respectively in SGC7901/DDP cells. The expression of RhoE in two groups was detected by qRT-PCR. As shown in Fig. 4, the expression of RhoE was significantly lower in pre-miR-200c transfected cells than that in pre-miR-NC transfected cells.

The Knockdown of RhoE Enhances the Sensitivity of SGC7901/DDP Cells

To explore the role of RhoE on sensitivity of chemotherapy in gastric cancer SGC7901/DDP cells, a series of studies were done and shown in Fig. 5. Figure 5a demonstrated that the RhoE was successful knocked out through transfecting si-RhoE. As shown in Fig. 5b, the IC_{50} of ADR, paclitaxel, 5-FU and DDP were (0.59±0.17)mg/L, (2.16±0.25)mg/L, (6.95±0.24)mg/L and (7.38±0.09)mg/L in si-RhoE transfected cells, and were (0.98±0.22)mg/L, (3.59±0.37)mg/L, (11.31±0.19)mg/L and (12.54±0.41)mg/L in si-NC transfected cells. The sensitivity of chemotherapy was significantly decreased in si-RhoE transfected cells. The proliferation of si-RhoE transfected cells was significantly inhibited compared to the negative control since the third day after transfection.

The Expression Change of Genes After Knockdown of RhoE in SGC7901/DDP Cells

To further explore the mechanism of RhoE in regulating drug resistance in gastric cancer, Western blot analysis was performed in SGC7901/DDP cells. The expression of ROCK1, PTEN and Bax were significantly increased in si-RhoE transfected cells,

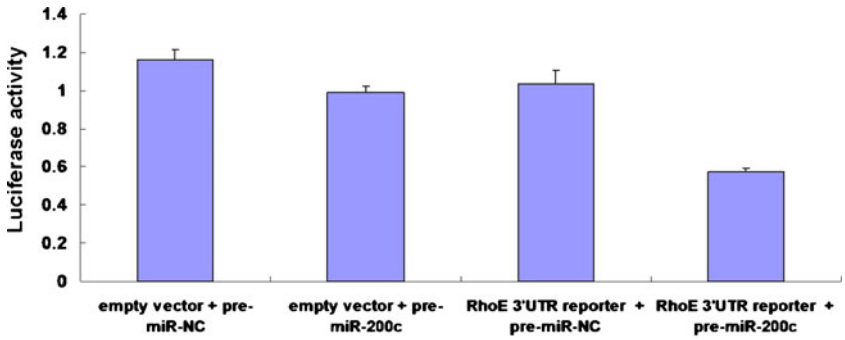

Fig. 3 miRNA-200c directly targets the 3'-UTR of RhoE. To understand the potential correlation between miRNA-200c and RhoE, a luciferase reporter assay was done in SGC7901/DDP cells

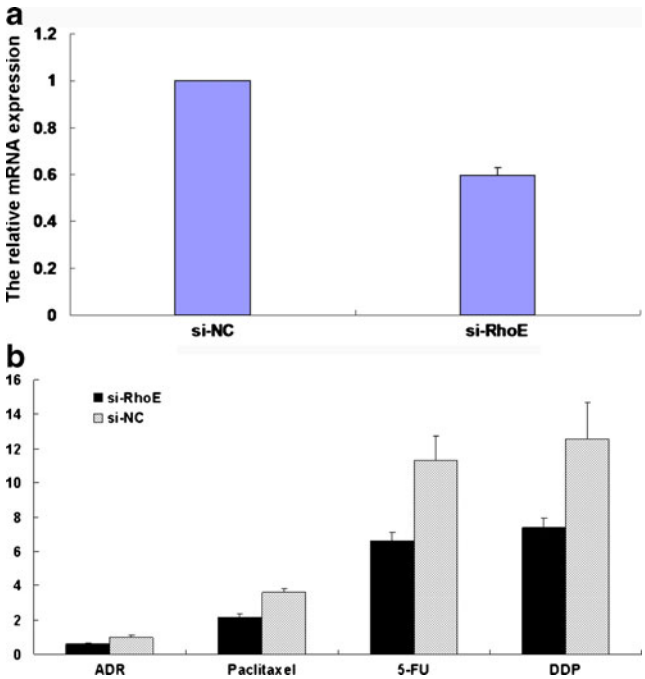

Fig. 5 The knockdown of RhoE enhances the sensitivity of SGC7901/DDP cells. **a** To explore the role of RhoE on sensitivity of chemotherapy in gastric cancer SGC7901/DDP cells, the IC50 of ADR, paclitaxel, 5-FU and DDP were detected. **b** The proliferation of cells was investigated

whereas the levels of p-Akt and Bcl-2 were significantly decreased (Fig. 6).

Discussion

MiRNAs are predicted to regulate up to 30 % of gene expression by binding with mRNAs of many genes. They have important roles in regulating cellular processes, such as proliferation, differentiation, apoptosis and especially oncogenesis, so they are key molecules in cancer diagnostics and therapeutics [17, 18]. Recent evidence indicated that miRNA-200 family has received much attention for suppressing epithelial-mesenchymal transition (EMT) that is a crucial process in cancer metastasis [19]. Bracken et al. reported that a double-negative feedback loop between ZEB1-SIP1 and the miRNA-200 family regulates EMT [20]. As a member of miRNA-200 family, miRNA-200c is paid more attention in recent studies. miRNA-200c can repress migration and invasion of breast cancer cells by targeting actin-regulatory proteins FHOD1 and PPM1F [21]. Lo et al. found that miRNA-200c attenuates tumour growth and metastasis of presumptive head and neck squamous cell carcinoma stem cells [22]. A recent study showed that miRNA-200c mitigates invasiveness and restores sensitivity to microtubule-targeting chemotherapeutic agents by reducing the target gene TUBB3 [23]. Leskelä et al. reported that miRNA-200c is associated with paclitaxel-based treatment response and progression-free survival in ovarian cancer patients [24]. However, it remains unclear that the role of miRNA-200c in drug resistance of gastric cancer. We tested the expression of miRNA-200c in gastric cancer tissues and cells by qRT-PCR. Down-regulated miRNA-200c was observed in cancerous tissues and SGC7901/DDP cells compared to matched normal tissues and SGC7901 cells. The results showed that miRNA-200c was bound up with diagnosis and sensitivity of chemotherapy of gastric cancer.

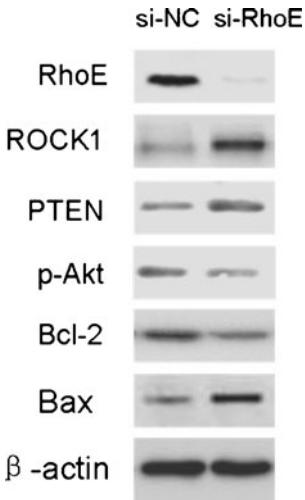

Fig. 6 The expression change of genes after knockdown of RhoE in SGC7901/DDP cells. Western blot analysis of ROCK1, PTEN, p-Akt and Bcl-2and Bax was performed in SGC7901/DDP cells

RhoE is an atypical member of Rho GTPase family and confirmed to inhibit cytoskeleton formation. Recently, studies reported that RhoE plays a key role in tumorigenesis and progression [25]. Poch et al. found that RhoE disrupts actin cytoskeleton organization and inhibits U87 glioblastoma cell proliferation [26]. Evidence indicated that overexpression of RhoE in non-small cell lung cancer (NSCLC) is associated with smoking and correlates with DNA copy number changes, and suggested that RhoE may serve as a molecular marker to identify high-risk individuals and assist in the identification of additional pathways of carcinogenesis [27]. Klein et al. observed that BRAF inhibitor treatments are associated with reduced expression of RhoE (Rnd3) in melanoma cell [28].

Our study found that RhoE may serve as a target gene of miRNA-200c through targetscan and pictar bioinformatics software. In this test, a luciferase reporter assay was done in SGC7901/DDP cells, and the results showed that miRNA-200c has target sites in the 3'-UTR of RhoE mRNA. We observed that transfection of pre-miR-200c reduces RhoE expression in SGC7901/DDP cells. Meanwhile, the knockdown of RhoE enhances the sensitivity of SGC7901/DDP cells by MTT and growth curve assay. The study demonstrated that RhoE may inhibit vincristine-induced apoptosis of gastric cancer cells by decreasing the expression of Bax [16]. Here, our results also found that the knockdown of

RhoE can change expression of genes in SGC7901/DDP cells, including up-regulated ROCK1, PTEN and Bax. Our findings showed that miRNA-200c directly targets the 3'-UTR of RhoE, and the knockdown of RhoE enhances the sensitivity of SGC7901/DDP cells and changes expression of some genes.

Conclusions

To summarize, these findings suggested that miRNA-200c regulates the sensitivity of chemotherapy to cisplatin (DDP) in gastric cancer SGC7901/DDP cells by directly targeting RhoE.

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Disclosure Statement There were no competing financial interests exist.

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