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Epigenetic silencing of miR-130b in ovarian cancer promotes the development of multidrug resistance by targeting colony-stimulating factor 1

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ABSTRACT

Objective. The purpose of this study was to investigate the role of miR-130b in the development of multidrug-resistant ovarian cancer.

Methods. The expression of miR-130b was assessed in ovarian tissues and cell lines by qRT-PCR. In vitro, miR-130b level was manipulated by transfection with mimics or inhibitors. Methylation level of miR-130b was evaluated by quantitative methylation-specific PCR (qMSP). CSF-1 expression in ovarian tissues and cells was determined by qRT-PCR, immunohistochemistry and ELISA, respectively. CSF-1 regulated by miR-130b was detected using Dual Luciferase Reporter system.

Results. Down-regulation of miR-130b in ovarian cancer was associated with FIGO III–IV clinical stages and poorer histological differentiation. MiR-130b was downregulated in multidrug resistant ovarian cancer cells. Restoration of miR-130b expression could sensitize these cells to anticancer drugs. MiR-130b hypermethylation was found in ovarian cancer tissues as well as in drug resistant cell lines and the methylation level was negatively correlated with its expression. Demethylation with 5-aza-CdR led to reactivation of miR-130b expression in drug resistant ovarian cancer cell lines concomitant with increase of sensibility to cisplatin and taxol. CSF-1 expression was negatively associated with miR-130b level in ovarian tissues and cell lines. Luciferase assay validated CSF-1 is a direct target of miR-130b. Knock-down of CSF-1 sensitized ovarian cancer cells to anticancer drugs and could partially attenuate the resistance inducing effect of miR-130b inhibitors.

Conclusions. Downregulation of miR-130b promotes the development of multidrug resistant ovarian cancer partially by targeting the 3′-UTR of CSF-1, and the silencing of miR-130b may be mediated by DNA methylation.

1. Introduction

Ovarian cancer is a common gynecologic malignancy and a leading cause of cancer death among women [1]. Most deaths of ovarian cancer are due to resistance to chemotherapies [2]. Generally, the molecular genetic basis of resistance to cancer therapeutics is complex, involving multiple processes, such as drug transport, drug metabolism, DNA repair and apoptosis [3]. Unfortunately, the factors that regulate the chemo-resistance of ovarian cancer remain poorly understood.

MicroRNAs (miRNAs) are endogenous, small non-coding RNAs (~22 nt) that negatively regulate gene expression via RNA interference (RNAi) machinery [4]. miR-130 family, including miR-130a and miR-130b, has been reported to be deregulated in some cancer types, such as hepatocellular carcinoma, lung carcinoma and gastric cancer [5–7]. Furthermore, down-regulation of miR-130a/b was observed in head and neck squamous cell carcinoma with docetaxel-induced drug resistance [8]. Currently, using miRNA microarray, Sorrentino et al. screened out miR-130b was one of the six miRNAs, which deregulated in all the four drug-resistant A2780 ovarian cancer cell lines used in their work [9]. Although these evidence implicated that miR-130 family is associated with carcinogenesis and development of MDR, the role of miR-130b in multidrug resistant ovarian cancer largely remains unexplored.

Besides gene expression studies, to define the role of miR-130b in multidrug resistant ovarian cancer, we explored mechanisms connected to its deregulation and identified a target gene of miR-130b involved in molecular pathway in drug resistance. Using real-time quantitative PCR (qRT-PCR), we analyzed miR-130b expression in malignant, benign and normal ovarian tissues from patients. On the cellular level, we investigated the role of miR-130b in aquisition of resistance to cisplatin and paclitaxel by functional experiments using specific mimics and inhibitors. Furthermore, we tested whether

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miR-130b silencing in ovarian cancer is associated with cytosine methylation in adjacent CpG island by performing a epigenetic association study. Finally, using bioinformatics prediction combined with gene expression assay and luciferase assay, we identified CSF-1 a direct target of miR-130b.

2. Material and methods

2.1. Human ovarian tissue specimens

One hundred and four human ovarian tissues were collected from women aged from 35 to 71 years old attending the Gynecology and Obstetrics Department of the Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between 2004 and 2010; patients who had undergone preoperative radiotherapy or chemotherapy were excluded. Among these cases, 81 were malignant ovarian tumors; 7 were benign ovarian tumors; 16 were normal ovarian tissues from non-cancerous prophylactic oophorectomy specimens. All of the tumor samples were obtained from primary tumor site. Diagnosis was confirmed by histopathology in all cases. The percentage of malignant part in each malignant sample was over 85%. Within the 81 malignant ovarian tumors, 22 cases were in stage I–II and 59 in stage III–IV, all based on the staging system of the International Federation of Gynecology and Obstetrics (FIGO stage).

2.2. Cell lines and cell culture

A2780 and SKOV3 were purchased from China Center for Type Culture Collection (CCTCC), Wuhan University, P. R. China. Cells were cultured in DMEM medium (Gibco BRL) supplemented with 50 IU/ml of penicillin, 50 μg/ml streptomycin and 10% fetal bovine serum (FBS). The cisplatin-resistant A2780 (A2780/CP), paclitaxel-resistant A2780 (A2780/TAX) and paclitaxel-resistant SKOV3 (SKOV3/TAX) cell lines were generated in our laboratory upon continuous exposure to cisplatin or paclitaxel [10].

2.3. miRNA expression analysis

Total RNA of cell lines and tissues were isolated using Trizol (Invitrogen, USA). Mature miRNAs were reversely transcribed with miScript Reverse Transcription Kit according to manufacturer's instruction (Qiagen, Germany). Real-time quantitative reverse transcription-PCR (qRT-PCR) was carried out using miScript SYBR-Green PCR Kit (Qiagen, Germany). The endogenous U6 snRNA was used as internal control. Each reaction was done in triplicate. The fold change for miRNA expression level was calculated using $2^{-\Delta\Delta CT}$ method [11].

2.4. Transfection of miR-130b mimics and inhibitors

Before transfection, cells were plated in 6-well plates (2×10^5/well). When the cells were 70% confluent, 100 pmol miR-130b mimics (target sequence CAG UGC AAU GAU GAA AGG GCA U, GenePharma, Shanghai, China) or inhibitors (target sequence AUG CCC UUU CAU CAU UGC ACU G, GenePharma, Shanghai, China) or their negative control siRNAs (GenePharma, Shanghai, China) were added. Transfection was performed with Lipofectamine 2000 Reagent following the manufacturer's protocol (Invitrogen, USA).

2.5. Drug sensitivity assay

The 50% inhibitory drug concentration (IC50) was determined using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazoliumbromide) assay. Cells were plated in 96-well plates (5000 cells/well), and after cellular adherence, the cells were exposed to paclitaxel or cisplatin in different concentrations. After incubation for 48 h, the cells were incubated with 20 μl MTT (at a final concentration of 0.5 mg/ml) at 37 °C for 4 h. The medium was removed and the precipitated formazan was dissolved in 100 μl DMSO. The absorbance at 490 nm was detected using microplate reader (BIO-RAD, USA). The IC50 was estimated by the relative survival curve. Each assay was performed in triplicate.

2.6. Cell viability assay

After cells were transfected with miR-130b mimics, inhibitors or CSF-1 shRNA plasmid, the viability was assessed by cell numbers counting after trypan blue staining.

2.7. 5-aza-CdR treatment and quantitative methylation specific PCR analysis

Using the CpG Island Searcher web tool (http://www.cpgislands.com/), a CpG island was identified upstream (−300 bp) of the region encoding miR-130b. A2780/TAX and A2780/CP cells were treated with 5 μM 5-aza-CdR or dimethyl sulfoxide (DMSO) for 72 h. For methylation analysis, genomic DNA was extracted from the tumor samples or the cells by using Genomic DNA extraction kit (Tiangen biotech, China). One microgram of genomic DNA was subjected to treatment with the CpGenome DNA Modification Kit (Chemicon, USA) according to the manufacturer's instructions. The bisulfite modified DNA was then suspended in 25 μl of deionized water and used immediately or stored at −80 °C until use. Methylation of the CpG island was analyzed using a SYBR Green-based quantitative methylation-specific PCR (qMSP). Two sets of PCR primers were designed using the MethPrimer program (http://www.urogene.org//methprimer). The primers for methylated sequence are 5′-AAA GAT GGA GTC GGT AGG C-3′ (forward) and 5′-AAA CGC GAA AAA TTA AAC GA-3′ (reverse), and the primers for unmethylated sequence are 5′-GTT AAA GAT GGA GTT GGT AGG T-3′ (forward) 5′-AAA CAC AAA AAA TTA AAC AAA AA-3′ (reverse). The PCR conditions were as follows: 95 °C for 15 min and then followed by 40 cycles of 94 °C for 30 s, 50 °C for 45 s and 72 °C for 1 min. The percentage of CpG methylation in a sample was estimated as Lu L et al. described [12].

2.8. Immunohistochemistry

Immunohistochemical staining was performed on formalin-fixed, paraffin-embedded tissues. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was carried out in citrate buffer (10 mM, pH-6) for 20 min at 100 °C. Tissue sections were incubated with the CSF-1 antibody (1:100, Boster, China) for 16 h at 4 °C and subsequently incubated with a secondary biotinylated antibody (1:200, Boster, China) for 2 h at 37 °C followed by

Fig. 1. Deregulation of miR-130b is associated with in ovarian cancer. A, box plots show results of qRT-PCR analysis of miR-130b expression in 81 malignant, 7 benign and 16 normal ovarian tissues. The vertical ordinate is logarithm of miR-130b expression level normalized to U6 RNA ($2^{-\Delta\Delta CT}$). Each box shows median, 75th and 25th percentile value. B, qRT-PCR analysis of miR-130b expression in ovarian cancer cells. The values correspond to the miR-130b/U6 RNA ratio ($2^{-\Delta\Delta CT}$). The miR-130b level in A2780 cells was set as 1. Student's *t* test, *** $P<0.001$, compared to parental cells. Bars, SD. C, qRT-PCR analysis of miR-130b expression in multidrug resistant ovarian cancer cell lines after transfection with mimics or inhibitors of miR-130b. Bars, SD. Student's *t* test, *** $P<0.001$, compared to NC. D, MTT analysis of cell viability after transfection with mimics or inhibitors of miR-130b. Data are represented as mean ± SD, each one done in triplicate. E, viable cell numbers were counted 48 h after treatment. Student's *t* test, * $P<0.05$.