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Down-regulated expression of miR-134 contributes to paclitaxel resistance in human ovarian cancer cells

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ABSTRACT

MiR-134 has been reported to have a role in the development and progression of various cancers. In this study, we found that miR-134 expression was significantly decreased in chemo-resistant serous epithelial ovarian cancer (EOC) patients. Over-expression of miR-134 enhanced the sensitivity of SKOV3-TR30 cells to paclitaxel, and increased paclitaxel-induced apoptosis. Further, Pak2 was identified as a direct target of miR-134, and Pak2-specific siRNA increased cell inhibition rate and promoted paclitaxal-induced apoptosis. By regulating Pak2 expression, miR-134 could mediate Bad phosphorylation at Ser112 and Ser136, which affected cell survival and apoptosis. In conclusion, our findings indicate that repression of miR-134 and consequent up-regulation of Pak2 might contribute to paclitaxel resistance.

1. Introduction

The incidence rate of ovarian cancer in the female population accounts for the second most common tumor and is ranked the number one cause of mortality [1]. At present, the international recommended treatment guideline or gold standard is surgery that is supplemented by paclitaxel and platinum based chemotherapy. However, most patients will relapse in one to 2 years and the 5-year survival rate is still low (30%) due to chemoresistance [2]. Therefore, searching for the molecular basis of chemoresistance is ongoing.

The MicroRNA (MiRNA) are 21–25 nucleotides small non-coding RNAs. In mammalian cells, miRNAs play a significant role in regulating gene expression at both transcriptions and at post-transcriptional level [3]. These transcripts are crucial regulators of fundamental cellular functions, including proliferation, apoptosis, invasion and differentiation [4,5]. Recently, several studies have shown that miRNA played an important role in chemo resistance of ovarian cancer. Mir-199b-5p is associated with acquired chemo resistance in ovarian cancer [6] and miR-93 contributes to cisplatin chemo sensitivity in ovarian cancer cells by regulating PTEN/Akt pathway [7]. Additionally, miR-29b and its signaling mechanism involved in patients with ovarian cancer [8]. The inhibition of miR-21 promotes apoptosis and, thus causes drug sensitivity in ovarian cancer [9]. The miR-197 contributes to paclitaxel resistance in ovarian cancer cells [10]. Huh [11] in their study found that dysregulation of miR-106a and miR-591 leads to paclitaxel resistance in ovarian cancer. Cittelly [12] discovered that restoration of miR-200c in ovarian cancer increased sensitivity to paclitaxel. MiR-134 has been reported to show down-regulation in multi-resistant small cell lung cancer and esophageal cancers [13–16]. Hirota found that miR-134 in lung cancer tissue decreased significantly; by inhibiting dihydro pyrimidine dehydrogenase (DPD) protein expression affects 5-fluorouracil sensitivity in lung cancer [15]. Kitamura et al. in their study found that miR-134 participates in TGF-β induced transformation of epithelial mesenchymal by regulating MAGI2 and causes gefitinib resistance in lung adenocarcinoma cancer cells [16]. However, the role of miR-134 in paclitaxel resistance of ovarian cancer is still relatively unknown.

In this study, we aim to search the significance of decreased expression of miR-134 in serous epithelial ovarian carcinoma and clarify that miR-134 by regulating Pak2 expression might contribute to paclitaxel resistance. In our study, we performed microarray analysis and found miR-134 was decreased in serous epithelial ovarian carcinoma chemo-resistant tissues. We further

Author contributions: Min Wang conceived and designed the experiments; Ting Shuang performed the experiments and wrote the paper; Cong shi and Ying ying Zhou analyzed the data; Dan dan Wang contributed analysis tools.

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searched the functional target Pak2 and identified the downstream effectors which might directly execute function of decreased expression of miR-134 in ovarian cancer paclitaxel resistance. Our study for the first time speculated that miR-134 regulating Pak2 expression contributes to ovarian cancer cell sensitivity maybe through Bad phosphorylation at Ser112 and Ser136.

2. Materials and methods

2.1. Tissue samples

Ovarian cancer tissue samples were obtained from routine surgery at Shengjing Hospital of China Medical University between 2010 and 2012 from female patients who received platinum-paclitaxel therapy after surgery. All patients were not pretreated with radiotherapy or chemotherapy prior to surgery. Patients with progressive disease during primary chemotherapy or those who suffered a recurrent disease within 6 months of completing primary chemotherapy were labeled as drug resistant (total of 24). Patients who had recurrences beyond 6 months or with no recurrence were drug sensitive (total of 24). All the samples selected were serous EOC (seen in Supplemental Tables 1 and 2). Samples were immediately frozen and stored in liquid nitrogen for analysis. The research study was reviewed by our ethics committee, and the study was approved by the institutional review board of Shengjing Hospital of China Medical University. Informed consent was obtained from all the female patients.

2.2. Cell culture

The ovarian carcinoma cell line SKOV3 was provided by Tumor Cell Bank Research Institute of the Chinese Academy of Medical Sciences (Beijing, China). The paclitaxel-resistant ovarian carcinoma cell line, SKOV3-TR30 cells was derived from SKOV3 cell line and provided by Zhejiang University affiliated Obstetrics and Gynecology Hospital (Hangzhou, China). SKOV3, SKOV3-TR30 cells were grown in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (Hyclone, Logan, UT, USA). SKOV3-TR30 cells were maintained with the addition of 30 nmol/L of paclitaxel (Sigma Aldrich, St. Louis, MO). Paclitaxel was withdrawn a week before the experiment. All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells in the logarithmic phase of growth were used for all studies described.

2.3. MicroRNA gene chip

Total RNA were extracted from 60 mg of frozen tumor tissues using Trizol (Invitrogen, Carlsbad, CA). We analyzed the expression profiles of miRNA on 4 pairs of the chemo-sensitive and chemo-resistant serous EOC tissue samples using FlashTag™ Biotin RNA Labeling Kit for Affymetrix® GeneChip® miRNA Arrays (Genisphere, FT30AFYB) (CaptialBio Corporation). The Cluster 3.0 software was used for the analysis of microRNA expression. The criteria for differentially expressed miRNA is $|\text{Score}\ (d)| \geqslant 2$, and fold change $\geqslant 2$ or $\leqslant 0.5$.

2.4. Plasmid generation

As acquired from previous hybrid PCR method [17], we found one potential binding site for miR-134 in the CDS (coding sequence) region of Pak2 gene (The coding region of a gene, also known as the coding sequence or CDS, is that portion of a gene's RNA, composed of exons, that codes for protein). We designed one pair of primers for cloning the fragment containing the potential binding sites of miR-134 (Primers used for the fragment containing were as following: Sense 5′ GGACTAGT ATG TCT GAT AAC GGA GAA CTG GAAG 3′, Antisense: 5′ CCCAAGCTT TCT CCA GTA ACA GCA TCAA 3′). The PCR products were then digested with Spe I and HindIII, followed by insertion into Spe I and, HindIII digested the pMIR-Report vector (Ambion, Carlsbad, CA) to obtain a luciferase construct. Then site-directed gene mutagenesis kit (ThermoFisher Scientific, Waltham, MA, USA) was applied to create the mutant counterpart, by using the mutant primers (the sequence were as following: Sense 5′ AGACCCTTTGTCAGCATACACGAGTTT GAAACCTTTGCC 3′, Antisense 5′ GGCAAAGGTTTCAAACTCGTG TATGCTGACAAAGGGTCT 3′). Pak2 gene over-expression plasmid was constructed into a pBI plasmid (Clonetech). To be specific, the complete CDS region was cloned with primer: Sense 5′ CGACGCGT ATGTCTGATAACGGAGAACTGGAAG 3′; Antisense 5′ ACGCGTCGAC ACGGTTACTCTTCATTGCTTCTTTA 3′. The PCR product was digested with Mlu, Sal, and then inserted into pBI plasmid to obtain Pak2 over-expression plasmid named as pBI-Pak2.

2.5. RNA isolation and quantitative reverse transcriptase PCR

Total RNA was isolated using Trizol agent (Invitrogen, Carlsbad, CA) and treated by TURBO DNA-free™ Kit (Ambion, Carlsbad, CA) according to the manufacturer's instructions. For miR-134 quantitative real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), cDNA was synthesized from 10 ng of total RNA using TaqMan™ miRNA hsa-miR-134 specific primers (Applied Biosystems Life Technologies, Beijing, China) and a TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems Life Technologies). The qPCR was performed on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems Life Technologies) and the U6 snRNA was used as an endogenous control and, fold change of expression was calculated according to the $2^{-\Delta\Delta CT}$ methods. All reactions were performed in triplicate.

For Pak2 mRNA expression, cDNA was generated using the PrimeScriptVR RT reagent Kit (Takara, Dalian, China). A quantitative real-time polymerase chain reaction (qRT-PCR) assay was performed to evaluate mRNA expression using the SYBR® Select Master Mix (Applied Biosystems Life Technologies) on the ABI PRISM 7300 Sequence Detection System (Applied Biosystems Life Technologies) Primer of Pak2 was design as Sense: TGGTCGGAACGCCATACTG, Antisense: TTCTGGGGTTCCATTAGTTGC (actin, Sense: CTCCATCCTGGCCTCGCTGT, Antisense: GCTGTCACCTTCACCGTTCC). The relative expression levels of each sample were measured using $2^{-\Delta\Delta CT}$ methods. All reactions were performed in triplicate.

2.6. Luciferase activity assay

Ovarian cancer paclitaxel resistance SKOV3-TR30 cells were applied for the luciferase activity assays. Cells were plated at 2×10^5 cells per well in a 24-well plate 24 h before transfection. Cells were transfected with luciferase reporter constructs (400 ng) and, miR-134 mimics (100 nM) with Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. The pRL-TK vector (promega, Madison, WI, USA) was co-transfected as an internal control to correct the differences in both transfection and harvest efficiencies. After 24 h cell lysate was prepared by using Dual-Luciferase® Reporter Assay Kit (Promega) and luciferase activity was analyzed using the Dual-Luciferase Reporter Assay System (Promega). Transfected wells were analyzed in triplicate for each group.