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Original Article

MicroRNA-136 inhibits cancer stem cell activity and enhances the anti-tumor effect of paclitaxel against chemoresistant ovarian cancer cells by targeting Notch3

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ABSTRACT

To identify microRNAs (miRNAs) regulating Notch3 expression in association with paclitaxel resistance, candidate miRNAs targeting Notch3 were predicted using TargetScan. We found that miR-136 directly targets Notch3, and miR-136 was significantly downregulated in OSC tissues relative to normal control tissues, and low expression of miR-136 correlated with poor overall in ovarian cancer patients. Artificial miR-136 overexpression significantly reduced cell viability, proliferation, Cancer stem cell (CSC) spheroid formation, and angiogenesis, and increased apoptosis in paclitaxel-resistant SKpac cells compared with the effects of paclitaxel alone. miR-136 overexpression downregulated cell survival- (survivin, DNA-PK, pS6, S6) and cell cycle- (Cyclin D1, NF-κB) related proteins, and anti-apoptotic proteins (BCL2, and BCL-XL), and upregulated pro-apoptotic proteins (Bim, Bid, and Bax).

Taken together, miR-136 targets the Notch3 oncogene and functions as a tumor suppressor. miR-136 overexpression resensitized paclitaxel-resistant ovarian cancer cells and reduced CSC activities, suggesting a promising new target for the treatment of chemoresistant ovarian cancers.

Introduction

Ovarian cancer is one of the leading causes of gynecological cancer-related death because of late diagnosis at an advanced stage and a high recurrence rate. The current standard treatment of ovarian cancer is cytoreductive surgery with platinum/paclitaxel (PTX) combined chemotherapy. Despite this multimodality treatment strategy, many patients experience recurrence and ultimately die from their disease. The development of resistance to anti-cancer drugs is one of the main reasons for recurrence and a major cause of poor prognosis [1,2], underscoring the need to identify new treatment strategies to control chemoresistance and recurrence.

The Notch signaling pathway plays an important role in cell fate determination, proliferation, differentiation, and regulation of normal and cancer cells [3]. Abnormal expression of Notch proteins and ligands has been reported in many cancers, including ovarian carcinomas [4,5]. Among Notch pathway components, *Notch3* is amplified and overexpressed in ovarian cancer, especially in high-grade ovarian serous carcinoma (OSC). OSC is the most common and lethal subtype of ovarian cancer, showing a high rate of cancer cell survival, growth, and adhesion [4,6]. Moreover, there are increasing evidences that *Notch3* plays a role in regulation of cancer stem cell (CSC) [7,8], which is one of major contributors to chemoresistance. Therefore, *Notch3* is a candidate novel target for the treatment of chemoresistant and recurrent ovarian cancer.

MicroRNAs (miRNAs) are noncoding regulatory RNAs of 21–25 nucleotides that function as critical regulators of post-transcriptional gene expression by binding to the 3′-untranslated region (3′-UTR) of specific mRNAs, leading to translational repression or mRNA degradation. miRNAs play important roles in development, proliferation, and differentiation by regulating the expression of up to 70% of human genes, suggesting that miRNAs are involved in regulating nearly every genetic pathway [9,10].

Here, we used miRNA prediction software to search for miRNAs targeting *Notch3* and identified miR-136 as a candidate miRNA.

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miR-136 has a tumor-suppressive function and promotes apoptosis in human glioma cells [11]. In lung adenocarcinoma cells, miR-136 suppresses metastasis by targeting Smad2 and Smad3 [12]. A recent study reported that miR-136 is downregulated and associated with primary cisplatin resistance in epithelial ovarian cancer [13]. However, the functional role of miR-136 in ovarian carcinoma, especially in relation to Notch signaling, remains largely unknown.

Here, we identified miR-136 directly targets *Notch3* and examined the expression levels and clinical significance of miR-136 in OSC samples and its functional role in OSC cells. The anti-tumor and CSC-regulating effects of miR-136 on chemoresistant OSC cells were investigated using gain-of-function experiments.

Materials and methods

Patients and tissue samples

Samples were obtained from 44 patients with high-grade OSC who underwent oophorectomy for ovarian carcinoma at CHA Bundang Medical Center. The tissues were fresh snap-frozen for quantitative RT-PCR. As a normal control, fallopian tubes were obtained from patients who underwent hysterectomy with salpingectomy for benign leiomyoma, which is known to be the origin of OSC [14,15]. Clinical and pathological data were retrieved from clinical databases and from the original pathology reports, including histological grade and tumor stage. Histopathological examination of samples was performed in the Department of Pathology according to the criteria of the International Federation of Gynecology and Obstetrics grading system. This study was approved by the Ethical Committee of CHA Bundang Medical Center, and informed consent was obtained from each patient prior to surgery.

Ovarian carcinoma cell lines

The human ovarian carcinoma cell line SKOV3 was obtained from the American Type Culture Collection (Manassas, VA, USA). Several PTX-resistant sublines (SKpac-10, -13, -16, and -17) were established by continuous exposure of SKOV3 cells to a stepwise increasing concentration of PTX for more than 8 months. These cell lines were maintained in McCoy's 5A medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Candidate miRNA prediction

Candidate miRNAs targeting *Notch3* were predicted using the algorithms TargetScan (http://www.targetscan.org/).

Quantitative real-time PCR

The expression of miR-136 was evaluated in serous carcinoma tissues and ovarian cancer cell lines by quantitative RT-PCR (qRT-PCR) to determine the clinical implications of miR-136 expression in ovarian cancer. We also performed qRT-PCR to compare the expression of cancer stem cell markers, including ALDH1, CD24, CD133, and c-KIT, in miR-136 mimic transfected and control miR-negative transfected SKpac cells. Total RNA was extracted from fresh tissues and cell lines using TRIzol reagent (Invitrogen). The stem-loop qRT-PCR for mature miRNAs was performed using a Bio-Rad CFX96 Real-Time PCR Detection System. All PCR reactions were run in triplicate and gene expression relative to RNU48 was calculated using the comparative threshold method ($2^{-\Delta\Delta Ct}$).

Transfection of the miR-136 mimic

A chemically modified RNA-based miRNA precursor, miR-136 mimic, was purchased from Ambion (Applied Biosystems, Foster City, CA). To transfect cells, 60 pmol of miR-136 mimic was diluted in 250 μl of serum-free McCoy's 5A medium with 5 μl of lipofectamine3000 (Invitrogen, Carlsbad, CA). miR-negative was used as a control. To validate the efficiency of transfection, miRNA expression was examined by qRT-PCR.

Luciferase assay

The 3′-UTR of *Notch3* was amplified by PCR from genomic DNA of SKpac cells. The putative binding sites for miR-136 within the 3′-UTR of *Notch3* were identified using the TargetScan algorithm (targetscan.org). The wild-type or mutated binding sites were cloned separately into the Nhel and Xhol sites of the pGL3-control vector (Ambion). The pGL3-control (100 ng) and pRL-TK plasmids (5 ng; for normalization) were transfected into SKpac cells seeded in 24-well plates (3×10^4 cells/well). Synthetic miR-136 mimic (Ambion) at a concentration of 20–60 nM was added to the above reactions. Luciferase activity was measured after 48 h on an Infinite 200pro series luminometer (Tecan Group, Zurich, Switzerland) using the Dual-Luciferase reporter assay system (Promega, Mannheim, Germany) according to the manufacturer's instructions. All experiments were performed in triplicate and normalized to Renilla luciferase activity.

Spheroid formation assay

Spheroid formation assays were performed to verify the effect of miR-136 on cancer stem cell (CSC) activation. SKpac cells were transfected with miR-136 mimic or miR-negative. Cells were plated at a density of 1000 cells/cm^2 in Ultra-Low Attachment Surface 6-well culture plates (Corning, Acton, MA, USA) in serum-free DMEM/F12 medium (Invitrogen) supplemented with 20 ng/ml epidermal growth factor (Invitrogen), 10 ng/ml basic fibroblast growth factor (Sigma–Aldrich, St. Louis, MO, USA), 0.4% bovine serum albumin (Sigma–Aldrich), and 5 μg/ml insulin (Sigma–Aldrich). Spheroid formation (50–100 cells per sphere) was assessed 7 days after seeding.

WST assay

Cytotoxicity was estimated using the WST assay. Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. The next day, cells were transfected with miR-136 mimic and incubated for 24 h and 48 h. Transfected cells were then replated in a 96-well culture plate at a density of 1×10^4 cells/well. To examine the growth inhibitory effect of PTX, cells were treated with 40 nM and 80 nM PTX for 24 h. Cell viability was measured according to the protocol of the Cell Counting Kit-8 (CCK-8) assay kit (Dojindo, Japan). Absorbance was measured at 450 nm using a microplate reader.

Colony-forming assay

Cells were seeded in 6-well plates at a density of 1×10^5 cells/well. The next day, cells were transfected with miR-136 mimic or miR-negative and incubated for 24 h. Transfected cells were then replated at a density of 300 cells/well in 6-well culture dishes. After 14 days, colonies were fixed with 4% paraformaldehyde for 10 min, visualized using hematoxylin, and counted. Groups of >50 cells were scored as colonies.

TUNEL assay

Apoptosis was assessed by flow cytometric detection using the TUNEL assay. SKpac cells were transfected with miR-136 mimic or miR-negative. After 24 h and 48 h, apoptotic cells were analyzed using the *In Situ* Cell Death Detection kit (Roche, Mannheim, Germany). Cells (2×10^7) were fixed with 75% ethanol for 2 h at −20 °C, washed twice with cold PBS, and incubated with 0.1% Triton X-100 and 0.1% sodium citrate for 2 min on ice. The cells were then incubated with a TUNEL labeling mixture for 1 h at 37 °C in the dark and analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA).

Wound healing assay

The wound healing assay was used to assess cell migration. The two groups of cells transfected with miR-136 mimic or miR-negative control were cultured to confluence (>90%) in 24-well plates. Then, a straight scratch wound was generated with a 200 μl sterile pipette tip. After 6 h, the cells were photographed, and cells migrating from the scratch line were counted. Assays were repeated three times.

Table 1
List of primary antibodies used in western blotting.

Antibody name	Company (Catalog No.)	Dilution
Anti-Notch3	Cell Signaling (5276S)	1:1000
Anti-NICD3	Cell Signaling (5276S)	1:1000
Anti-HES1	Novus Biologicals (NBP1-47791)	1:1000
Anti-HEY1	Proteintech (19929-1-AP)	1:1000
Anti-HEY2	Proteintech (10597-1-AP)	1:1000
Anti-Survivin	Epitomics (2463-1)	1:1000
Anti-DNA-PK	Epitomics (1579-1)	1:1000
Anti-pS6	Epitomics (2268-1)	1:10000
Anti-S6	Cell Signaling (2217S)	1:10000
Anti-Cyclin D1	Thermo (RM-9104-S0)	1:1000
Anti-Cyclin D3	Cell Signaling (DCS22)	1:1000
Anti-NF-kB	Cell Signaling (8242)	1:5000
Anti-p21	Epitomics (2990-1)	1:500
Anti-p27	Cell Signaling (3688)	1:1000
Anti-BCL-W	Epitomics (T0195)	1:500
Anti-BCL-2	Lab Vision (MS-123-P0)	1:1000
Anti-BCL-XL	Epitomics (1018-1)	1:1000
Anti-Bad	Epitomics (1541-1)	1:1000
Anti-Bax	Cell Signaling (5023)	1:1000
Anti-B-actin	Santacruz (SC-47778)	1:10000