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Original article

MiR-939 promotes the proliferation of human ovarian cancer cells by repressing APC2 expression

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A B S T R A C T

Aberrant activation of the Wnt/β-catenin signal pathway is frequently observed in various human cancers. Therefore, it was speculated that adenomatous polyposis coli 2 (APC2) could play important roles in activating the Wnt/β-catenin pathway. In this present study, miR-939 expression was markedly upregulated in ovarian cancer tissues and ovarian cancer cells. In functional assays, Overexpression of miR-939 promoted the proliferation and anchorage-independent growth of ovarian cancer cells, whereas inhibition of miR-939 inhibited this effect. Bioinformatics analysis further revealed APC2, a putative tumor suppressor as a potential target of miR-939. Result of luciferase reporter assays showed that miR-939 directly binds to the 3′-untranslated region (3′-UTR) of APC2 mRNA. Furthermore, we demonstrated that miR-939 could reduce the Wnt/β-catenin signal pathway by suppressing APC2 directly, resulting in increasing expression of CyclinD1, MYC and TCF. In functional assays, APC2-silenced in miR-939-in-transfected ES-2 cells have positive effect to promote cell proliferation, suggesting that direct APC2 downregulation is required for miR-939-induced ovarian cancer cell proliferation. In sum, our data provided compelling evidence that miR-939 functioned as a potential tumor promoter by regulating the Wnt/β-catenin signal pathway through direct suppression of APC2 expression and might sever as a potential therapeutic target for ovarian cancer patients.

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1. Introduction

Ovarian cancer (OC) is the most lethal gynecological malignancy and the fifth leading cause of cancer death in women [1]. The reason of high mortality rate is the lack of an early detection method for OC. Thus, there is an urgent need to elucidate the novel and efficient diagnostic and prognostic molecular biomarkers for OC.

MicroRNAs (miRNAs) were discovered to be the endogenous non-coding small RNA, are involved in multiple biological processes such as cell proliferation, apoptosis, invasion and differentiation in various cancers [2–5]. MiRNAs play essential roles in development of cancer by targeting 3′-untranslated region (3′-UTR) of target mRNA, and then triggering either mRNA degradation or translational repression [3,6–8]. The Wnt signaling pathway regulates cellular processed and plays key roles in tumorigenesis [9,10]. Adenomatous polyposis coli 2 (APC2), a tumor suppressor, plays a critical role in the activation of the canonical Wnt/β-catenin signaling pathway [11,12]. However, the related mechanism still needs to be clarified. This study explores the targeted regulation relationship of the miR-939 with the APC2 in OC cells, and investigates its function in cell proliferation.

2. Materials and methods

2.1. Clinical specimens

Eight pairs of primary ovarian carcinomas tissues and the matched tumor adjacent normal tissues (TAT) were collected from patients in Department of Gynecology, State Key Laboratory of Oncology in South China, Sun Yat-sen University Cancer Center (Guangzhou, People's Republic of China). The study was approved

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by the ethics committee of Sun Yat-sen University Cancer Center (Guangzhou, People's Republic of China). Written informed consent was obtained from all patients. Tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored until total RNAs or proteins were extracted.

2.2. Cell culture

Human ovarian cancer cell lines HO-8910, A2780/DDP, SKOV-3, CAOV-3 and ES-2 were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Sigma, USA), 100units/ml of penicillin-streptomycin (Invitrogen, Carlsbad, CA), and HOSE (Pricells, Wuhan, China), a cell derived from human ovary surface epithelial cells, act as the normal control cell, was maintained in cell culture medium consisting of 1:1 Medium199 (Sigma–Aldrich) and MCDB105 medium (Sigma–Aldrich) with 10% heat-inactivated fetal bovine serum (FBS, Gibco) and 10 ng/ml epidermal growth factor (Sigma–Aldrich). All cell lines were cultured in a humidified incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air.

2.3. Plasmids, small interfering RNA and transfection

For ectopic expression of APC2, the prediction of APC2 mRNA as a target of miR-939 was made with TargetScan (http://www.targetscan.org/) programs. APC2 ORFs with 3′-UTR was amplified using PCR and subcloned into pEGFP-N3 (Invitrogen). The miR-939 mimics, negative control, miR-939 inhibitor and miR-939-mut were purchased from Genecopoeia (Genecopoeia Co. Ltd.) and transfected into OC cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

For APC2 depletion, small interfering RNA (siRNA-APC2, HSH000364) was synthesized and purified by GeneCopoeia Co. (Guangzhou, Guangdong, China). Transfection of siRNAs was performed using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

2.4. RNA extraction and real-time quantitative PCR

Total RNA including microRNAs was extracted from culture cells and patient samples using mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's instructions, reverse transcription and quantitative PCR were performed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China) by using the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The relative miR-939 expression levels after normalization to U6 small nuclear RNA were calculated using $2^{-[(\text{Ct of miR-939}) - (\text{Ct of U6})]}$. Quantitative PCR was performed by SYBR Kit (Qiagen, China) using a Light Cycler system. The primers were synthesized by GeneCopoeia Co., APC2 (HQP000364), MYC (HQP011597), TCF (HQP017965), GAPDH (HQP006940). Expression data were normalized to the geometric mean of GAPDH to control the variability in expression levels and calculated as $2^{-[(\text{Ct of APC2, CyclinD1, Myc and TCF}) - (\text{Ct of GAPDH})]}$.

2.5. MTT assays and Colony formation

For cell proliferation analysis, ES-2 cells after transfection were seeded in 96-well plates approximately 3000 cells/well. Cultures were stained after 1, 2, 3, 4 and 5 days, respectively. The absorbance at 490 nm was measured in a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA) after incubation of the cells with 20 μl of 5 mg/ml MTT solution (Sigma–Aldrich) at 37 °C for 4 h and the resulting MTT formazan was solubilized in 150 μl of DMSO.

For colony formation assay, ES-2 cells were plated into three 6-cm cell culture dishes (1×10^3 cells per well) and incubated in medium containing 10% FBS. After 14 days, the colonies were fixed with 10% formaldehyde for 20 min, washed with PBS and stained with 1.0% crystal violet for 30 s. The number of colonies, defined as >50 cells/colony were counted.

2.6. Anchorage-independent growth assay

Cells were trypsinized, and 1000 cells were resuspended in 2 ml complete medium plus 0.3% agar (Sigma). The agar-cell mixture was plated on top of a bottom layer consisting of 1% agar in complete medium. Cells were incubated at 37 °C. After 14 days, the colonies were stained with 0.5% Crystal Violet for counting under microscope and cell colonies were photographed at an original magnification of 100×. Only cell colonies containing more than 50 cells were counted.

2.7. Luciferase assays

The pGL3-luciferase reporter gene plasmids pGL3-APC2-3′-UTR, or the control-luciferase plasmid were cotransfected into the cells with the control pRL-TK Renilla plasmid (Promega) using Lipofectamine 2000 Reagent (Invitrogen). Cells were collected at 24 h after transfection. Luciferase and Renilla activities were assayed, the cells were lysed and the fluorescence intensity was detected using the dual luciferase assay kit.

2.8. Western blotting

Protein lysates were prepared, and protein concentration was measured using the BCA Protein Assay kit (Beyotime, China). Equal quantities of protein were separated on SDS-PAGE and transferred onto PVDF membranes for 2 h at 4 °C, and then blocked for 2 h 5% non-fat milk. The membrane was incubated overnight with anti-APC2, anti-β-Cactenin, anti-Cyclin D1 and anti-C-Myc (1:1000 dilution; Cell Signaling Technology) and washed with tris buffered saline and Tween-20 (TBST) three times, for 5 min each time. To control sample loading, the blotting membranes were stripped and re-probed with an anti-a-tubulin antibody (Sigma–Aldrich). After being washed with TBST and incubation with horseradish peroxidase-conjugated antibody (Sigma–Aldrich) for 2 h at room temperature, immunocomplexes were visualized using the enhanced chemi-luminescence (ECL) following the manufacturer's protocol.

2.9. Statistical analysis

All statistical analyses except for microarray data were subjected to statistical analysis by SPSS 16.0 software. Student's *t* test was used to evaluate the significance of the differences between two groups of data in all the pertinent experiments. A $P < 0.05$ (using a two-tailed paired *t*-test) was thought to be significantly different for two groups of data.

3. Result

3.1. MiR-939 expression was upregulated in OC cell lines and OC tissues

To investigate the role of miR-939 in ovarian cancer development, we first evaluated the expression levels of miR-939 in OC cell lines and ovarian cancer tissues by qRT-PCR (Fig. 1A). As shown in Figure 1A, miR-939 was significantly increased in OC cell lines. It was also shown that miR-939 was up-regulated in OC tissues (Fig. 1B). Together, these results suggest that miR-939 was abnormally up-regulated in human OC cell lines and OC tissues.

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