MiR-630 promotes epithelial ovarian cancer proliferation and invasion via targeting KLF6

S. ZHANG1, J.-Y. ZHANG1, L.-J. LU2, C.-H. WANG1, L.-H. WANG3

1Department of Gynecology, Zhangjiagang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, Jiangsu Province, China
2Department of Obstetrics and Gynecology, Suzhou Hospital of Traditional Chinese Medicine, Suzhou, Jiangsu Province, China
3Reproductive Medicine, Zhangjiagang Hospital of Traditional Chinese Medicine Affiliated to Nanjing University of Traditional Chinese Medicine, Jiangsu Province, China

Abstract. – OBJECTIVE: MicroRNAs play critical roles in post-translational gene expression. The current study was to investigate the effects of miR-630 in epithelial ovarian cancer.

PATIENTS AND METHODS: Thirty epithelial ovarian cancer tissue and thirty normal ovarian tissue samples were collected and were detected miR-630 expression level with qRT-PCR. MiR-630 mimics, inhibitors and negative controls were transfected into SKOV3 and Cell Counting Kit-8 (CCK-8) assay, and transwell experiment were performed to detect the proliferation rate and migration, respectively. The luciferase reporter assay was utilized to identify miR-630’s target gene. Balb/c nude mice were utilized to verify the effect of miR-630 in vivo.

RESULTS: QRT-PCR showed a significantly high miR-630 expression in epithelial ovarian cancer relative to normal ovarian tissue. The miR-630 overexpression promoted epithelial ovarian cancer cell SKOV3 proliferation and migration. Krüppel-like factor 6 (KLF6) was predicted as the target of miR-630. In vivo study also verified that miR-630 overexpression stimulated ovarian cancer growth.

CONCLUSIONS: We propose that targeting miR-630 might be a promising therapeutic approach for ovarian cancer.

Key Words: Micro RNAs, Ovarian cancer, KLF6, miR-630.

Introduction

Ovarian cancer is known as one of the most common gynecological cancers, and epithelial ovarian cancer is one of the most common, which accounts for about 85-90%. Most patients were diagnosed at advanced stage because there are no early symptoms in the early stage of ovarian cancer. Due to the aggressive metastasis and the recurrence as well as chemotherapy resistance, the five-year survival rate is only about 30%.

MicroRNAs (miRNAs) are a cluster of very small, noncoding and single-stranded RNAs which were highly conserved in plants, animal, and even some viruses, and they regulate gene expression post-translationally. MiRNAs function via base-pairing with complementary sequences within mRNA molecules partially or completely. The dysregulation of miRNAs interacts with a lot of disorders. It was reported that the mutation of miRNAs could be found in some inherited diseases such as hereditary progressive hearing loss and skeletal and growth defects. In recent years, increasing miRNAs are found to be linked with cancers. It was reported that miR-21, miR-125a, miR-125b and miR-99a were differentially expressed in serous ovarian cancer and were related with the prognosis of ovarian carcinoma. Wang et al observed that MiR-630 played different roles in different neoplasms. Patients with bladder urothelial carcinoma with high miR-630 had poorer prognosis than those with lower miR-630 expression. While in esophageal squamous cell carcinoma, miR-630 knockdown enhanced proliferation, invasion, metastasis. To date, little is known about the role of miR-630 in epithelial ovarian cancer.

We investigated the miR-630 expression and functions in ovarian cancer in the current research. Results from this work indicated high miR-630 expression in ovarian cancer tissue samples when compared with normal ovarian tissue and that the overexpression of miR-630 enhances...
ovarian cancer cell proliferation and migration via targeting Krüppel-like factor 6 (KLF6). Our results indicated a promising therapy target for ovarian cancer.

**Patients and Methods**

**Human Tissue Samples Collection**

We collected thirty ovarian cancer samples and thirty normal ovarian tissues from patients who received surgeries in our hospital. All patients agreed and signed the consent and this study was fully approved by the Ethics Committee of our Hospital. The pathologic type of all samples was confirmed by two independent pathological experts. Tissues were frozen at -80°C immediately.

**Cell Line Culture and Transfection**

Roswell Park Memorial Institute-1640 (RPMI-1640) medium which was added with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin was used to culture human epithelial ovarian cancer cell line SKOV3 under the atmosphere incubator of 5% CO2 at 37°C. SKOV3 was plated in 6-well plates and was transfected with Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) and Opti-MEM (Grand Island, NY, USA). Mimics, inhibitors and negative controls of miR-630 were respectively transfected into SKOV3. In order to overexpress or downregulate KLF6, we purchased KLF6 open reading frame which did not contain the miR-630-responsive 3’-UTR from FulenGen (Guangzhou, China). KLF6 siRNA was synthesized by RiboBio Company (Guangzhou, China). An empty plasmid was utilized as a negative control.

**Cell Proliferation Assay**

We seeded transfected SKOV3 in 96-well plates at a density of 5×10^3 cells each well. 48 hours later, 100 µL RPMI-1640 medium mixed with 10 µL CCK-8 solutions were supplemented to each well. Then, the absorbance of each well was detected at 450 nm after incubation at 37°C for 30 min. The analysis was repeated for at least three times.

**Luciferase Reporter Assay**

After cells reached about 90%, pEZX-MT01 vector was transfected to SKOV3 where wild or mutant type of 3’UTR of KLF6 was cloned in special medium (reduced serum as well as antibiotics-free OptiMEM) supplemented with oligofectamine 2000 for 6 hours. Firefly luciferase and renilla luciferase were the reporter gene and tracking gene, respectively. In addition, mimics, inhibitors or negative controls of miR-630 were transfected to cells. 24 hours later, luciferase and renilla strength were detected in cell lysates by a Dual Luciferase Reporter Assay kit (Promega, Madison, WI, USA).

**qRT-PCR Analysis**

We purchased TaqMan miRNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA). Total RNAs were extracted from cells with TRIzol and reverse transcripted to get cDNAs. TaqMan Universal PCR Master Mix plusing with MicroRNA Assay Mix together with cDNAs were mixed together and synthesized for DNAs with the StepOnePlus Real-time PCR systems. U6 was used internal control. Total RNAs were extracted from cells and reversed for cDNAs. The relative mRNA levels of KLF6 were evaluated with GAPDH as an internal reference.

**Western Blot Assay**

We abandoned the medium and washed cells with ice-cold phosphate buffered saline (PBS). Cells were lysed with radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China) to get total protein. We determined protein concentration with a protein assay kit. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins. Next, proteins were shifted to polyvinylidene fluoride (PVDF) membranes. 5% fat-free milk was used to block non-specific protein interactions in tris-buffered saline and tween 20 (TBST) buffer. The membranes loaded with proteins were incubated with primary antibodies at 4°C. These membranes were incubated with secondary antibodies for 1 hour at room temperature. TBST buffer was used to wash membranes for three times. Then, we developed the membranes using chemiluminescence to detect antibodies concentration and took GAPDH as our internal control. The antibodies, anti-KLF6 and anti-GAPDH, were purchased from Abcam (Cambridge, MA, USA).

**Transwell Cell Migration Detection**

Transfected cells suspended with 200 µL serum-free medium were seeded in the upper chamber of 24-well Boyden chambers. About 600 µL medium supplemented with 20% fetal bovine serum (FBS) were supplemented to the lower
chamber. The chambers were fixed in formaldehyde (4%) 24 hours later and stained with 1% crystal violet. We randomly selected 5 fields in each membrane and analyzed migrated cells.

**Animals Experiment**

Balb/c female mice of five-week were purchased from the Slaccs Experimental Animals Center (Shanghai, China). SKOV3 transfected with inhibitors, mimics or negative control of miR-630 were injected subcutaneously in the flank back (n=6). We measured and calculated the volume of tumor every three days (V=a*b*b/2, a: the longest diameter, b: the shortest diameter) until the fifteenth day. All experiments were fully approved by the Ethics Committee of Animal Experiments in our hospital.

**Statistical Analysis**

SPSS11.0 (Version X; IBM, SPSS Inc., Armonk, NY, USA) was used to analyze our data. Quantitative data was expressed as mean ± SD. Non-paired t-test was utilized to analyze data between groups. A \( p < 0.05 \) was determined as statistically significant.

**Results**

**MiR-630 Showed a High Expression in Epithelial Ovarian Cancer Tissue**

We performed qRT-PCR to detect the miR-630 expression level in epithelial ovarian cancer tissue relative to normal ovarian tissue. Results showed that among the 30 ovarian cancer samples analyzed, the relative miR-630 expression was significantly upregulated as compared with that of the normal ovarian tissues (Figure 1).

**MiR-630 Overexpression Enhances SKOV3 Proliferation and Migration**

SKOV3 was transfected with inhibitors, mimics and negative controls of the miR-630 and qRT-PCR were used to confirm the transfection effect (Figure 2A). CCK-8 assay and the transwell experiment were used to evaluate the proliferation and migration of SKOV3 cells, respectively. As shown in Figure 2, the miR-630 overexpression stimulated SKOV3 cell proliferation, whereas the miR-630 downregulation inhibited SKOV3 proliferation (Figure 2B). Results from the transwell experiment indicated miR-630 overexpression enhanced SKOV3 migration while miR-630 knock-down blocked SKOV3 cells migration (Figure 2C). Our findings showed that the miR-630 upregulation promoted SKOV3 proliferation and migration.

**MiR-630 Targets KLF6 Directly**

To illustrate the mechanisms of miR-630 promoting SKOV3 proliferation and migration, miRanda (http://www.microrna.org) and Target Scan (http://www.targetscan.org) were utilized to identify promising target genes. Finally, KLF6 concerned among those potential targets. According to the prediction tools, 3’UTR of KLF6 was the target of miR-630. Then, a luciferase reporter construct involving the 3’UTR and mutations in the site of KLF6 was used to test whether miR-630 could directly exert influence on KLF6. The reporter gene’s luciferase activity was suppressed by upregulation of miR-630, whereas this suppression effect was abolished by mutations in the mRNA binding site (Figure 3A). To further directly verify this target, SKOV3 was transfected with mimics of miR-630 and qRT-PCR assay as well as Western blot revealed the downregulated KLF6 mRNA levels compared with control group (Figure 3B).

**MiR-630 Overexpression Stimulates Ovarian Cancer Proliferating In Vivo**

In order to test the effects of miR-630 overexpression on ovarian cancer in vivo further, SKOV3 transfected with inhibitors, mimics, as well as negative controls of miR-630, were injected subcutaneously in the flank back (n=5).
By measuring the volume of tumors every three days in each group, we found that the volume of tumors in miR-630 mimics group increased rapidly compared with that in control group (Figure 4A). Tumors were dissected after all mice were sacrificed. Compared with control group, tumors in miR-630 mimics group were larger in volume and heavier in weight (Figure 4B).

**Figure 2.** MiR-630 overexpression promotes SKOV3 proliferation and migration. (A) SKOV3 cells were transfected with miR-630 mimics, inhibitors and negative control. QRT-PCR confirmed the corresponding transfection effects. (B) CCK-8 assay detected the cell viability of transfected cells in each group. (C) Transwell assay detected the migrated cells in each group. U6 was used as the internal control. *p < 0.05, ***p < 0.001, compared with negative control.

**Figure 3.** MiR-630 targets KLF6 directly. (A) Effect of miR-630 over-expression on a dual luciferase reporter plasmid containing the KLF6-3’ UTR was analyzed. Firefly and renilla luciferases were measured in cell lysate. (B) QRT-PCR and Western blot were used to measure the expression of KLF6 in miR-630-overexpressed SKOV3 cells. GAPDH was used as an internal control. **p < 0.01, compared with negative control.
Currently, we examined the role of miR-630 in epithelial ovarian cancer. An inverse expression pattern of miR-630 and KLF6 was confirmed in clinical epithelial ovarian cancer samples. Consisting with the prediction tools, Luciferase reporter assay determined the KLF6 as the effective direct target of miR-630. We concluded miR-630 overexpression promoted epithelial ovarian cancer proliferation and migration while anti-miR-630 attenuated this effect both in vitro and in vivo. The tumor-suppressive effect of KLF6 was confirmed further in epithelial ovarian cancer cells by overexpressing and knocking down KLF6.

In recent years, an increasing miRNAs have been reported to function as malignancy oncogenes or suppressors in the process of tumor initiation and progression and even influencing the chemosensitivity. MiR-630 has been studied in various carcinomas. However, the differential expression level of miR-630 was reported inconsistently. Jin et al. revealed that miR-630 was down-regulated in esophageal squamous cell tissue samples and ectopic miR-630 expression inhibited proliferation, invasion and metastasis. However, in colorectal cancer, miR-630 expression was markedly increased and high miR-630 expression was related with cancer invasion, metastasis and TNM stage. Until now, it remains unclear the association of miR-630 and epithelial ovarian cancer. KLF6 is known as a tumor repressor gene and was reported inactivated in various malignances. KLF6 plays role in regulating cell cycle, apoptosis and differentiation. However, the function of KLF6 in epithelial ovarian cancer invasion and metastasis has not been investigated.

Conclusions

We showed that miR-630 promoted epithelial ovarian cancer proliferation and invasion by targeting KLF6. Moreover, the overexpression of miR-630 stimulated tumor growth in vivo. Hence, we put forward that targeting miR-630 might be a promising therapy for epithelial ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References


