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ORIGINAL ARTICLE

MicroRNA-375 inhibits the growth, drug sensitivity and metastasis of human ovarian cancer cells by targeting PAX2

Shuli Yang[1], Ruiqi Yang[1], Ruixin Lin[2], Lihui Si[1]

[1]Department of Obstetrics and Gynecology, and [2]Department of General Surgery, Second Hospital of Jilin University, Changchun, 130041, P.R. China.

Summary

Purpose: *Ovarian cancer is responsible for a significant number of deaths in women and there is urgent need to develop efficient treatment strategies for this disease. Studies have shown microRNAs (miRs) are involved in diverse cellular processes and exhibit therapeutic implications. Herein, the role of miR-375 in ovarian cancer was explored.*

Methods: *OVACAR-3 cell line was mainly used in this research. Expression analysis was performed by qRT-PCR. Cell viability was determined by MTT assay. Cell cycle analysis was carried out by flow cytometry. Transwell assay was used for cell migration and invasion. Western blot analysis was used to determine the protein expression.*

Results: *Gene expression analysis carried out by qRT-PCR of ovarian cancer cell lines and tissues revealed significant downregulation of miR-375. Ectopic expression of miR-375 halted the growth of the OVACAR-3 cells by triggering G2/M cell cycle arrest. Moreover, miR-375 also caused a significant decrease in the migratory and invasive potential of the OVACAR-3 cells and enhanced their chemosensitivity to cisplatin. Bioinformatic analysis and the dual luciferase showed that miR-375 targets PAX2 in OVACAR-3 cells. Suppression of PAX2 inhibits the growth of the OVACAR-3 cells while PAX2 overexpression could avoid the growth inhibitory effects of miR-375 in OVACAR-3 cells.*

Conclusion: *miR-375 may prove to be an important therapeutic target in ovarian cancer and warrants further research endeavors.*

Key words: *ovarian cancer, microRNA, Cell cycle arrest, proliferation*

Introduction

Being the seventh most prevalent type of cancer in women, ovarian cancer causes significant mortality [1]. Ovarian cancer constitutes 2.5% of all cancers in women and 5% of the cancer-related deaths [2]. Approximately, 22000 new ovarian cancer cases and 14000 deaths due to this disease were reported in United States in 2018 [3]. The 5-year survival rate of ovarian cancer is less than 45%, indicating a need for the identification of novel treatment strategies for this malignancy [4]. It has been reported that early diagnosis and development of efficient and novel chemotherapeutic agents may help to curb the incidence of ovarian cancer and to improve the 5-year survival [5]. Utilisation of microRNAs (miRs) for cancer therapy is currently one of the promising fields in cancer research and treatment [6]. MiRs contain around 18-25 nucleotides long endogenous molecules that control the expression of the target genes by binding to 3´UTR and causing degradation of mRNA or repression of translation [7]. MiRs play vital roles in fundamental cellular processes which include but are not limited to proliferation, development, differentiation, apoptosis and autophagy [8]. There is strong evidence showing that many miRs show dysregulation in cancer tissues and play important part in the development of cancer [9]. Hence, it is believed that miRs may serve as therapeutic targets

Corresponding author: Lihui Si, MD. Department of Obstetrics and Gynecology, Second Hospital of Jilin University, No.218 Ziqiang Street, Changchun 130041, P.R.China.
Tel & Fax: +86 0431 81136555, Email: SophieMaysmsy@yahoo.com
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and will allow targeted therapy for the treatment of cancer [10]. MiR-375 has been shown to control the growth of different cancers types. For example, miR-375 suppresses the proliferation of colorectal cancer cells [11]. He et al reported that miR-375 inhibits the growth of liver cancer cells under both in vitro and in vivo conditions [12]. Nonetheless, the role of miR-375 has not been studied in ovarian cancer. Against this background, the present study investigated the function and therapeutic utility of miR-375 in ovarian cancer.

Methods

Cell lines and culture conditions

The human ovarian cancer cell lines (PA-1, OVACAR-3, SW-626, Caov-3) and normal ovarian cell line (SV40), were procured from Sun Yat-sen University Cancer Center (Guangzhou, China). The cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, USA) which was also supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA). These cell lines were incubated in a humidified atmosphere at 37°C with 5% CO2.

Expression analysis by qRT-PCR and transfections

Total RNA from the normal and the ovarian cancer cell lines and tissues was isolated by TRIzol Reagent (Invitrogen) following the manufacturer's instructions. The complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and amplified with Platinum SYBR Green qPCR SuperMix-UDG reagents (Invitrogen, Carlsbad, USA) using the CFX96 sequence detection system (Bio-Rad, Hercules, CA, USA). All transfections were carried out by Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) as per the manufacturer's protocol.

MTT assay

The OVACAR-3 cells were cultured in a 96-well tissue culture plates with approximately 2500 cells/well. The viability of the cells was evaluated at different time intervals by Vybrant MTT Cell Proliferation Assay (Invitrogen Carlsbad, USA) as per the manufacturer's guidelines. Finally, the optical density (OD) was measured at 570 nm via spectrophotometer.

Transfection

The OVACAR-3 cells were cultured to 80% confluence and then 10 pmol negative control (miR-NC) and miR-375 mimics from Shanghai GenePharma (Shanghai, China; 10 pmol), small interfering (si)-RNA-YAP1 and pcDNA-YAP1 (2 μg; Taijin Saier Biotechnology, Inc., Xiaozhan, China) were transfected using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol.

Flow cytometric cell cycle analysis

After transfection the OVACAR-3 cells were cultured for 24 h at 37°C. The OVACAR-3 cells were harvested and PBS-washed. Afterwards, the OVACAR-3 cells were stained with propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Cell migration and invasion assay

Transwell chambers with Matrigel were employed to monitor the OVACAR-3 cell invasion. Briefly, the cells were transfected with appropriate constructs and 48 h post-transfection the cells were harvested and suspended in fresh RPMI-1640 medium, while 200 μL of the cell suspension containing approximately 5 x 104 cells was placed onto the upper compartment and a fresh 500 μL RPMI-1640 medium was placed in the lower compartment. After 24 h cells present at the upper compartment were removed by swabbing, while cells that invaded to the lower surface were fixed and then subsequently stained with 0.05% crystal violet. Finally, 10 random fields were selected to determine the invasion under light microscope. The cell migration assay was also performed by following the same procedure as that of cell invasion but Matrigel was not used in case of cell migration assay.

Western blotting

The ovarian cancer tissues and cell lines were lysed and the protein concentration in each sample was

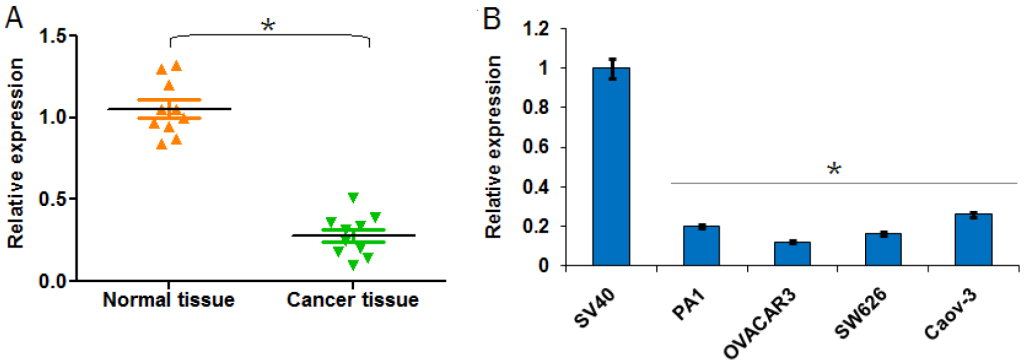

Figure 1. (A): Expression of miR-375 in ovarian cancer and normal adjacent tissues. **(B):** Expression of miR-375 in normal SV40 cells and different ovarian cancer cell lines. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

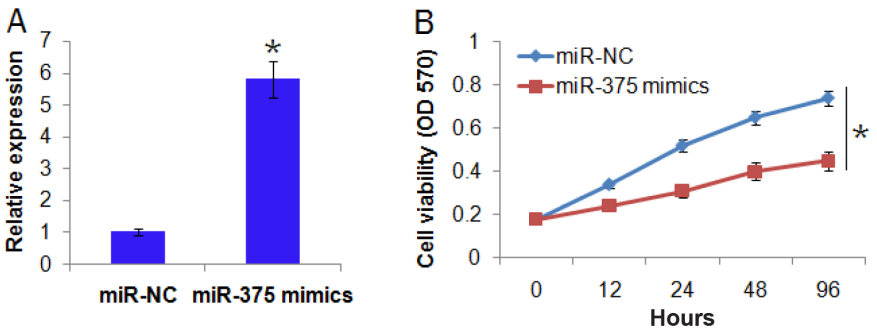

Figure 2. (A): Expression of miR-375 in miR-NC and miR-375 mimics transfected OVACAR-3 cells. **(B):** Cell viability of the miR-NC and miR-375 mimics transfected OVACAR-3 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

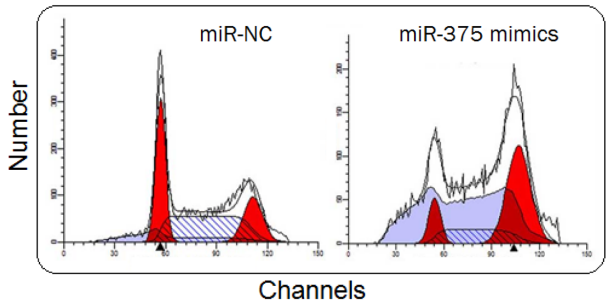

Figure 3. Cell cycle analysis of the miR-NC and miR-375 mimics transfected OVACAR-3 cells as determined by flow cytometry, showing induction of G2/M cell cycle arrest. The experiments were performed in triplicate.

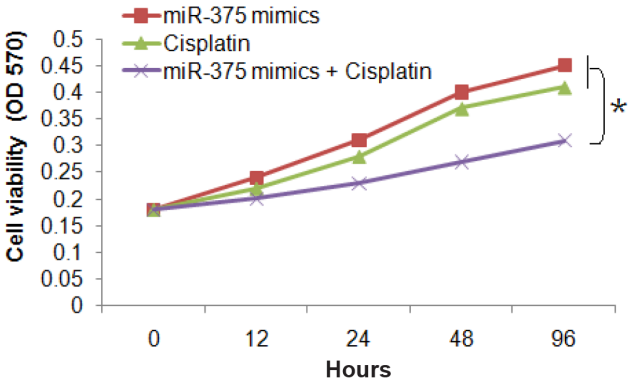

Figure 4. Overexpression of miR-375 enhances the chemosensitivity of OVACAR-3 cells to cisplatin. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

measured by Bradford assay. Equal concentrations of the proteins from each sample were loaded on 10% SDS polyacrylamide gel, followed by shifting to polyvinylidene fluoride membranes. Blocking of the membrane was then performed by fat-free milk (5%). This was followed by incubation with primary antibody for 24 h at 4°C. Subsequently, a secondary antibody was added at 25°C for about 2 h. The bands of interest were finally observed by chemiluminescence.

Statistics

Data are shown as mean ± SD of 3 independent experiments. Statistical analysis was done using Students t-test with GraphPad prism 7software. Values of p<0.05 were taken as significant difference.

Results

miR-375 is suppressed in ovarian cancer

The gene expression of miR-375 was determined in ovarian cancer tissues and normal adjacent tissues of 10 patients. The qRT-PCR results revealed miR-375 expression was significantly suppressed in ovarian cancer tissues (Figure 1A). Gene expression analysis of miR-375 in ovarian cancer cells showed miR-375 to be significantly (up to 9) downregulated in ovarian cancer cell lines (Figure 1B).

The highest downregulation was observed in OVACAR-3 cells. This cell line was therefore used for further studies.

miR-375 inhibits proliferation of OVACAR-3 cells through G2/M arrest

Next, we sought to explore the role of miR-375 in ovarian cancer and for that we overexpressed miR-375 in OVACAR-3 cells (Figure 2A). The MTT assay showed a remarkable decline in the growth of OVACAR-3 cells upon miR-375 overexpression (Figure 2B). Next, we carried out flow cytometric analysis of the miR-375 mimics-transfected OVACAR-3 cells and found that miR-375 overexpression caused arrest of the OVACAR-3 cells at the G2/M phase of the cell cycle (Figure 3).

miR-375 enhances the chemosensitivity of the ovarian cancer cells

The impact of miR-375 was evaluated on the cisplatin sensitivity of OVACAR-3 cells. The results showed that miR-375 caused remarkable enhancement in the cisplatin sensitivity of OVACAR-3 cells (Figure 4).

MiR-375 supresses the metastasis of OVACAR-3 cells

The transwell assay was used to assess the impact of miR-375 on the metastatic potential of OVACAR-3 cells. We found that the OVACAR-3 cell migration and invasion were suppressed upon increase in miR-375 expression. The migration and invasion of the OVACAR-3 cells was suppressed by 72 and 80% in comparison to control respectively (Figure 5).

The miR-375 targets PAX2 in ovarian cancer cells

The bioinformatic approaches via TargetScan were employed to identify miR-375 targets. The TargetScan revealed PAX2 to be the potential target of miR-375 (Figure 6A). Next, dual luciferase assay also confirmed the interaction between miR-375 and PAX2 (Figure 6B). We also examined the PAX2 expression in the all ovarian cancer cell lines and the qRT-PCR revealed PAX2 to be aberrantly

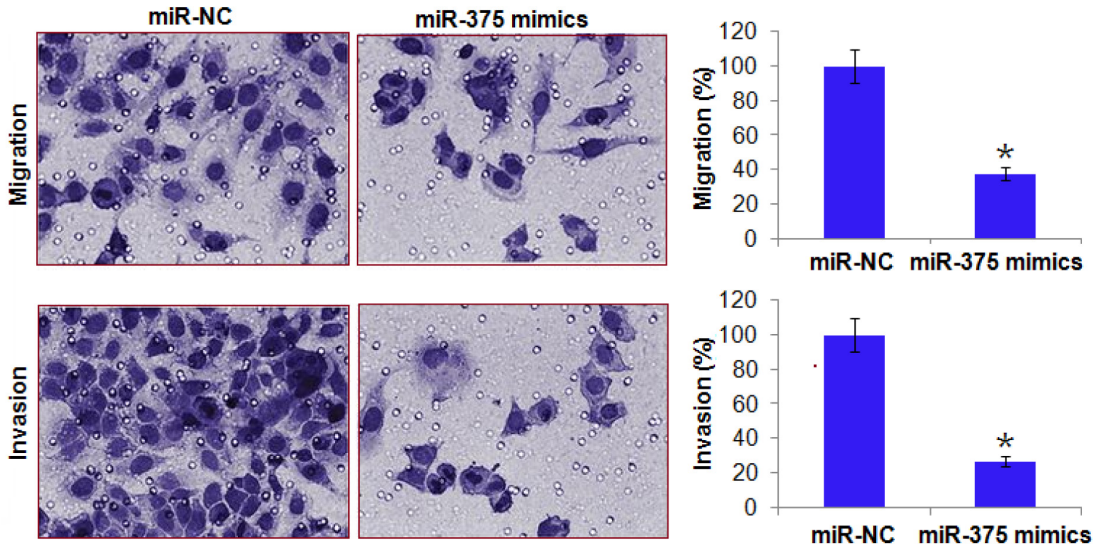

Figure 5. Transwell assay showing migration and invasion of the miR-NC and miR-375 mimics transfected OVACAR-3 ovarian cancer cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

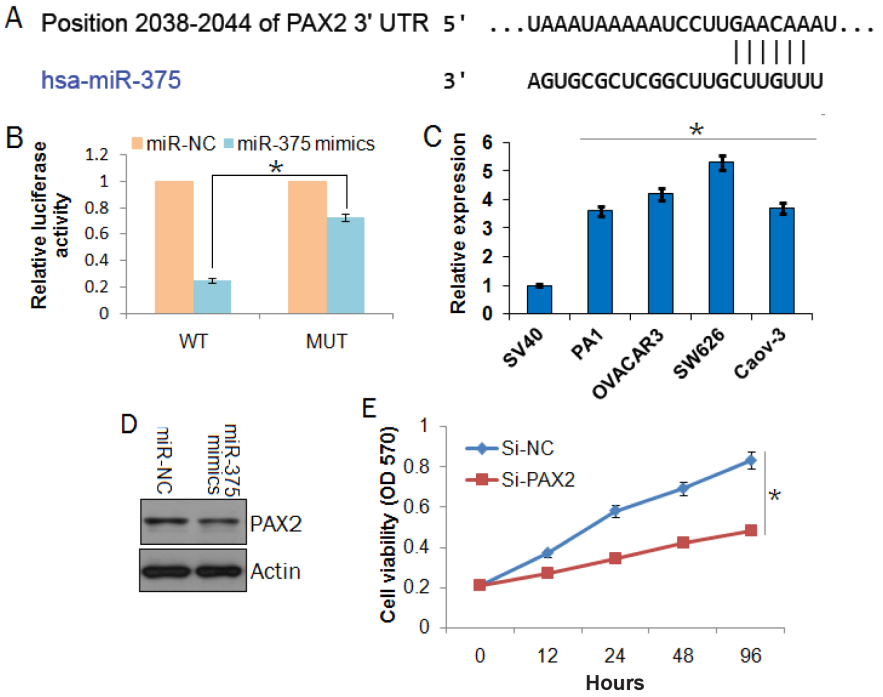

Figure 6. (A): TargetScan analysis showing PAX2 as the target of miR-375. **(B):** Dual luciferase assay. **(C):** Expression of miR-PAX2 in normal SV40 cells and different ovarian cancer cells. **(D):** Expression of PAX2 in miR-NC or miR-375 mimics transfected OVACAR-3 cells. **(E):** Cell viability of si-NC or si-PAX2 transfected OVACAR-3 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

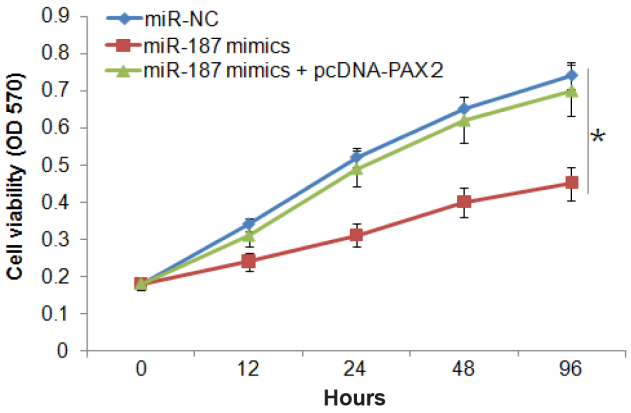

Figure 7. Overexpression of PAX2 avoids the tumor suppressive effects of miR-375 mimics transfected OVACAR-3 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

upregulated in all the cell lines (Figure 6C). However, miR-375 overexpression could cause decline in PAX2 expression in the OVACAR-3 cells (Figure 6D). We also sought to ascertain if silencing of PAX2 caused similar effects on OVACAR-3 cells. We found that PAX2 silencing caused a remarkable decline in the growth of OVACAR-3 cells (Figure 6E). The impact of PAX2 overexpression was also investigated on the growth of the OVACAR-3 cells overexpressing miR-375. We observed that overexpression of PAX2 in the OVACAR-3 cells overexpressing miR-375 promoted their proliferation, thereby avoiding the growth inhibitory effects of miR-375 (Figure 7).

Discussion

MicroRNAs (miRs) have shown great promise as therapeutics for cancer treatment [13]. Since the discovery of the first miR in early 1990s, several miRs have been reported to play vital roles in different cellular processes, such as proliferation, cell death, metastasis via modulation of post-gene expression post-transcriptionally [14]. The miRs have been shown to act as tumor suppressors as well as oncomiRs and many of the miR-based therapies are being evaluated for cancer treatment [15]. The miR-375 has been found to act as tumor suppressor but its therapeutic implications have not been explored in ovarian cancer. Herein, we determined the therapeutic implications of miR-375 in ovarian cancer. We found miR-375 expression was considerably suppressed in ovarian cancer. These findings are complemented by earlier studies wherein miR-375 has been found to be downregulated in lung cancer [16]. Next, miR-375 was overexpressed in ovarian cancer and we found that miR-375 ectopic expression halted the proliferation of OVACAR-3 cells by G2/M cell cycle arrest. Studies carried out earlier have reported that miR-375 halted the proliferation of liver and colorectal cancer cells [11,12]. The impact of miR-375 overexpression was also examined on the cisplatin sensitivity and we found that miR-375 enhances the cisplatin sensitivity of the ovarian cancer cells. In addition, earlier studies have also shown that miR-375 enhances the cisplatin sensitivity of the gastric cancer cells [17]. The transwell assays showed that miR-375 overexpression suppressed the metastasis of the ovarian cancer cells. These findings are in agreement with a previous study wherein miR-375 has been reported to inhibit the migration and invasion of the esophageal cancer cells [18]. The miRs have been reported to exert their effects by targeting different genes. Herein, we found that miR-375 targets PAX2 in ovarian cancer and suppression of PAX2 could inhibit the growth of ovarian cancer cells. However, PAX2 overexpression could nullify the tumor-suppressive effects of miR-375.

Conclusion

The findings the present study indicate that miR-375 is overexpressed in ovarian cancer and targets PAX2 to control the growth and metastasis of the ovarian cancer cells. Taken together, miR-375 may be utilised as therapeutic target for ovarian cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

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