MicroRNA-126 affects ovarian cancer cell differentiation and invasion by modulating expression of vascular endothelial growth factor

JIANQIAO LUO1,2, CAIDAN ZHU2, HONGYA WANG2, LI YU2 and JIANWEI ZHOU2

1Department of Gynecology, Shaoxing Shangyu Women and Children Hospital, Shaoxing, Zhejiang 312000; 2Department of Gynecology, The Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310009, P.R. China

Received April 17, 2016; Accepted November 2, 2017

DOI: 10.3892/ol.2018.8025

Abstract. Primary ovarian cancer is the main cause of gynecological cancer-associated mortality. However, the mechanism behind the spread of ovarian cancer requires elucidation. The present study aimed to investigate the effects of microRNA-126 (miR-126) on differentiation and invasion, and its mechanism in primary ovarian cancer. Ovarian cancer SKOV3 cells transfected with LV3-has-miR-126 mimics and LV3-has-miR-126 inhibitor were produced; it was revealed that LV-miR-126 mimics could induce cell cycle arrest at G1 phase, suppress cell invasion through Matrigel-coated membranes and downregulate the expression of vascular endothelial growth factor (VEGF). Furthermore, LV-has-miR-126 inhibitor-transfected cells could increase the number of cells in S phase, induce cell invasion and upregulate the expression of VEGF. The present study, to the best of our knowledge, is the first to report that miR-126 may serve tumor suppressor roles by inducing G1 cell cycle arrest and suppressing invasion in ovarian cancer cells, at least in part by targeting VEGF expression.

Introduction

Primary ovarian cancer is the leading cause of gynecological cancer-associated mortality cause of death in patients with and most cases are diagnosed at an advanced stage with lymph node metastasis (1-3). Overall 5-year survival rates of patients with advanced ovarian cancer undergoing comprehensive surgical procedures remain low, owing to the spread of ovarian cancer cells throughout the peritoneal cavity (4). The majority of women are diagnosed with stage III or IV cancer (5,6).

Vascular endothelial growth factor (VEGF) is a potent mediator of angiogenesis that induces the formation of novel blood vessels, and the growth and metastasis of cancers (7,8). The growth and spread of tumors depends on the formation of an adequate vascular support and amoeboid mode of tumor cell invasion (9). Furthermore, several studies have indicated that VEGF serves a role in promoting endothelial cell proliferation and migration, and induces a direct effect on cell proliferation and invasiveness (3,10,11). Previous studies have indicated that suppressing the expression or activity VEGF in ovarian cancer cells may represent a potential anticancer therapy (12-14).

MicroRNAs (miRNAs/miRs) are 22-nucleotide-long non-coding RNAs that regulate the expression of target mRNAs and are involved in cellular processes, including proliferation, differentiation, apoptosis and development (15-17). miR-126 is upregulated in the endothelium and originates from a common precursor structure located within the epidermal growth factor-like protein 7 gene (18,19). Several online databases are available to identify potential miRNA targets. Analysis performed using the PicTar algorithm (http://www.pictar.org/) (20), miRBase (www.mirbase.org) (21) and TargetScan target prediction (version 6.2; www.targetscan.org) (22) tools revealed that VEGF was the putative target of miR-126 that may serve a role in angiogenesis owing to regulation of VEGF signaling (23,24). Alteration of expression of miR-126 was also reported in various breast, lung and prostate cancer cells (24-26). However, the cellular mechanisms underlying the effect of miR-126 on ovarian cancer cells remains to be elucidated.

The present study aimed to characterize the role of miR-126, and the signaling pathway that it may be involved in, in the pathogenesis of ovarian cancer cells. Using SKOV3 ovarian cancer cells with up or downregulated miR-126, the present study attempted to identify the role of miR-126 in ovarian cancer cells.

Materials and methods

Cell culture. The ovarian cancer SKOV3 cell line (American Type Culture Collection, Manassas, VA, USA) was used in the present study. Cells were maintained and propagated in vitro by
Cells were subsequently washed three times with PBS, and incubated for 5 min at 37°C. SKOV3 cells were treated with LV3-has-miR-126 inhibitor and LV3-NC as LV3-has-miR-126 mimic group, LV3-has-miR-126 inhibitor group and NC group respectively, while uninfected SKOV3 cells were the untreated SKOV3 cells group. The SKOV3 cells of the first three groups were infected at a multiplicity of infection (MOI) of 15 in the presence of 5 µg/ml polybrene (Shanghai GenePharma Co., Ltd.). Efficiency of infection was ~90% as assessed by GFP and fluorescent microscopy. Cells were used for subsequent experimentation 72 h after transfection.

Cell cycle assay. Cells from all treatment groups were harvested at 48 h following transfection, fixed in 70% ice-cold ethanol overnight at 0°C and washed with 1X Buffer A (centrifugation at 4,200 x g for 5 min at 0°C). Subsequently, 5 µl propidium iodide (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) was added into 0.5 ml of cell suspension (5x10^5 cells) for 15 min at room temperature. Experiments were performed in triplicate for each sample and analyses were performed using a flow cytometer (FACScanto II; BD Biosciences, Franklin Lakes, NJ, USA) in accordance with the manufacturer's protocol. Multicycle software for Windows (Phoenix Flow Systems, San Diego, CA, USA) was used to determine the percentage of cells in G1, G2, and S stages.

Cell invasion assay. A total of 48 h following transfection, the invasiveness of ability cells was assayed using a Transwell invasion assay (8-mm pore size). Transwell inserts were put into the 24-well plates. A total of 0.1 ml Matrigel (50 mg/ml; BD Biosciences) was added onto the plate surface and incubated for 4-5 h. SKOV3 cells were freshly trypsinized (0.25% trypsin) at 37°C for 3 min and washed with PBS, then suspended in DMEM with 0.1% BSA (Gibco; Thermo Fisher Scientific, Inc.). Subsequently, 0.1 ml cell suspension (1x10^5 cells) was added to the upper chamber of each insert coated with Matrigel. A total of 0.5 ml DMEM containing 20% FBS was added into the lower chamber and cells were allowed to invade for 24 h at 37°C in a 5% CO2 humidified incubator. Following the incubation, cells on the membrane were removed using a cotton swab and 0.05% crystal violet stain was used to identify migratory cells at 20°C for 30 min. The number of migratory cells on the lower surface of the membrane was counted under Nikon Labophot 2 light microscope (Nikon Corp., Tokyo, Japan; 10 fields of vision) at a magnification of x200.

Immunofluorescence staining and western blot analysis. Analysis preformed using the PicTar algorithm (http://www.pic.tar.org/) (20), miRBase (www.mirbase.org) (21) and TargetScan target prediction (version 6.2; www.targetscan.org) (22) tools revealed that VEGF was the putative target of miR-126. Therefore, the expression of VEGF was explored in all groups. A total of 48 h after transfection, cells were fixed in 4% paraformaldehyde for 30 min at 20°C, washed three times with PBS, and incubated for 5 min at -20°C in 95% ethanol (v/v in PBS). Cells were subsequently washed three times with PBS, blocked for 1 h at 20°C in 5% normal goat serum (Wuhan Boster Biological Technology, Ltd., Wuhan, China) in PBS with 0.1% Triton X-100 and incubated overnight with anti-VEGF antibodies (cat no. BA0407; 1:200; Wuhan Boster Biological Technology, Ltd.) at 4°C. Cells were subsequently seeded into a 15-cm dish. pGLV3–miR-126 or pGLV3 vector and packing plasmids, including pGag/Pol, pRev and pVSV-G analysis by Shanghai GenePharma Co., Ltd. The resulting vectors were named LV3–has-miR-126, LV3–has-miR-126 inhibitor and LV3–NC. All sequences of resulting vectors were verified with sequence analysis by Shanghai GenePharma Co., Ltd.
from the G0 phase revealed that SKOV3 cells were promoted to 34.44% in the miR-126 mimic group compared with the NC group (P<0.05). These results indicate that the overexpression of miR-126 in SKOV3 cells induced G0 cell cycle arrest. Cell cycle analysis was also conducted in the miR-126 inhibitor group. Following transduction with the LV3-miR-126 inhibitor for 48 h, flow cytometry analysis revealed that SKOV3 cells were promoted from the G1 phase into the S phase. The percentage of cells in G1 phase was from 44.47% in the NC group and 9.42% in the miR-126 inhibitor group, while the percentage of cells in S phase was 53.12% in the NC group and 90.58% in the miR-126 inhibitor group (P<0.05). There were no significant alterations in cell cycle ratios between the untreated group and the NC group (P>0.05; Fig. 1). The results demonstrated that miR-126 mimic arrested SKOV3 cells at the G1 phase while the miR-126 inhibitor induced cell cycle progression.

**Cell invasion assay.** The number of SKOV3 cells in the miR-126 mimic group that invaded through the Matrigel-coated membranes was significantly lower than that in the untreated group (164.6±25.8 vs. 253.1±14.9 cells, respectively; P<0.05; Fig. 2). Furthermore, the depletion of miR-126 significantly promoted SKOV3 cell invasion through Matrigel-coated membranes, compared with the untreated group (290.9±13.2 cells vs. 253.1±14.9 cells, respectively; P<0.05; Fig. 2). There were no significant differences between the number of invaded NC cells and that of the untreated cells (256.5±15.2 vs. 253.1±14.9 cells respectively; P>0.05; Fig. 2). These results indicated that downregulation of miR-126 may promote cell invasion, whereas upregulation of miR-126 inhibits the invasion of cells through Matrigel-coated membranes.

**Immunofluorescence staining and western blot analysis.** Immunofluorescence double staining and semi-quantitative confocal laser scanning analysis revealed that miRNA vectors and VEGF were expressed in the four treatment groups. Green fluorescence, indicating vector-transfected cells, was detected in all cell nuclei and only certain cytoplasmic areas. The red fluorescence detected in the cytoplasm of SKOV3 cells indicated VEGF expression. The results of immunofluorescence staining demonstrated that transfection with miR-126 mimics mediated the inhibition of VEGF expression. The mean immunofluorescence intensity of VEGF in the miR-126 inhibitor group was significantly increased compared with that in the control group (P<0.05; Fig. 3). Furthermore, the expression level of VEGF decreased as a result of overexpression of miR-126 in LV3-has-miR-126-transfected SKOV3 cells, compared with untreated and negative control cells (Fig. 3). These results further indicated that miR-126 has an inhibitory role on VEGF expression in SKOV3 cells.

VEGF protein was detected in all treatment groups as a single band at ~28 kDa. Densitometry analysis of the VEGF bands revealed a significant increase in expression levels (110.4±6.8%) in the LV3-has-miR-126 inhibitor group and a significant decrease (31.2±2.2%) in the LV3-has-miR-126 mimic group, compared with the untreated group (71.4±2.8%; both P<0.05; Fig. 4). There was no significant difference between the NC control group and the untreated group (70.9±3.8 vs. 71.4±2.8%, respectively; P>0.05; Fig. 4). The above results indicate that VEGF was upregulated in SKOV3 cells transfected with LV3-has-miR-126 inhibitor, whereas VEGF expression was inhibited following LV3-has-miR-126 mimic transfection.

**Discussion**

miR-126 has been reported to be downregulated in human breast, lung, stomach, cervix and pancreas cancer (24,27-30). Furthermore, patients with non-small cell lung cancer and...
LUO et al: miR-126 INHIBITS OVARIAN CANCER BY MODULATING EXPRESSION OF VEGF

primary breast tumors exhibiting low miR-126 expression displayed poor survival compared with patients with high miR-126 levels (25). These studies indicated the presence of an association between the expression of miR-126 and metastasis-free survival. The present study demonstrated that ovarian cancer cells transfected with miR-126 exhibited a significantly increased frequency of cells in G1 phase and a lower frequency of cells in S phase. The percentage of cells in G1 phase in the miR-126 mimic group was markedly higher than that in the NC group. These results demonstrated that miR-126 could induce ovarian cancer cell cycle arrest, whereas inhibition miR-126 promoted cell cycle progression and induced ovarian tumor cell proliferation.

Previous studies demonstrated that VEGF serves a role in the modulation of cellular functions, including angiogenesis, tumor cell proliferation and invasiveness (31-33). VEGF increases vascular permeability and leads to malignant ascites in later stages of ovarian cancer, indicating disease progression and treatment failure (31,34). Therefore, anti-VEGF treatment could aid in improving patient survival (35). Clinical trials have demonstrated that certain anti-VEGF agents, including bevacizumab, can increase the risk of treatment-associated death caused by pulmonary hemorrhage, hypertension, proteinuria or bleeding, owing to disruption of normal vasculature (36,37). These side effects limit the therapeutic usage of anti-VEGF agents. The targeting of endogenous miRNAs provides an alternative approach to anti-VEGF treatment (38). Online accessible algorithms, including PicTar, miRBase and TargetScan,
demonstrated that one of the potential targets of miR-126 is VEGF (18,19). The present study demonstrated that miR-126 was a target of VEGF and the upregulation of miR-126 markedly inhibited VEGF expression. The present study aimed to elucidate the interaction between miR-126 and VEGF in ovarian cancer cells. The results demonstrated that SKOV3 cells infected with LV-miR-126 can efficiently reduce the expression of VEGF. Furthermore, SKOV3 cells infected with LV-miR-126 inhibitor exhibited overexpression of VEGF and the cell proliferation as 90.58% cells of S phase vs. 53.12% in the NC group.

Invasion through the basement membrane is one of the features of aggressive tumors (39); therefore, the present study further investigated the association between the expression of miR-126 and ovarian cell invasion. Cells transfected with LV-has-miR-126 inhibitor exhibited increased invasiveness compared with the untreated group. The results also demonstrated that increasing the expression of miR-126 in ovarian cancer cells could reduce the invasiveness. We hypothesize that low expression of miR-126 promotes VEGF expression and cell invasion, leading to the development of ovarian cancer.

In conclusion, to the best of our knowledge, the present study is the first to present data indicating that miR-126 is a tumor suppressor that can induce ovarian cancer cell cycle arrest and suppress invasion, at least in part, by targeting VEGF.
expression. miR-126 may be used as a potential marker for clinical prognosis and treatment of ovarian cancer in the future.

Acknowledgements

The present study was supported by the Natural Science Foundation of Zhejiang Province (grant no. LY13H160018) and the National Natural Science Foundation of China (grant no. 81371881). The authors would like to thank Dr Faisal Rehman (Zhejiang University, Zhejiang, China) for critical proofreading of the manuscript and language correction.

Competing interests

The authors declare that they have no competing interests.

References