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microRNA-126 suppresses PAK4 expression in ovarian cancer SKOV3 cells

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Abstract. Primary ovarian cancer is one of the predominant causes of mortality from gynecological cancer. The suppression of serine/threonine p21-activated kinases (PAKs), proteins involved in cell morphology and cytoskeletal reorganization, has been hypothesized to improve the survival of patients with ovarian cancer. However, the association between microRNA-126 (miR-126) and PAK4 in the inhibition of ovarian cancer cell invasion remains to be established. The present study demonstrated changes in the level of PAK4 expression in ovarian cancer SKOV3 cells with altered miR-126 compared with normal SKOV3 cells. The SKOV3 cells that were transfected with LV3-miR-126 to increase miR-126 expression exhibited significantly downregulated expression levels of PAK4 ($P<0.05$), whilst transfection with the LV3-hsa-miR-126 inhibitor increased the expression of PAK4 in these cells ($P<0.05$), as assessed by immunofluorescence staining. Furthermore, western blot analysis revealed a significant increase in PAK4 expression in the SKOV3 cells transfected with the LV3-hsa-miR-126 inhibitor, and a decrease in those transfected with LV3-hsa-miR-126. The present study provides an experimental foundation for miR-126 as a potential tumor suppressor that may decrease PAK4 expression to inhibit ovarian cancer cells.

Introduction

Epithelial ovarian cancer is one of the most common causes of mortality among females (1). The high mortality rate of ovarian cancer patients (9.30 out of every 100,000 patients each year) is a consequence of late-stage diagnosis, and the five-year survival rate (<50% for patients >64 years) for the advanced stages is extremely poor in the USA, Europe and Japan (2). A large tumor burden and extensive metastatic lesions of the abdominal cavity also contribute to the poor prognosis and the high rate of mortality of this disease (3). Tumor cell migration/invasion is a complex process involving cytoskeletal reorganization and membrane ruffling. The suppression of cytoskeletal reorganization and the redistribution of actin fibers may lead to the formation of non-adhesive membrane protrusions and therefore, dysregulated cellular adhesion capacity; this has been hypothesized to improve the survival of patients with ovarian cancer (4).

The actin cytoskeleton is essential for cell motility and cell invasion (5,6). Serine/threonine p21-activated kinases (PAKs) are effector proteins for the Rho GTPases Cdc42 and Rac, which are important for cell morphology and cytoskeletal reorganization (7,8). PAK4 was initially identified due to its regulation of cytoskeletal reorganization (9,10). Subsequent studies indicated that PAK4 is a key integrator of cell migration, invasion and apoptosis (11,12). Furthermore, PAK4 is upregulated in the majority of cancer cell lines, while previous studies have revealed that PAK4 is strongly linked to the progression of ovarian tumors and breast cancer. Additionally, overexpression of PAK4 in mammary epithelial cells leads to tumorigenesis in mice. Therefore, this protein may be a valuable molecular prognostic marker and therapeutic target in a number of cancers (13-16).

microRNAs (miRNA/miR), are non-coding RNAs of ~22 nucleotides, and are involved in various cellular processes, including proliferation, differentiation, apoptosis and invasion (17-19). miR-126 originates from a common precursor structure located within the EGFL7 gene, and its expression levels have been reported to vary in a number of human cancers; patients with low miR-126 expression exhibit poor survival compared with patients with high miR-126 levels (20-23). It has been proposed that miR-126 is essential in the inhibition of the invasive growth of cancer cells. Thus, the current study investigated whether the up- or downregulation of miR-126 modulates PAK4 expression in human ovarian cancer cells.

Materials and methods

Cell culture. SKOV3 cells (American Type Culture Collection, Rockville, MD, USA) were used as the ovarian cancer cells in the present study. The cells were maintained and propagated *in vitro* by serial passage in Dulbecco's modified

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Eagle's medium (DMEM; Gibco, Life Technologies Corporation, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Life Technologies Corporation), 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37˚C. All procedures were performed according to the internationally accepted ethical guidelines and approved by the Institutional Review Board of the Second Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China).

Plasmid construction, lentivirus packaging and cell infection. pGLV3/H1/green fluorescent protein (GFP)+Puro (pGLV3; Shanghai GenePhama Co., Ltd., Shanghai, China), a lentiviral vector, was used to construct the pGLV3-miR-126 plasmid. The miR-126 mimic, miR-126 inhibitor and negative control (NC) oligonucleotides were chemosynthesized by Shanghai GenePhama Co., Ltd. The oligonucleotide sequences were as follows: miR-126, 5'-TCG TACCGTGAGTAATAATGCG-3'; hsa-miR-126 inhibitor, 5'-CGCATTATTACTCACGGTACGA-3'; and microRNA NC, 5'-TTCTCCGAACGTGTCACGT-3'. The miR-126 small hairpin (sh)DNA double chain template sequence was synthesized artificially, and inserted into the pGLV3-miRNA lentivirus plasmid. The miR-126 mimic sequence was constructed as follows: (Forward) hsa-miR-126-*Bam*HI, GATCCGTCGTACCGTGAGTAATAATGCGTTCAAGAG ACGCATTATTACTCACGGTACGACTTTTTTG; (reverse) hsa-miR-126-*Eco*RI, AATTCAAAAAAGTCGTACCGT GAGTAATAATGCGTCTCTTGAACGCATTATTACTCA CGGTACGACG. The miRNA-126 inhibitor sequence was constructed as follows: (Forward) hsa-miR-126-*Bam*HI, GATCCGAGCATGGCACTCATTATTACGCTTCAAGAG AGCGTAATAATGAGTGCCATGCTCTTTTTTG; (reverse) hsa-miR-126-*Eco*RI, AATTCAAAAAAGAGCATGGCA CTCATTATTACGCTCTCTTGAAGCGTAATAATGAGTGCC ATGCTCG. pGLV3-shDNA-NC was used as a negative control, with the following sequence: (Forward) NC-*Bam*HI, GATCCGTCGTACCGTGAGTAATAATGCGTTCAAGAG ACGCATTATTACTCACGGTACGACTTTTTTG; (reverse) shNC-*Eco*RI, AATTCAAAAAAGTCGTACCGTGAGTAA TAATGCGTCTCTTGAACGCATTATTACTCACGGTACG ACG.

The 293T producer cell line (Cell Bank of Chinese Academy of Science, Beijing, China) was maintained in DMEM, with 10% FBS, 4.0 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. One day prior to transfection, the cells were seeded into a 15-cm dish. pGLV3-miR-126 or pGLV3 vectors and packing plasmids, including pGag/Pol, pRev and pVSV-G (Shanghai GenePhama Co., Ltd.) were co-transfected using RNAi-mate (Shanghai GenePhama Co., Ltd.), according to the manufacturer's instruction. At 72 h post-transfection, the supernatant was harvested, cleared by centrifugation (2,200 x g at 4˚C for 4 min), passed through a 0.45-μm syringe filter, and cleared by centrifugation again (20,000 rpm at 4˚C for 2 h). The titer of the virus was measured according to the expression level of GFP, following the manufacturer's instructions. The packaged lentiviruses were designated LV3-hsa-miR-126, LV3-hsa-miR-126 inhibitor and LV3-NC. The sequences of the resulting vectors were verified by sequence analysis.

The SKOV3 cells were infected with LV3-hsa-miR-126, LV3-has-miR-126 inhibitor or LV3-NC, at a multiplicity of infection ratio of 15, in the presence of 5 μg/ml polybrene (Shanghai GenePhama Co., Ltd.); the infection efficiency was 80-90%, as assessed by microscopic analysis of GFP fluorescence.

Immunofluorescence staining and western blot analysis. At 48 h post-transfection, the cells were fixed in 4% paraformaldehyde, washed three times with phosphate-buffered saline (PBS), and incubated for 5 min at -20˚C in 95% ethanol (vol/vol in PBS). The cells were subsequently washed three times with PBS, blocked for 1 h in 5% normal goat serum in PBS with 0.1X Triton X-100, and incubated overnight with polyclonal rabbit anti-human PAK4 antibodies (Abcam, Cambridge, MA, USA; dilution, 1:200) at 4˚C. The following day, the cells were washed three times with PBS and incubated for 40 min at 37˚C with the corresponding secondary antibody [polyclonal goat anti-rabbit immunoglobulin (Ig)G (H+L)-tetramethylrhodamine (TRITC); dilution 1:200; SouthernBiotech, Birmingham, AL, USA], then washed and mounted. Immunostained SKOV3 cultures were examined under a laser scanning confocal microscope (LSM 510 Meta; Carl Zeiss Microscopy GmbH, Jena, Germany) for detection of the TRITC-fluorophore. Each group was photographed at x400 magnification with the aid of a digital camera attached to the microscope, and the expression of PAK4 was assessed by calculating the percentage of positive cells and the optical density, subsequent to defining a threshold for background correction.

For the western blot analysis, proteins were extracted from the SKOV3 cells, solubilized in radioimmunoprecipitation assay buffer, separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Wuhan Boster Ltd., Wuhan, China) and electro-transferred onto polyvinylidene difluoride membranes (Invitrogen Life Technologies, Carlsbad, CA, USA). The membranes were blocked in 5% skimmed milk powder prepared in Tris-buffered saline with Triton X-100 (TBS-T) for 30 min. For PAK4 detection, the membranes were incubated at 4˚C overnight with anti-PAK4 antibodies (Abcam; dilution 1:500). The membranes were washed three times for 10 min in TBS-T and incubated with a 1:5,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG for 2 h. Finally, the membranes were washed six times for 20 min each in TBS-T, prior to development with a standard enhanced chemiluminescence kit (KeyGEN Biotech, Nanjing, China). The densitometric analysis of the PAK4 and β-actin bands was assayed by Quantity One software (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis. All data are presented as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS statistical software, version 17.0 (SPSS, Inc., Chicago, IL, USA) for Windows. The significance of any differences between groups was evaluated using one-way analysis of variance. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Immunofluorescence double staining and semi-quantitative confocal laser scanning analysis detected the expression of

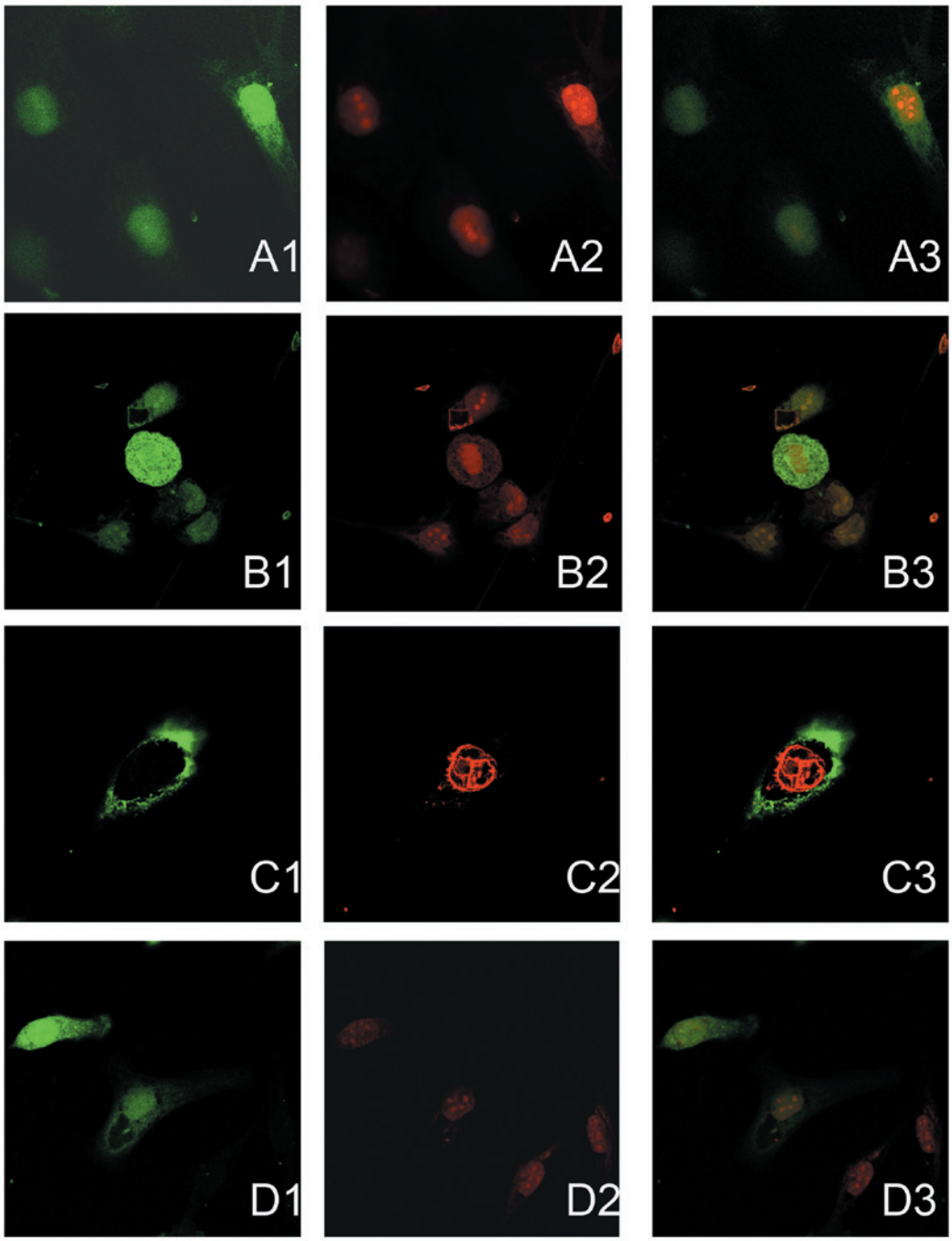

Figure 1. Immunofluorescence staining of PAK4 in four groups (magnification, x400): (A) Untransfected SKOV3 cells; (B) SKOV3 cells transfected by LV3 negative control; (C) SKOV3 cells transfected by LV3-hsa-miR-126 inhibitor; and (D) SKOV3 cells transfected by LV3-hsa-miR-126. (1) Green fluorescent protein-positive cells are indicated by green signals; (2) PAK4-positively stained cells are indicated by red signals; (3) merged images. The mean immunofluorescence intensities of PAK4 in the miR-126 inhibitor group were significantly higher (C2) than that of control group cells (A2), while the expression of PAK4 in the miR-126 upregulated group (D2) was significantly lower compared with that in the control group cells ($P<0.05$). PAK, serine/threonine p21-activated kinase.

the miRNA vectors and PAK4 in the following four groups of SKOV3 cells: Untransfected cells, LV3-NC-transfected cells, LV3-hsa-miR-126-transfected cells and LV3-hsa-miR-126 inhibitor-transfected cells. The expression of PAK4 was indicated by red immunofluorescence staining, and the GFP expressed by the miRNA vectors (green fluorescence) highlighted successfully transfected cells; green fluorescence was detected in all of the nuclei, but only in certain cytoplasmic regions of the SKOV3 cells in the NC, miR-126 inhibitor and miR-126 mimic groups. The mean immunofluorescence intensity of PAK4 in the miR-126 inhibitor group was significantly higher (Fig. 1, C2) compared with that of the untransfected SKOV3 cells (Fig. 1, A2). Furthermore, the expression level of PAK4 was effectively decreased by the overexpression of miR-126 in the LV3-hsa-miR-126-transfected cells (Fig. 1, D2) compared with that of the untransfected SKOV3 cells (Fig. 1, A2), and particularly compared with that of LV3-hsa-miR-126 inhibitor-transfected cells (Fig. 1, C2). Furthermore, as shown in Fig. 1 C3, the cells transfected with LV3-hsa-miR-126 inhibitor (green) exhibited greater expression of PAK4 (red), whilst cells transfected with

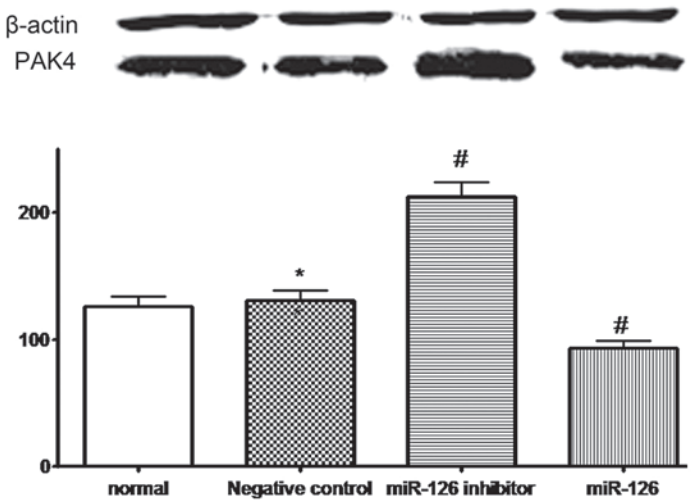

Figure 2. Western blot analysis of PAK4 expression in four groups. Data are presented as the mean ± standard deviation. Normal, the group of untransfected SKOV3 cells; negative control, the group of SKOV3 cells transfected by LV3 negative control; miR-126 inhibitor, the group of SKOV3 cells transfected with LV3-hsa-miR-126 inhibitor; miR-126, the group of SKOV3 cells transfected by LV3-hsa-miR-126. *P>0.05, vs. normal group, #P<0.05, vs. normal group. miR/miRNA, microRNA; PAK, serine/threonine p21-activated kinase.

LV3-hsa-miR-126 (green) exhibited reduced expression of PAK4 (red) (Fig. 1 D3).

PAK4 protein expression in the four groups of cells was also evaluated by western blotting. PAK4 was visible as bands of ~64 kDa. A densitometric analysis of the PAK4/β-actin bands revealed a significant increase in PAK4 expression in the SKOV3 cells transfected with LV3-hsa-miR-126 inhibitor (mean ± SD, 215.1±10.5 vs. 128.6±8.2%; P=0.001) and a decrease in PAK4 expression in the SKOV3 cells transfected with LV3-hsa-miR-126 (mean ± SD, 91.6±7.7 vs. 128.6±8.2%; P=0.002), compared with the untransfected SKOV3 cells (Fig. 2). No significant difference was observed between the expression in the SKOV3 cells in the NC group and those that were untransfected (mean ± SD, 130.9±9.1 vs. 128.6±8.2%; P=0.706; Fig. 2). Therefore, it is proposed that LV3-has-miR-126 inhibitor increases the expression of PAK4, whereas LV3-hsa-miR-126 attenuates this expression.

Discussion

In the present study, changes in PAK4 expression were demonstrated in ovarian cancer cells with up- or downregulated miR-126 (induced by the transfection of LV-miR-126 or LV-has-miR-126 inhibitor) when compared with normal ovarian cancer cells. The SKOV3 cells transfected with LV-hsa-miR-126 exhibited reduced expression of PAK4, while the cells transfected with LV-hsa-miR-126 inhibitor exhibited increased expression. These findings suggest that miR-126 is a potential tumor suppressor, with the ability to decrease the level of PAK4 in ovarian cancer SKOV3 cells.

The invasive ability of malignant cancer cells depends upon the altered regulation of cell migration by the membrane protrusion formation in response to chemotactic and migratory stimuli (6). Membrane protrusions are formed by polymerization of submembrane actin filaments. The PAK family comprises important signaling proteins that are indicated to be involved in a variety of cellular functions, including cell proliferation, migration and cytoskeletal organization (7,24). The family consists of six members, categorized into two groups: Group A, PAKs 1, 2 and 3; and group B, PAKs 4, 5 and 6 (7,25). PAK4 has been indicated to be involved in several types of cancer, and strong links have been observed between PAK4 and ovarian cancer (26). Analysis of cell migration and invasion in *in vitro* and *in vivo* studies has highlighted the contribution of PAK4 to the progression and metastasis of ovarian cancer; this is consistent with the role of PAK4 in the reorganization of the cytoskeleton and the migration of cells, which is at least in part executed in the cytoplasm (26). PAK4 expression and activation are important in cancer progression, and increased PAK4 expression has been shown to be associated with metastasis, progression to late stages of the disease, reduced patient survival and increased resistance to chemotherapy (13,14,27). The mechanisms by which PAK4 affects ovarian cancer cell progression include the control of cell migration, invasion and proliferation. PAK4 may act via the regulation of c-Src, mitogen-activated protein kinase kinase/extracellular signal-regulated kinases 1/2, matrix metalloproteinase-2, and c-Src/epidermal growth factor receptor. Inhibition of PAK4 may therefore be a potentially valuable therapeutic target (16,28).

miR-126 is a non-coding RNA that is involved in various cellular processes, including proliferation, differentiation, apoptosis and invasion (17,21,29). miRNAs that are upregulated in cancer may function as oncogenes through the negative regulation of tumor suppressor genes, whilst miRNAs that are downregulated may function as tumor suppressor genes and inhibit cancer by regulating oncogenes (30,31). miR-126 acts as a metastatic suppressor in a number of human cancers (21,23). However, the expression and function of miR-126 in ovarian cancer remains unclear. In the present study, the association between miR-126 and PAK4 was investigated in ovarian cancer cells. The results demonstrated that transfection with LV3-miR-126 may efficiently reduce the expression of PAK4 in SKOV3 cells. Furthermore, the LV-miR-126 inhibitor was observed to upregulate the expression of PAK4 in these cells.

In conclusion, as PAK4 is essential for ovarian cancer cell invasion, the present study provides an experimental foundation for the use of miR-126 as a potential tumor suppressor;

this miRNA may potentially be used to decrease expression levels of PAK4, leading to the inhibition of ovarian cancer cell invasion. However, further studies are required to elucidate the mechanisms involved in the suppression of PAK4 by miR-126.

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