Siva 1 inhibits proliferation, migration and invasion by phosphorylating Stathmin in ovarian cancer cells

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Received April 6, 2016; Accepted March 3, 2017

DOI: 10.3892/ol.2017.6307

Abstract. Ovarian cancer is one of the most common types of gynecologic malignant tumor, with high incidence and high mortality rates. It is difficult to diagnose ovarian cancer early due to the complex structure and function of the ovaries. Siva 1 is a well-known pro-apoptosis protein that functions in multiple types of cancer cells: There are several studies demonstrating that Siva 1 arrests apoptosis and facilitates cancer development in osteosarcoma and non-small cell lung cancer. Whether Siva 1 functions in ovarian cancer remains unknown. In the present study, it was established that Siva 1 was stably overexpressed in ovarian cancer cell lines, and demonstrated that the overexpression of Siva 1 inhibited proliferation, promoted apoptosis and suppressed migration and invasion by facilitating phosphorylation of Stathmin and polymerization of α-tubulin in ovarian cancer cells. These data provide specific novel insights into the molecular mechanism of ovarian cancer, and may be of significance for the clinical diagnosis and therapy of ovarian cancer.

Introduction

Ovarian cancer is one of the most common types of gynecologic malignant tumor, whose incidence rate ranks third in cancers of the female reproductive system, and mortality ranks first (1). The etiology of ovarian cancer remains unknown, and may be associated with a number of internal and external factors. Early diagnosis of ovarian cancer is difficult due to the complicated structure and function of the ovary. In addition, late-stage tumors often recur and metastasize subsequent to surgery, and eventually evolve resistance to chemotherapy. The five-year survival rate of patients with ovarian cancer is <30% (1,2). Therefore, it is necessary to identify molecules that function in the occurrence, development and metastasis of ovarian cancer, and to develop more effective diagnosis and therapy in clinic.

Siva 1 is a pro-apoptotic protein first obtained by Prasad et al from a HeLa cell library via yeast two-hybrid screening with CD27, a member of the tumor necrosis factor receptor superfamily, as the bait (3). Siva 1 exists in a wide variety of tissues and cells, and serves a crucial role in certain extrinsic and intrinsic apoptosis signaling pathways, for example: Siva 1 interacts with CD27 and induces apoptosis via the caspase-independent mitochondrial pathway in T lymphocytes (4); Siva 1 maybe stimulated by thromboxane receptor and aggravates apoptosis of HeLa cells induced by cisplatin (5). Siva 1 binds to B-cell lymphoma-extra large (Bcl-XL) and inhibits Bcl-XL-mediated apoptosis protection from UV radiation in breast cancer cells (6). Additionally, Siva 1 participates in virus infection-associated apoptosis: It has been demonstrated that Siva 1 promotes apoptosis of A549 cells induced by influenza A virus and sensitizes CD4+ cells to HIV-1 envelope-induced apoptosis in a caspase-dependent manner (7,8).

However, Siva 1 has been demonstrated to serve opposite roles in other previous studies. In KRASG12D-derived non-small cell lung cancer (NSCLC), Siva 1 is highly expressed and facilitates tumorigenesis by regulating metabolism and autophagy (9); the knockdown of Siva 1 inhibits human fetal lung cell proliferation and induces cell cycle arrest via the alternative reading frame tumor protein (p) 53 pathway (10); Siva 1 destabilizes p53 and promotes its degradation, suppresses p53-mediated apoptosis in osteosarcoma cells, and the knockdown of Siva 1 inhibits tumorigenesis (11). In addition, Li et al revealed that Siva 1 phosphorylated Stathmin at Ser16, attenuated its microtubule-destabilizing activity and suppressed epithelial-mesenchymal transition and metastasis of breast cancer cells (12). Stathmin is a microtubule destabilizer, participating in cell mitosis and migration by regulating microtubule stability. Additionally, it is highly expressed in a number of malignant tumors, including ovarian cancer (13), suggesting a cancer-promoting effect.

In conclusion, the effect of Siva 1 is different in a number of cell types, which is a result of the combined action of multiple signaling pathways. At present, Siva 1 is pro-apoptotic.
and carcinostatic in colorectal (14), cervical (5) and breast cancer (12) and acute leukemia (15), but anti-apoptotic and carcinogenic in osteosarcoma (11) and NSCLC (9). However, it remains unknown whether Siva 1 functions in ovarian cancer. In the present study, the effect of Siva 1 on ovarian cancer cells and the underlying mechanism of its action in these cells were investigated.

Materials and methods

Cell culture, transfection and establishment of stable cell line. Ovarian cancer SKOV3 and OVCAR-3 cell lines were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences and cultured with RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA USA) supplemented with 10% fetal bone serum (FBS; Hyclone; GE Healthcare; Logan, UT, USA) at 37°C in 5% CO₂. A2780 cells were purchased from Cell Preservation Center of Wuhan University and cultured with Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented 10% FBS at 37°C in 5% CO₂. Siva 1 overexpression plasmid pCMV3-Siva 1 or the control pCMV3 [China National Pharmaceutical Group (CNPGC); Sinopharm, Beijing, China] were transfected into A2780 cells with lipofectamine reagent (Invitrogen; Thermo Fisher Scientific, Inc.). A total of 24 h after transfection, 200 µg/ml G418 (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the medium, and the culture medium was renewed every 2 days. Approximately 1 week later, monoclonal cell clusters were observed and selected for culture. Thereafter, the Siva 1 stably expressed A2780 cell line and its control were used for subsequent experiments.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was isolated from the cells by total RNA rapid isolation kit (BioTeke Corporation, Beijing, China) according to the manufacturer's protocol. The concentration was measured by the OD samples using a NanoDrop 2000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA). The RNA was reverse transcribed with Moloney Murine leukemia virus (M-MLV) reverse transcriptase (BioTeke Corporation), with oligo-deoxy-thymidine nucleotides and random primers (Sangon Biotech Co., Ltd., Shanghai, China) as the RT primers. The negative control was the RNA in pCMV3-transfected cells. For the RT-PCR, the following items were added with RNA samples into each tube: 1 µg RNA, 1 µl oligo (dT) primers, 1 µl random primers, 2 µl dNTP and ddH₂O to make a final reaction volume of 14.5 µl. Each tube was heated at 70°C for 5 min, cooled on ice for 2 min, and the following items were added: 4 µl 5X buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 50 mM DTT; Dongsheng Biotech, Guangzhou, China), 0.5 µl RNasin ribonuclease inhibitor (Tianz, Inc., Beijing, China) and 1 µl M-MLV reverse transcriptase. The thermocycler conditions were as follows: 25°C for 10 min, then at 42°C for 50 min, heated at 95°C for 5 min, and finally cooled on ice for several min. There was 20 µl cDNA sample in each reaction. All the instruments used in this step were treated with Surface RNase Erasol (Tianz, Inc.) and all the reagents used in this protocol were RNase-free.

The produced cDNA (1 µg) was used for qPCR by Taq PCR MasterMix (BioTeke Corporation) supplemented with SYBR Green (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) to detect Siva 1 or Stathmin using the following procedure: 95°C for 10 min, 40 cycles of 95°C for 10 sec, 60°C for 20 sec and 72°C for 30 sec, and finally 4°C for 5 min, with β-actin as the internal control. The primer sequences are provided in Table I. The data were analyzed by the 2−ΔΔCq method (16), with β-actin as the reference gene.

Western blot analysis. Protein samples from SKOV3, OVCAR-3 and A2780 cell lines were extracted using a total protein extraction kit (Wanleibio Co., Ltd., Beijing, China) according to the manufacturer's protocol. Subsequent to quantification of the content using a bicinchoninic acid protein quantification kit (Wanleibio Co., Ltd.), the protein was isolated by SDS-PAGE using 40 µg protein in each lane and transferred to polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% skim milk (YILI, Hohhot, Inner Mongolia, China) at room temperature for 1 h, diluted by TBS TWEEN (TBST), the PVDF membrane was incubated with the following antibodies at 4°C overnight: rabbit anti-human anti-Siva 1 polyclonal antibody (dilution, 1:200; Santa Cruz Biotechnology, Inc., Dallas, TX, USA, cat. no., sc-48767), rabbit anti-human anti-cleaved caspase-3 polyclonal antibody (dilution, 1:1,000; Abcam, Cambridge, UK, cat. no., ab2302), goat anti-human anti-cleaved caspase-9 polyclonal antibody (dilution, 1:200; Santa Cruz Biotechnology, Inc., cat. no., sc-22182), rabbit anti-human anti-Bcl-2-like protein 4 (Bax) polyclonal antibody (dilution, 1:400; Boster, Hubei, Wuhan, China, cat. no., BA0315), rabbit anti-human anti-Bcl-2 polyclonal antibody (dilution, 1:400; Boster, cat. no., BA0412), rabbit anti-human anti-Stathmin polyclonal antibody (dilution, 1:500; Bioss Antibodies, Beijing, China, cat. no., bs-1902R), rabbit anti-human anti-phosphorylated Stat3 polyclonal antibody (dilution, 1:500; Bioss Antibodies, cat. no., bs-3431R), rabbit anti-human anti-α-tubulin polyclonal antibody (dilution, 1:1,000; Abcam, cat. no., ab178484) or mouse anti-human anti-actin AT-α-tubulin monoclonal antibody (dilution, 1:1,000; Abcam, cat. no., ab24610). Subsequent to washing with TBST 4 times for 5 min each, the PVDF membrane was incubated with goat anti-rabbit polyclonal IgG horseradish peroxidase (HRP; dilution, 1:5,000; Wanleibio Co., Ltd., cat. no., WLA024), goat anti-mouse polyclonal IgG-HRP (dilution, 1:5,000; Wanleibio Co., Ltd., cat. no., WLA024) or donkey anti-goat polyclonal IgG-HRP (dilution, 1:5,000; Beyotime Institute of Biotechnology, China, cat. no., BA001) at 4˚C overnight: rabbit anti-human anti-Siva 1 polyclonal antibody (dilution, 1:1,000; Santacruz Biotechnology, Inc., cat. no., sc-47778) and goat anti-rabbit polyclonal IgG-HRP (dilution, 1:5,000; Wanleibio Co., Ltd.) to detect the internal control, β-actin. The bands were analyzed with Gel-Pro-Analyzer software (version 4, Media Cybernetics, Silver Spring, MD, USA), and experiments were performed in triplicate.

Immunofluorescence. A2780 cells were seeded onto glass slides prior to this protocol. When cell confluence reached 70-80% the cells were fixed in 4% paraformaldehyde.
Seeded. Clones with >50 cells were considered positive. The cell migration rate was calculated as follows: clone number/2-3 times, cell clones were counted and the clone formation rate was calculated as follows: clone number of cells/well. Cell viability was detected at 24, 48, 72, and 96 h after seeding. The CCK-8 assay (Wanleibio Co., Ltd.) was added into the culture medium at 10 µl/well. Cell viability was measured using a microplate reader (Biotek Instruments, Winooski, VT, USA). The overexpression of Siva 1 in ovarian cancer cells, Siva 1 expression level was first detected in three ovarian cancer cell lines, A2780, OVCAR-3 and SKOV3. The western blot analyses results demonstrated that the expression level of Siva 1 in A2780 cells was markedly lower compared with OVCAR-3 cells and SKOV3 cells (Fig. 1A and B). The overexpression with Antifade Mounting Medium (BeyotimeInstitute of Biotechnology) and observed with fluorescence microscopy (Olympus Corporation) at magnification, x400, and analyzed with CellSens version 1.6 (Olympus Corporation).

**Wound healing assay.** A wound healing assay was performed to measure cell migration ability. A2780 cells were cultured with serum-free medium supplemented with 1 µg/ml mitomycin C (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 1 h. The wound was caused by 200 µl pipette tip, and images of the wound sizes 0, 24 and 48 h subsequent to wounding were captured with an inverted phase contrast microscope (Motic, Xiamen, Fujian, China) at magnification, x100. The number of cells/wound size at 0 h/48 h/wound size at 0 h; the cell migration rate at 48 h was calculated as (wound size at 0 h-wound size at 24 h)/wound size at 0 h.

**Transwell assay.** A2780 cell invasion was detected by Transwell assay supplemented with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) in vitro, with A2780 cells transfected with pCMV3 vector used as the negative control. All experiments were performed in triplicate. A total of 40 µl 2.5 mg/ml Matrigel was added into the Transwell chamber (Corning Incorporated, Corning, NY, USA) and preheated at 37°C to solidify. A total of 200 µl serum-free cell suspension with 1x10^5 cells was seeded into the upper chamber, and 800 µl medium with 20% FBS (Hyclone; GE Healthcare; Logan, UT, USA) was added into the lower chamber. Subsequent to culturing for 24 h, the Transwell chamber was removed and the cells in upper chamber were wiped away. The cells in lower chamber were fixed with 4% paraformaldehyde (CNPGC; Sinopharm) at room temperature for 1 h, and stained with DAPI (Biosharp, Hefei, China) for 5 min, and mounted with an anti-fade solution (Beijing Solarbio Science & Technology Co., Ltd.) in the dark. The slides were observed and images were captured with a fluorescence microscope (Olympus Corporation, Tokyo, Japan) at magnification, x400, and analyzed with Image pro-plus v6.0 (IPP) software (Media Cybernetics, Rockville, MD, USA).

**Colony formation.** A2780 cells were seeded onto 35-mm petri dishes with 500 cells per dish, and cultured at 37°C in 5% CO₂. Approximately 2 weeks later, the majority of the clones were able to be seen by the naked eye. The cells were fixed with 4% paraformaldehyde (CNPGC; Sinopharm) at room temperature for 20 min and stained with Wright-Giemsa stain reagent (Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China) for 5-8 min. Subsequent to rinsing with PBS 2-3 times, cell clones were counted and the clone formation rate was calculated as follows: clone number/number of cells seeded. Clones with >50 cells were considered positive.

**Hoechst staining.** A2780 cells were seeded on microscope slides in a 12-well plate with 3x10⁴ cells/well. When cell confluence reached 60-70%, the cells were fixed with 4% paraformaldehyde (CNPGC; Sinopharm) at room temperature for 20 min and stained with Hoechst 33258 stain reagent (Wanleibio Co., Ltd.) for 5 min in the dark. Cells were blocked with an anti-human anti-Siva 1 polyclonal antibody (dilution, 1:50; Santa Cruz Biotechnology, Inc., cat. no., sc-48767) at 4°C over night. Subsequent to rinsing with PBS, the cells were incubated with goat anti-rabbit polyclonal IgG-Cy3 (1:200) (Beyotime Institute of Biotechnology, cat. no., A0516) for 60 min, stained with DAPI (Biosharp, Hefei, China) for 5 min, and mounted with an anti-fade solution (Beijing Solarbio Science & Technology Co., Ltd.) in the dark. The slides were observed and images were captured with a fluorescence microscope (Olympus Corporation) at magnification, x400, and analyzed with Image pro-plus v6.0 (IPP) software (Media Cybernetics, Rockville, MD, USA).

**Table I. Sequences of real-time PCR primers.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5' to 3')</th>
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<tbody>
<tr>
<td>Siva</td>
<td>F: CCAAGCGAICTCTGTTCCTC</td>
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<tr>
<td></td>
<td>R: CCAATCGAGCATCGCCAC</td>
</tr>
<tr>
<td>Stathmin</td>
<td>F: TCGCTTGTCTTCTATTCACCA</td>
</tr>
<tr>
<td></td>
<td>R: CTTCCTTCTGTCCTGTGTT</td>
</tr>
<tr>
<td>β-actin</td>
<td>F: CTTAGTTGCCTTGACCTTTCCTTG</td>
</tr>
<tr>
<td></td>
<td>R: TGTCAACCTTCCAGGTTCAGTT</td>
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(F, forward; R, reverse.)
plasmid pCMV3-Siva 1 was then constructed and transfected into A2780 cells. By screening with G418, a monoclonal cell line was obtained, with A2780 cells transfected with pCMV3 as the control and untreated A2780 cells as the parental line. qPCR and western blot analyses showed that mRNA and protein levels of Siva 1 in pCMV3-Siva 1 cells increased 3.39-fold (P=0.0004) and 3.68-fold (P=0.0019), respectively, compared with pCMV3 (Fig. 1C-E). The immunofluorescence assay results demonstrated the same increase, and Siva 1 was distributed in the cytoplasm and in the nuclei (Fig. 1F).

**Siva 1 inhibits the growth and proliferation of ovarian cancer cells.** CCK-8 and colony formation assays were performed to detect cell viability and colony formation abilities of the cell lines. As shown in Fig. 2A, Siva 1 impaired cell viability by 21% (P=0.0056), 25% (P=0.0005), 30% (P<0.0001), 32% (P=0.0001) and 31% (P=0.0001) in 24, 48, 72, 96 and 120 h, respectively. The colony formation assay indicated that Siva 1 decreased the colony formation ability of A2780 cells by 44% (P=0.0025) (Fig. 2B). Therefore, these data suggest that overexpression of Siva 1 inhibited the proliferation of ovarian cancer cells.

**Siva 1 promotes apoptosis of ovarian cancer cells.** To determine the effect of Siva 1 on apoptosis of ovarian cancer cells, flow cytometry and Hoechst staining were performed. Flow cytometry detection demonstrated that Siva 1 increased the apoptosis rate by 4.55-fold (P<0.0001) (Fig. 3A and B). Using Hoechst staining, bright nuclei in were observed in the pCMV3-Siva 1 cells (Fig. 3C), which indicates chromosome breakage and nucleus shrinkage in apoptosis progress. In addition, there are a number of signaling molecules participating in intrinsic apoptosis, which may be detected as apoptosis markers. In the present study, the expression levels of executioners of apoptosis, cleaved caspase-3 and cleaved caspase-9, pro-apoptotic protein Bax and anti-apoptotic protein Bcl-2 were detected. The western blot analyses results demonstrated that in cells that expressed stable levels of Siva 1, the protein levels of cleaved caspase-3 increased 1.96-fold (P=0.0016), cleaved caspase-9 increased 2.03-fold (P=0.0002), Bax increased 2.02-fold (P=0.0005) and Bcl-2 decreased by 44% (P=0.0004) (Fig. 3D and E). These data indicate that Siva 1 promoted the apoptosis of ovarian cancer cells.

**Siva 1 suppresses the migration and invasion of ovarian cancer cells.** Since migration and invasion are other vital aspects of cancer cell malignancies, the migration and invasion potential of ovarian cancer cells was measured. The wound healing assay demonstrated that subsequent to Siva 1 overexpression, migration rate decreased by 41% (P=0.0052) and 49% (P=0.001) after 24 and 48 h, respectively (Fig. 4A and B). The Transwell assay with Matrigel found that Siva 1 decreased the invasion potential of ovarian cancer cells by 35% (P=0.0001) (Fig. 4C and D).

**Siva 1 enhances the phosphorylation of Stathmin.** The effects of Siva 1 on Stathmin in ovarian cancer cells were then investigated. qPCR and western blot analyses results demonstrated that overexpression of Siva 1 did not increase Stathmin expression, but promoted its phosphorylation (Fig. 5A-C). As the polymerized tubulin was acetylated, the level of acetyl-α-tubulin was also measured to determine whether the microtubule-destabilizing activity of
Figure 2. Siva 1 inhibits proliferation of ovarian cancer cells. (A) Overexpression of Siva 1 inhibited A2780 cell viability as detected by CCK-8 assay. (B and C) Overexpression of Siva 1 represses colony formation of A2780 cells as detected by plate colony formation assay. **P<0.01 vs. pCMV group; ***P<0.001 vs. pCMV group; OD, optical density.

Figure 3. Siva 1 promotes apoptosis of ovarian cancer cells. (A and B) Expression of Siva 1 promotes apoptosis of A2780 cells detected by flow cytometry. (C) Apoptosis of A2780 cells detected by Hoechst staining. (D and E) Expression level of apoptosis-associated proteins, cleaved caspase-3, cleaved caspase-9, Bax and Bcl-2 detected by western blot. **P<0.01 vs. pCMV group; ***P<0.001 vs. pCMV group; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4.
Siva 1 inhibits malignancies of ovarian cancer cells

Stathmin was impaired. Western blot analyses revealed that acetyl-α-tubulin levels were elevated, but the levels of α-tubulin were not in cells with an overexpression of Siva 1, which indicates that Siva 1 stabilized microtubules by inhibiting Stathmin activity.

Taken together, these data suggest that Siva 1 inhibited cell growth and proliferation, promoted apoptosis and suppressed migration and invasion by promoting Stathmin phosphorylation and stabilizing microtubules in ovarian cancer cells.

Discussion

There have been several studies demonstrating that Siva 1 facilitated or suppressed apoptosis in different types of cancer.
cells previously. In the present study, it was revealed that Siva 1 promoted intrinsic apoptosis and inhibited the proliferation of ovarian cancer cells. In this process, the levels of apoptosis executors activated caspase-3, caspase-9 and pro-apoptosis protein Bax increased, and the level of the anti-apoptosis protein Bcl-2 decreased. Additionally, the phosphorylation of Stathmin and acetylation of α-tubulin increased, which represented an increase in the number of microtubules produced.

Stathmin, also known as oncoprotein 18, is a microtubule destabilizing protein. It binds to tubulin and maintains the dynamic process of continuous microtubule synthesis and degradation (17). Microtubules, composed of α- and β-tubulin, constitute cytoskeleton, spindle, cilia and flagella, and participate in support, material transport, cell movement, mitosis and other functions. During the mitosis process, tubulin is rapidly depolymerized and re-polymerized into spindle fibers. Once Stathmin was phosphorylated and inactivated, cell proliferation was inhibited (18,19). In the present study, Siva 1 inhibited cell proliferation and promoted apoptosis by facilitating the phosphorylation of Stathmin and decreasing its activity.

Metastasis is another important contributor to the high mortality rates and difficulty in curing malignant tumors. Stathmin was highly expressed in a number of malignant tumors (20). It reduced the stability of microtubules and provided tumor cells with improved motility. Stathmin has been used as a therapeutic target and prognostic indicator in prostate and cervical cancer, oral squamous cell carcinoma and medulloblastoma (21-24). In the present study, it was revealed that Siva 1 promoted the phosphorylation of Stathmin, increased the level of acetylated α-tubulin, which was present only in polymerized tubulin (25), and therefore inhibited the migration and invasion of ovarian cancer cells.

Siva 1 serves different roles in various cells, and the mechanism of action also relies on the specific types. In the present study, Siva 1 was overexpressed in the ovarian cancer A2780 cell line, the changes in cell phenotype were detected, and the potential mechanism was studied. The results of the present study demonstrated that Siva 1 inhibited proliferation, promoted apoptosis, suppressed migration and invasion by facilitating phosphorylation of Stathmin and polymerization of tubulin in ovarian cancer cells. These data provide novel insights into the molecular mechanism of ovarian cancer pathogenesis, and provide a basis for additional studies investigating the clinical diagnosis and treatment methods of ovarian cancer.

References