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Id1 knockdown induces the apoptosis and inhibits the proliferation and invasion of ovarian cancer cells

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Abstract. – OBJECTIVE: To explore the role of Id1 in ovarian cancer cell proliferation, invasion and apoptosis.

MATERIALS AND METHODS: Lentivirus-based shRNA vectors were constructed to knockdown Id1 expression in SKOV3 ovarian cancer cells. The proliferation, invasion ability and apoptosis of SKOV3 cells were evaluated by CCK-8 assay, transwell assay and flow cytometry, respectively.

RESULTS: Compared to control cells, cell proliferation and invasion were significantly inhibited in SKOV3 cells depleted of Id1, while apoptosis was significantly increased in SKOV3 cells depleted of Id1.

CONCLUSIONS: Id1 functions to promote ovarian cancer cell proliferation and invasion, and Id1 is a promising therapeutic target for ovarian cancer.

Key Words:
Id1, Ovarian cancer, Invasion, shRNA.

Introduction

Inhibitor of DNA binding 1 (Id1) is a member of the helix-loop-helix (HLH) family of transcription factors that act as dominant negative transcriptional repressors of basic HLH (bHLH) factors[1-2]. Id1 has been implicated in the progression and metastasis of a variety of cancers, such as prostate cancer, breast cancer, gastric cancer, esophageal cancer and uterine cervical cancer[3-9].

Ovarian cancer is one of the most deadly gynecological cancers with very poor prognosis[10]. Despite the developments of surgery and chemotherapy, advanced ovarian cancer still has the highest mortality rate of all gynecologic cancers[11]. In a previous study, Id-1 was shown as an independent prognostic factor of ovarian cancer and the overexpression of Id-1 was associated with aggressive behaviors of ovarian cancer cells[12]. To further investigate the role of Id1 in ovarian cancer cells, in this study we employed RNA interference (RNAi) technology to knockdown Id1 in ovarian cancer cells and examined the effects on the apoptosis, proliferation, and invasion of ovarian tumor cells.

Materials and Methods

Cell line and Reagents

Human ovarian cancer SKOV3 cell line was purchased from the European Collection of Cell Cultures (ECACC) and cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA).

siRNA Design

siRNA was designed using the software of Ambion based on Id1 gene sequence (No. NM-002165.3). The following three sites were chosen: siRNA1 ACGTGCTGCTCTACGACAT (188-206); siRNA2 GGTGAGCAAGGTGGAGATT (264-282); siRNA3 CTCGGAATCCGAAGTTGGA (330-348). siRNA-negative control (siRNA-NC) with a random sequence of GAAGCCAGATCCAGCTTCC did not target any known mammalian gene.

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Construction of Recombinant Vector pSIH1-H1-GFP-shRNA

DNA templates of siRNAs (two separate oligonucleotides for each site) were synthesized and complementary strands were annealed. The double-stranded oligonucleotides were inserted into pSIH1-H1-GFP vector via BamHI and EcoRI sites. The recombinant vectors were verified by sequencing.

Real-time RT-PCR

The total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The specific primers for Id1 (forward: 5'-CGA GGT GGT GCG CTG TCT GT-3', reverse: 5'-TCC AAC TGA AGG TCC CTG ATG TAG-3', product 237 bp) and for beta-actin (forward: 5'-CCT GTA CGC CAA CAC AGT GC-3', reverse: 5'-TCC AAC TGA AGG TCC CTG ATG TAG-3', product 237 bp) were designed and synthesized by Invitrogen (Carlsbad, CA, USA). Real-time PCR was performed using SYBR premix Ex Taq kit (Takara, Dalian, China) according to the manufacturer's protocol. PCR reactions were as follows: denatured at 95°C for 10 s, followed by 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 20 s. At the end of the amplification, melting curve (disassociation curve) was run to ensure that only a single specific product was amplified. The modification of the 2-∅∅Ct method was used to calculate the relative expression of Id1 normalized to β-actin.

Western Blot Analysis

The cells were collected and lysed in ice-cold lysis buffer containing 50 mmol/L Tris-Cl (pH 7.5), 150 mmol/L NaCl, 0.2 mmol/L EDTA, 1 mmol/L PMSF and 1% (v/v) Nonidet-P40. The lysates were centrifuged at 13,000 rpm for 5 min at 4°C and the supernatants were collected. Protein content was measured by bicinchoninic acid (BCA) method. Equal amounts of 10 μg protein were separated by sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked with 10% (w/v) nonfat milk powder at room temperature for 1 h, and then incubated with antibodies against Id1 and the second antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), respectively. The immune reaction was visualized with chemiluminescent detection kit (Zhongshan Biotechnology, Beijing, China) and exposed to X-ray films.

The Package of and Infection of Lentiviral Vectors

The 293 cells of logarithmic growth phase were digested by trypsin and seeded in 10 cm dishes. When the cells grew to 80% confluence, they were cotransfected with Id1-shRNA vector and viral packaging system. Viral supernatant was harvested 48 h after transfection and stored frozen overnight. Next, SKOV3 cells were infected by lentiviral particles and divided into four groups: SKOV3 group, SKOV3+Lentivirus Control group (cells were infected with empty lentivirus), SKOV3+Lv-NC (cells were infected with lentivirus containing shRNA-negative control), SKOV3+Lv-Id1-RNAi (cells were infected with lentivirus containing Id1 shRNA3).

Cell Proliferation Assay

SKOV3 cells were inoculated in 96-well plates at 2×10^4 cells/well and cultured in the atmosphere with 5% CO_2 at 37°C. The cell viability was measured by cell counting kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). 10 μl CCK-8 solutions were added to each well at 0 h, 24 h, 48 h, or 72 h after culture. The cells were incubated at 37°C for 4 h, and the absorbance value at 450 nm was measured on a microplate reader (Bio-Rad, Hercules, CA, USA).

Cell Invasion Assay

The invasion of SKOV3 cells was measured using 24-Well cell invasion assay kit (Millipore, Billerica, MA, USA) according to the manufacturer's protocol. 1.0×10^6 cells in 250 μl of the serum-free medium were seeded into the upper chamber and 500 μl medium containing 10% FBS were added to the lower one. After incubation at 37°C for 48 h, the invaded cells were stained with CyQuant GR, and counted by using fluorospectrophotometer.

Apoptosis Assay

SKOV3 cells were collected and washed with PBS twice, and then the cells were incubated with 5 μL AnnexinV-FITC and 5 μL of propidium iodide (BD Bioscience, San Jose, CA, USA) in the dark for 5 min at room temperature. The samples were analyzed immediately by flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Statistical Analysis

Data were presented as mean ± standard deviation (SD) and analyzed by using the SPSS13.0 program (SPSS Inc., Chicago, IL, USA). Stu-

dent's *t*-test was used for the comparison among several groups. $p < 0.05$ was considered as a significant difference.

Results

Analysis of Id1 Expression Level in 293 cells Transfected with Id1 shRNA

To evaluate the efficiency of Id1 shRNA to knockdown Id1 expression, we first transected 293 cells with different Id1 shRNA constructs. RT-PCR analysis showed that compared with negative and blank control groups, mRNA expression of Id1 was significantly decreased in 293 cells transfected with pSIH1-H1-GFP-Id1-shRNAs ($p < 0.01$), especially in cells transfected with pSIH1-H1-GFP-Id1-shRNA3 (Figure 1A).

Next we performed Western blot analysis to confirm that Id1 shRNA could knockdown Id1 expression. The results showed that protein expression of Id1 was decreased in 293 cells transfected with pSIH1-H1-GFP-Id1-shRNAs, especially in cells transfected with pSIH1-H1-GFP-Id1-shRNA3, compared to control cells (Figure 1B). Taken together, these data demonstrate that the shRNAs we designed are efficient to knockdown Id1 expression. Because Id1 shRNA3 was the most efficient to knockdown Id3 expression, we chose it for the following experiments.

shRNA Mediated Knockdown of Id1 in SKOV3 cells

Next we infected SKOV3 ovarian cancer cells with Id1 shRNA3 lentivirus and control lentivirus. Western blot analysis showed that the level of Id1 protein in the skov3+Lv-Id1-shRNA3 group was decreased compared to negative and blank control groups (Figure 2).

Id1 Knockdown Inhibits the Proliferation of SKOV3 Cells

To characterize the role of Id1 in ovarian cancer growth, we employed loss of function approach by the depletion of Id1 in SKOV3

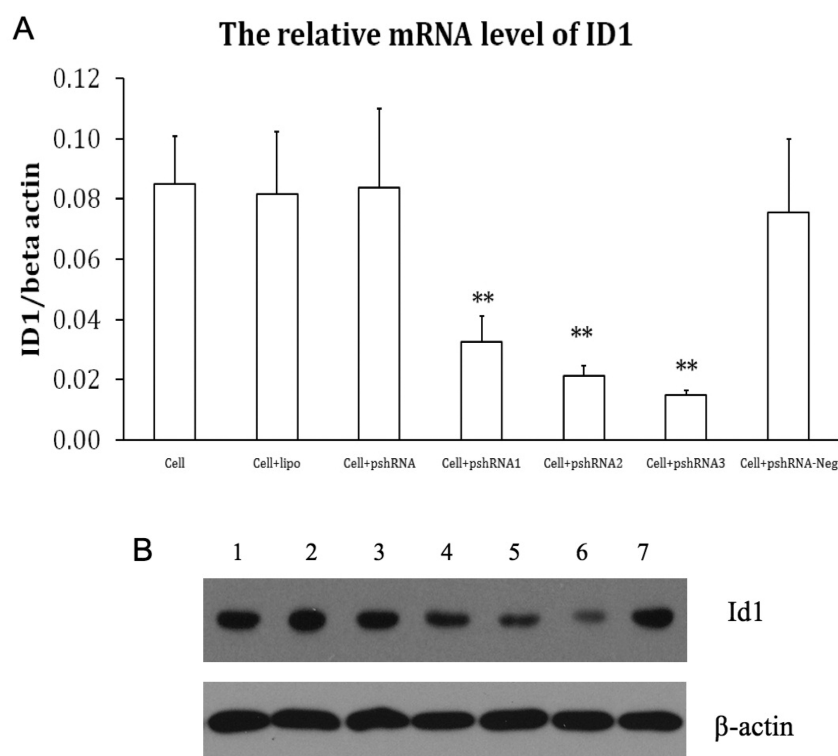

Figure 1. Id1 shRNA mediated silencing of Id1 expression in 293 cells. ***A,*** Real-time PCR analysis of Id1 mRNA levels in the cells. $^{**}p$ <0.01 vs. cells transfected with shRNA-NC. ***B,*** Western blot analysis of Id1 protein levels in the cells. 1: Untransfected cells; 2: Cells treated with Lipofectamine; 3. Cells transfected with pSIH1-H1-GFP; 4. Cells transfected with Id1 shRNA1; 5. Cells transfected with Id1 shRNA2; 6. Cells transfected with Id1 shRNA3; 7. Cells transfected with shRNA-NC. Shown were representative blots from three independent experiments. β-actin was loading control.

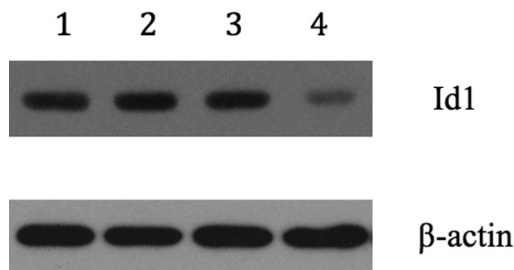

Figure 2. Id1 shRNA mediated silencing of Id1 expression in SKOV3 cells. Western blot analysis of Id1 protein levels in the cells. 1: Untransfected SKOV3 cells; 2: Cells infected with empty lentivirus; 3. Cells infected with shRNA NC lentivirus; 4. Cells infected with Id1 shRNA3 lentivirus. Shown were representative blots from three independent experiments. β-actin was loading control.

cells. CCK8 assay showed that compared to uninfected cells, the proliferation of SKOV3 cells was inhibited after the infection of Id1-shRNA3 lentivirus, but not after the infection of empty lentivirus or lentivirus containing shRNA NC (Figure 3). These results suggest that Id1 functions to promote the proliferation of ovarian cancer cells.

Id1 Knockdown Promotes the Apoptosis of SKOV3 Cells

To elucidate other mechanisms by which Id1 affects the growth of SKOV3 cells, we detected the apoptosis of the cells. Flow cytometry analysis showed that the number of apoptotic SKOV3 cells was increased after the infection of Id1-shRNA3 lentivirus, compared to cells infected with empty lentivirus or lentivirus containing shRNA NC (Figure 4). These results suggest that Id1 functions to inhibit the apoptosis of ovarian cancer cells.

Id1 Knockdown Disrupts the Invasion of SKOV3 Cells

To characterize the role of Id1 in ovarian cancer metastasis, we employed loss of function approach by the depletion of Id1 in SKOV3 cells. The *in vitro* cell invasion assay showed that the number of invaded SKOV3 cells was decreased after the infection of Id1-shRNA3 lentivirus, but not after the infection of empty lentivirus or lentivirus containing shRNA NC (Figure 5). These results suggest that Id1 functions to promote the invasion of ovarian cancer cells.

Discussion

In this study, we reported that shRNA mediated knockdown of Id1 in ovarian cancer cell line SKOV3 led to decreased cell proliferation and invasion and increased apoptosis. These results are consistent with the current view that Id1 plays an important role in tumor cell differentiation, proliferation, apoptosis and metastasis[3-9,12].

Several studies in other tumor types have shown that Id1 functions as an oncogene to promote cancer growth, metastasis and angiogenesis, and the resistance to chemotherapy-induced apoptosis and cell senescence[13-17]. In ad-

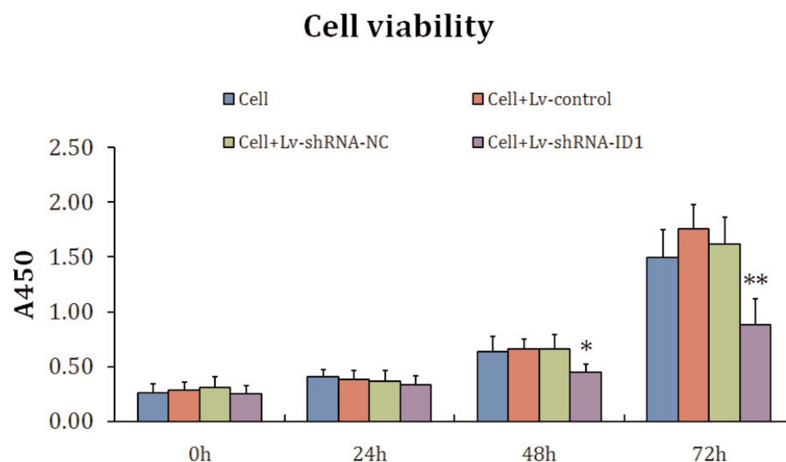

Figure 3. Id1 shRNA inhibits the proliferation of SKOV3 cells. The proliferation of SKOV3 cells was determined by CCK-8 assay at the indicated time points. Data were presented as mean ± SD (n = 6). $^{*}p < 0.05$, $^{**}p < 0.01$ compared to cells infected with shRNA NC lentivirus.

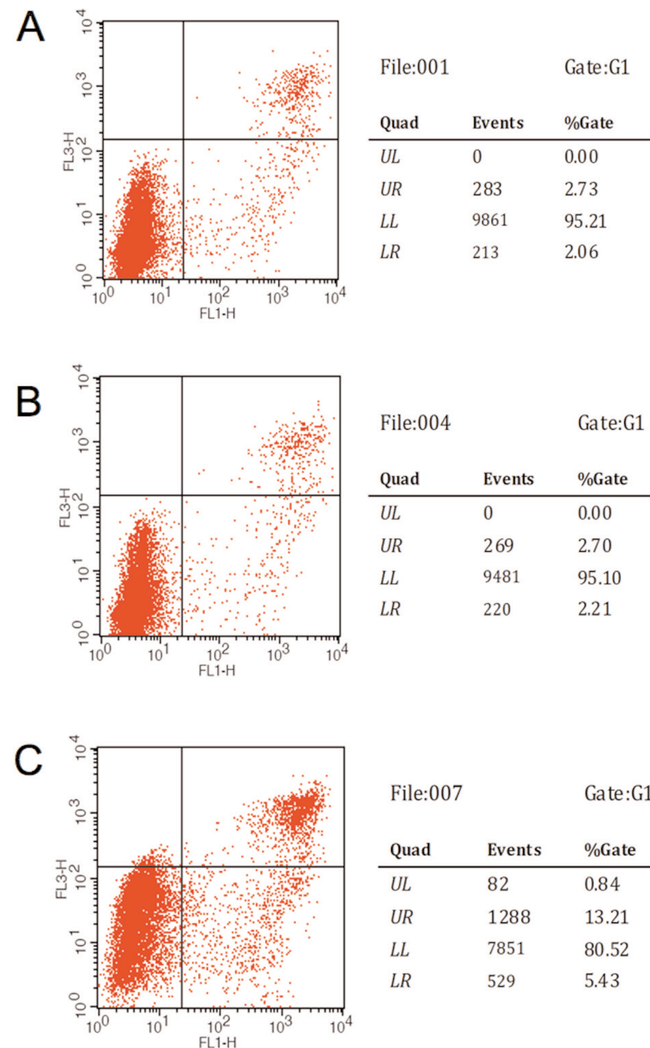

Figure 4. Id1 shRNA promotes the apoptosis of SKOV3 cells. The number of apoptotic SKOV3 cells was determined by flow cytometry. ***A,*** Shown were cells infected with empty lentivirus. ***B,*** Shown were cells infected with shRNA NC lentivirus. C. Shown were cells infected with Id1 shRNA3 lentivirus.

dition, the overexpression of Id-1 was associated with aggressive behaviors of ovarian cancer cells[12]. However, the underlying mechanisms are not clear.

To elucidate the oncogenic role of Id1 in ovarian cancer, in this study we employed a loss-of-function approach to knockdown Id1 expression in ovarian cancer cell line SKOV3. We designed shRNAs against Id1 and constructed recombinant plasmids harboring Id1 shRNAs. After the transfection of the recombinant plasmid into 293 cells, we detected Id1 expression in these cells at both mRNA and proteins levels. RT-PCR and Western blot analysis showed that the shRNAs against Id1 could efficiently knockdown Id1 expression in 293 cells.

Next we constructed lentivirus harboring Id1-shRNA and lentivirus harboring scramble sequence (shRNA-NC) as negative control because lentivirus has a very high efficiency to deliver foreign genes or siRNAs into the cells. When we infected SKOV3 cells with recombinant lentivirus, we found that shRNA mediated knockdown of Id1 could inhibit the proliferation and induce the apoptosis of SKOV3 cells. These may explain how Id1 promotes ovarian cancer growth. Furthermore, we characterzied the invasion ability of SKOV3 cells depleted of Id1, and observed that the cell invasion ability was significantly inhibited, compared to control SKOV3

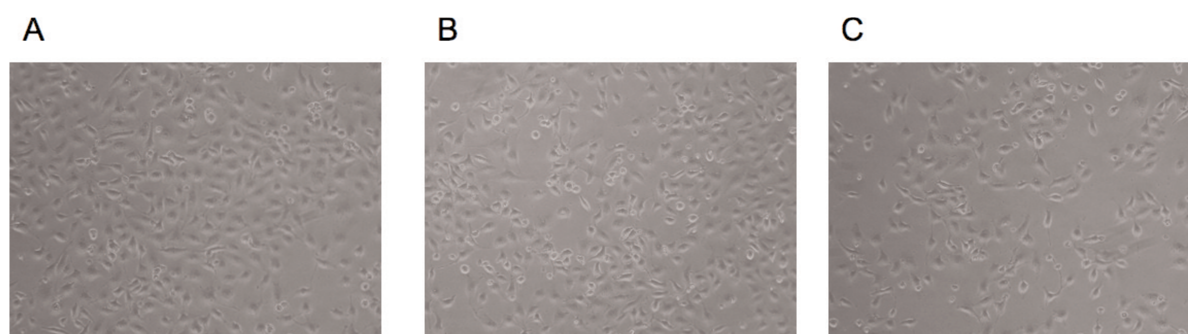

Figure 5. Id1 shRNA inhibits the invasion of SKOV3 cells. The invasion of SKOV3 cells was determined by in vitro cell invasion assay at 48 h after incubation. ***A,*** Shown were cells infected with empty lentivirus. ***B,*** Shown were cells infected with shRNA NC lentivirus. ***C,*** Shown were cells infected with Id1 shRNA3 lentivirus.

cells. Taken together, these data strongly suggest that Id1 promotes the proliferation and invasion of ovarian cancer cells.

The limitations of this study should be pointed out. First, we only used one ovarian cancer cell line. Second, we characterized oncogenic role of Id1 in *in vitro* settings. Further *in vivo* studies are needed to confirm oncogenic role of Id1 in ovarian cancer. Third, recent data suggest that the resistance to chemotherapy-induced apoptosis is crucial to the malignant behaviors of ovarian cancer cells[18,19]. It will be important to investigate the role of Id1 in the resistance to chemotherapy-induced apoptosis in ovarian cancer cells.

Conclusions

In this study we made important findings that knockdown of Id1 inhibited the proliferation and invasion of SKOV3 cells. These results suggest that Id1 overexpression contributes to the progression and metastasis of ovarian cancer and reveal Id1 as a novel therapeutic target for malignant ovarian cancer.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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References

1) Perk J, Iavarone A, Benezra R. Id family of helix-loop-helix proteins in cancer. Nat Rev Cancer 2005; 5: 603-614.
2) Lasorella A, Uo T, Iavarone A. Id proteins at the cross-road of development and cancer. Oncogene 2001; 20: 8326-8333.
3) Fong S, Debs RJ, Desprez PY. Id genes and proteins as promising targets in cancer therapy. Trends Mol Med 2004; 10: 387-392.
4) Sikder HA, Devlin MK, Dunlap S, Ryu B, Alani RM. Id proteins in cell growth and tumorigenesis. Cancer Cell 2003; 3: 525-530.
5) Yuen HF, Chua CW, Chan YP, Wong YC, Wang X, Chan KW. Id proteins expression in prostate cancer: high-level expression of Id-4 in primary prostate cancer is associated with development of metastases. Mod Pathol 2006; 19: 931-941.
6) Jang KS, Han HX, Paik SS, Brown PH, Kong G. Id-1 overexpression in invasive ductal carcinoma cells is significantly associated with intratumoral microvessel density in ER-negative/node positive breast cancer. Cancer Lett 2006; 244: 203-210.
7) Jang TJ, Jung KH, Choi EA. Id-1 gene downregulation by sulindac sulfide and its upregulation during tumor development in gastric cancer. Int J Cancer 2006; 118: 1356-1363.
8) Yuen HF, Chan YP, Chan KK, Chu YY, Wong ML, Law SY, Srivastava G, Wong YC, Wang X, Chan KW. Id-1 and Id-2 are markers for metastasis and prognosis in oesophageal squamous cell carcinoma. Br J Cancer 2007; 97: 1409-1415.
9) Maw MK, Fujimoto J, Tamaya T. Expression of the inhibitor of DNA-binding (ID)-1 protein as an angiogenic mediator in tumour advancement of uterine cervical cancers. Br J Cancer 2008; 99: 1557-1563.
10) Chiang YC, Chen CA, Chiang CJ, Hsu TH, Lin MC, You SL, Cheng WF, Lai MS. Trends in incidence

and survival outcome of epithelial ovarian cancer: 30-year national population-based registry in Taiwan. J Gynecol Oncol 2013; 24: 342-351.

11) Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. CA Cancer J Clin 2008; 58: 71-96.

12) Schindl M, Schoppmann SF, Strobel T, Heinzl H, Leisser C, Horvat R, Birner P. Level of Id-1 protein expression correlates with poor differentiation, enhanced malignant potential, and more aggressive clinical behavior of epithelial ovarian tumors. Clin Cancer Res 2003; 9: 779-785.

13) Yu X, Xu X, Han B, Zhou R. Inhibitor of DNA binding-1 overexpression in prostate cancer: relevance to tumor differentiation. Pathol Oncol Res 2009; 15: 91-96.

14) Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes RO, Zhuang Y, Manova K, Benezra R. Id1 and Id3 are required for neurogenesis, angiogenesis and vasculariza-tion of tumour xenografts. Nature 1999; 401: 670-677.

15) Gupta GP, Perk J, Acharyya S, de Candia P, Mittal V, Todorova- Manova K, Gerald WL, Brogi E, Benezra R, Massagué J. ID genes mediate tumor reinitiation during breast cancer lung metastasis. Proc Natl Acad Sci U S A 2007; 104: 19506-19511.

16) Di K, Ling MT, Tsao SW, Wong YC, Wang X. Id-1 modulates senescence and TGF-betal sensitivity in prostate epithelial cells. Biol Cell 2006; 98: 523-533.

17) Cummings SD, Ryu B, Samuels MA, Yu X, Meeker AK, Healey MA, Alani RM. Id1 delays senescence of primary human melanocytes. Mol Carcinog 2008; 47: 653-659.

18) Zhang J, Yin XJ, Xu CJ, Ning YX, Chen M, Zhang H, Chen SF, Yao LQ. The histone deacetylase SIRT6 inhibits ovarian cancer cell proliferation via down-regulation of Notch 3 expression. Eur Rev Med Pharmacol Sci 2015; 19: 818-824.

19) Zhao JX, Liu H, Lv J, Yang XJ. Wortmannin enhances cisplatin-induced apoptosis in human ovarian cancer cells in vitro. Eur Rev Med Pharmacol Sci 2014; 18: 2428-2434.