

Gli3 silencing enhances cyclopamine suppressive effects on ovarian cancer

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Abstract: Ovarian cancer is a leading gynecological malignancy associated with high mortality. Hedgehog signaling has been found to be important for cell proliferation and tumor growth for multiple cancers, including ovarian cancer. The present study showed that the drug cyclopamine, which blocks the hedgehog signaling pathway, could reduce cancer cell growth and proliferation and induce cell apoptosis. In addition, the silencing of the glioma-associated oncogene (Gli)3, a downstream component of the hedgehog signaling pathway, could further enhance the antitumor effects of cyclopamine. Our results suggest that Gli3 may act as resistance to cyclopamine's effect on tumor growth. The combined treatment of cyclopamine application and Gli3 silencing therapy, therefore, may provide novel directions for clinical management of ovarian cancer.

Keywords: ovarian cancer, hedgehog, cyclopamine, proliferation, apoptosis, Gli3

Introduction

Ovarian cancer is a leading gynecological malignancy associated with high mortality. The management of ovarian cancer with pharmacological compounds is however still limited. Hedgehog signaling represents an important target in controlling cancer cell proliferation and progression, including that of ovarian cancer cells.¹⁻⁵ For instance, the cyclopamine that blocks hedgehog signaling by antagonizing smoothened (smo) function has been employed to induce cancer cell apoptosis in different types of cancer cell lines, including those of prostate cancer, pancreatic cancer, ovarian cancer cells, and breast cancer.⁶⁻¹¹

Downstream to smo protein, recent studies revealed the role of the glioma-associated oncogene (Gli) in the hedgehog signaling pathway.¹²⁻¹⁴ The Gli family includes several homologs such as Gli3, which has been proposed to be important in cancer development and tumor maintenance.¹⁵⁻¹⁷ Gli protein signaling has been recognized in the development of ovarian cancer;¹⁸ however, the exact role of Gli3 protein in hedgehog signaling in ovarian cancer proliferation and maintenance has not been fully investigated.

The present study examined the involvement of Gli3 signaling in the suppressive effects of cyclopamine on human ovarian cancer cell lines. The results suggest novel directions in targeting ovarian cancer at various molecular levels.

Materials and methods

Ethics statement

The study was approved by the Liaoning Medical College Animal Research Committee, and all experimental procedures were carried out in accordance with the guidelines of medical research in Liaoning Medical College.

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Cell culture

The human ovarian SKOV3 cancer cell line was obtained from the Liaoning Medical College Cell Research Center and maintained in 10% fetal bovine serum, antibiotics, and RPMI 1640 culture medium. Cyclopamine at 20 μ M was used to induce cell proliferation arrest and apoptosis as previously described.⁶

Cell proliferation analysis

The Premix WST-1 Cell Proliferation Assay (Takara Bio, Shiga, Japan) was employed for cell proliferation evaluation according to the manufacturer's instructions in order to determine the cell proliferation rates. The assays were repeated three times.

Cell viability assay and apoptosis analysis

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was employed to examine cell viability, using a Millipore MTT kit (Billerica, MA, USA). The MTT assay was repeated five times.

To detect cell apoptosis in culture, the TUNEL kit (Roche, Basel, Switzerland) was used, and TUNEL-positive cells were counted using Hoechst counterstain for all the rest of the cells. The ratios of TUNEL positive to normal cells were calculated.

Small interfering RNA for Gli3

Gli3-specific small interfering RNAs (siRNAs) were obtained as previously described¹⁶ (siRNA-G1, 5'-UGA AUG GAA UGU UUC CGC GAC UGA A-3'; siRNA-G2, 5'-CCA UUG CAU AUG ACU UCC GCC UUA U-3'; 60 nM) (Shanghai Shengji Biotechnol, Shanghai, People's Republic of China). Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA, USA) was used for cell culture transfection. The transfection was performed 48 hours prior to the experiment.

Xenograft

SKOV3 cancer cells (2×10^6) in 200 μ L saline were injected into the intraperitoneal cavity of adult male nude mice 2 weeks prior to treatment to promote tumor growth. Mice were divided into four groups with 12 animals per group: control group received saline injection every 3 days, cyclopamine treatment group (25 mg/kg every 3 days), cyclopamine plus G1 siRNA group, and cyclopamine plus G2 siRNA group, also every 3 days. Cyclopamine was injected intraperitoneally while G1 and G2 were administered via the tail vein.

Mice were sacrificed at 4 weeks (6 animals in each group) and 7 weeks (6 animals in each group) after grafting of cancer cells, and tumor sizes were measured. Sections of the tumors were prepared for TUNEL staining, and the percentage of apoptotic cells was counted using a Zeiss microscope (Oberkochen, Germany).

Statistics

The data were represented as mean \pm standard deviation and analyzed with SPSS 17.0 software (IBM Corporation, Armonk, NY, USA). The results between different groups were analyzed by Student's *t*-test and ANOVA. $P < 0.05$ was considered significantly different.

Results

Cyclopamine suppresses cancer cell proliferation

We found that the cyclopamine treatment significantly decreased cell proliferation after time lengths of 12, 24, and 48 hours (Figure 1).

Cyclopamine decreases cell viability and induces cell apoptosis

We further examined if cyclopamine decreased cell proliferation by the induction of cell apoptosis. Indeed, we found that accompanying the decrease in cell viability, TUNEL-positive cells (apoptotic) increased in a time-dependent manner in cell culture (Figure 2). Given the clear effects of cyclopamine at 24 hours after treatment, we selected 24 hours as the time point for mechanistic investigations in the following studies.

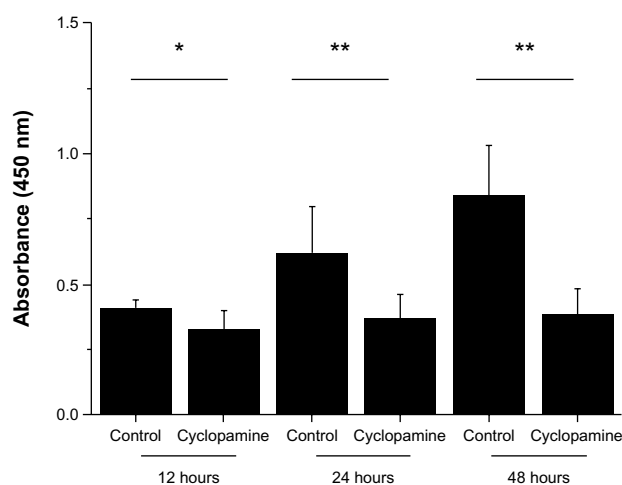


Figure 1 Cyclopamine suppresses cancer cell proliferation at 12-, 24-, and 48-hour time points.

Notes: Light absorbance at 450 nm; *represents $P < 0.05$; **represents $P < 0.01$ compared to the control group.

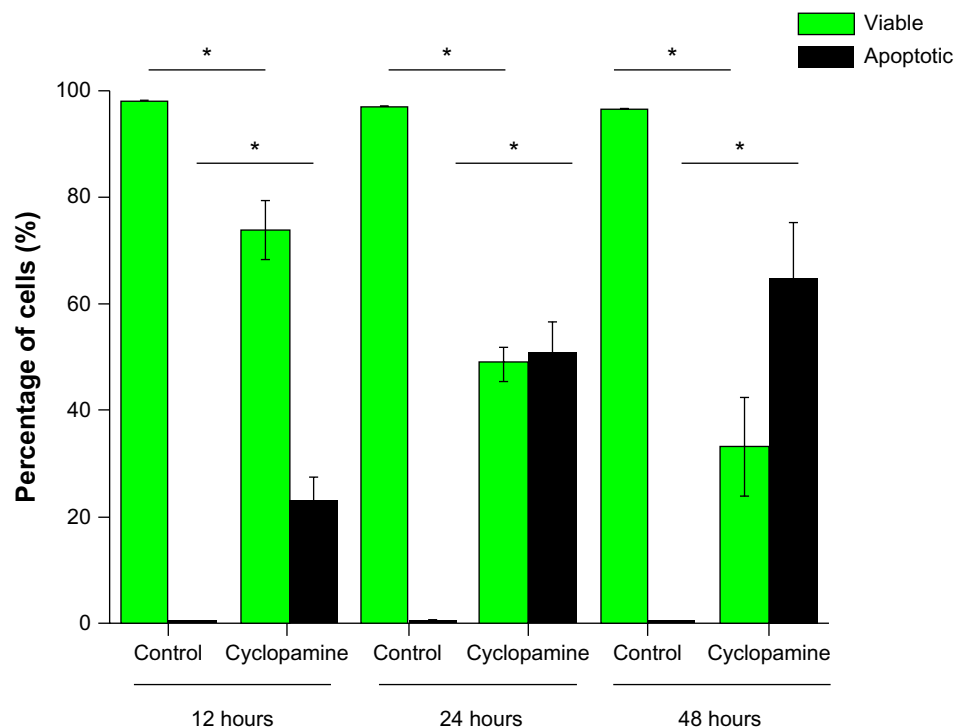


Figure 2 Cyclopamine decreases cell viability and induces cell apoptosis at 12-, 24-, and 48-hour time points.

Note: *Represents $P < 0.01$.

Gli3 silencing further enhances cyclopamine effects

We employed two siRNAs for Gli3 (siRNA-G1 and siRNA-G2) to silence the expression of Gli3. We confirmed that the two siRNAs did reduce the expression levels of Gli3 to less than 15% in our preliminary experiments. Interestingly, this silencing of Gli3 further enhanced the cyclopamine effects to suppress the cancer cell proliferation at the 24-hour time point (Figure 3). Gli3 silencing did not affect the baseline cell proliferation rate however (Figure 3).

We further examined the usefulness of Gli3 silencing on cyclopamine effects on cell viability and apoptosis at 24 hours. Gli3 silencing did not affect the baseline cell viability or apoptosis rate, yet both G1 and G2 siRNA treatment enhanced the cyclopamine-induced cell apoptosis of cultured cells (Figure 4).

Gli3 silencing in vivo enhances the antitumor growth effect of cyclopamine

In order to further validate the effects we observed on cultured cancer cells, we performed xenograft experiments and systematically infused siRNA-G1 and siRNA-G2 via the tail vein. We found that cyclopamine treatment could effectively induce tumor size decrease (Table 1) as well as cell apoptosis

in the tumor tissue (Figure 5). Both effects were enhanced with G1 and G2 treatment (Figure 5).

Discussion

Ovarian cancer is a challenging malignancy for women's health. Recent studies highlighted the role of hedgehog sig-

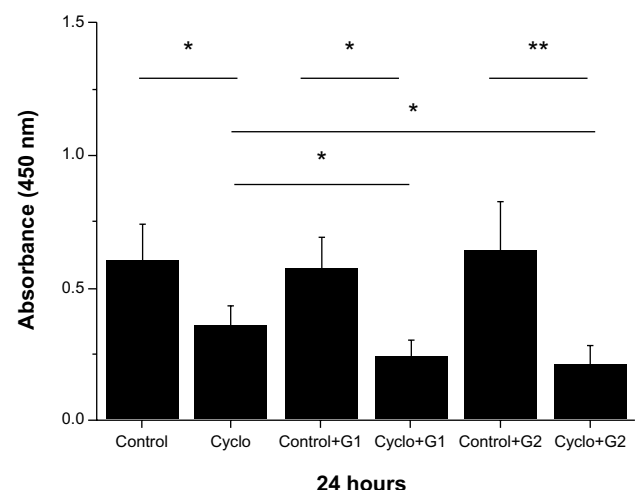


Figure 3 Gli3 silencing with two independent siRNAs further suppressed cancer cell proliferation.

Notes: Light absorbance at 450 nm; *represents $P < 0.05$; **represents $P < 0.01$ compared to the relevant group.

Abbreviations: Cyclo, cyclopamine; G1, siRNA-G1 (5'-UGA AUG GAA UGU UUC CGC GAC UGA A-3'); G2, siRNA-G2 (5'-CCA UUG CAU AUG ACU UCC GCC UUA U-3'); siRNA, small interfering RNA.

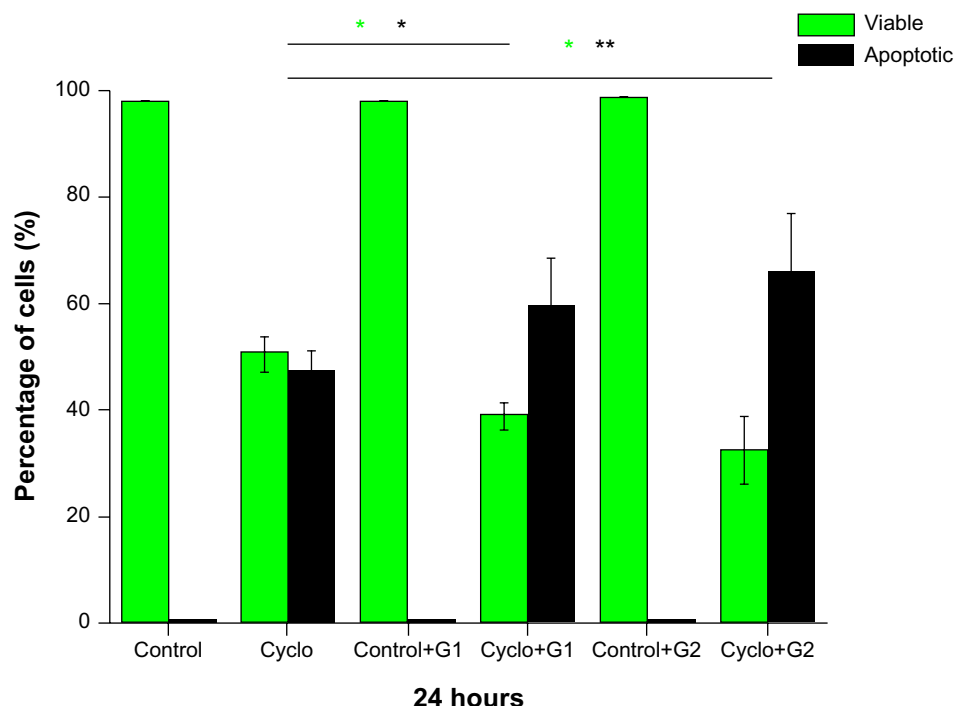


Figure 4 Gli3 silencing with two independent siRNAs further decreased cell viability and induced cell apoptosis in addition to the cyclopamine effects at 12-, 24-, and 48-hour time points.

Notes: *Represents $P < 0.05$; **represents $P < 0.01$ compared to the relevant group.

Abbreviations: Cyclo, cyclopamine; G1, siRNA-G1 (5'-UGA AUG GAA UGU UUC CGC GAC UGA A-3'); G2, siRNA-G2 (5'-CCA UUG CAU AUG ACU UCC GCC UUA U-3'); siRNA, small interfering RNA.

naling in ovarian cancer development and progression.^{18,19,21} Cyclopamine has been applied for different diseases, with proven roles in antitumor growth as well as an inducer of cell apoptosis.^{1,2,20,21} The present study demonstrated that Gli3 may act to exert “resistance” downstream to the hedgehog signaling pathway, thereby partly antagonizing the effects of cyclopamine. The synergistic effect of cyclopamine application and Gli3 knockdown, therefore, represents a novel strategy for clinical management of ovarian cancer.

A previous study revealed that the differences in sensitivity to cyclopamine across different pancreatic cancer cell lines were mediated by Gli3 function.¹⁶ This is in line with the current results. It will be interesting to perform gain of

function studies on Gli3 to see if this could promote tumor cell growth at the baseline level. Interestingly, in our study, Gli3 silencing did not cause much change in cell proliferation, cell viability, and apoptosis compared to the baseline level. It is possible that at baseline level, Gli3 is not actively involved in tumor growth; however, Gli3 is recruited when the

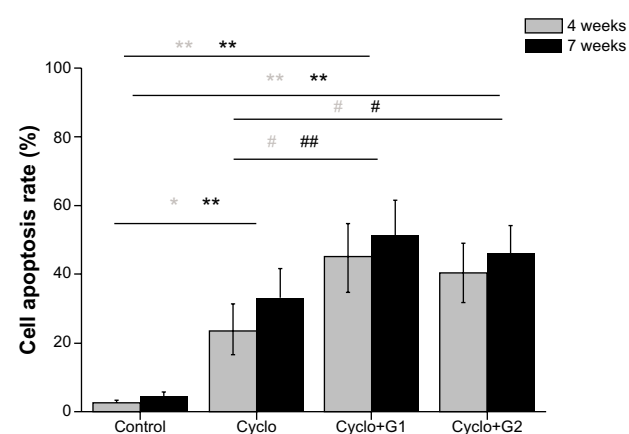


Figure 5 Gli3 silencing in vivo increased the antitumor growth effect of cyclopamine through upregulation of cell apoptosis at both the 4-week and 7-week time points.

Notes: *Represents $P < 0.05$; **represents $P < 0.01$ compared to the control group; #represents $P < 0.05$; ##represents $P < 0.01$ compared to the cyclopamine group.

Abbreviations: Cyclo, cyclopamine; G1, siRNA-G1 (5'-UGA AUG GAA UGU UUC CGC GAC UGA A-3'); G2, siRNA-G2 (5'-CCA UUG CAU AUG ACU UCC GCC UUA U-3'); siRNA, small interfering RNA.

Table 1 Gli3 silencing in vivo enhances the antitumor growth effect of cyclopamine

Tumor size	Control	Cyclopamine	Cyclo + G1	Cyclo + G2
4 weeks	645±114	504±106*	320±76**##	339±82***##
7 weeks	862±190	622±127**	394±93***##	427±115**.

Notes: Tumor size in mm³; *represents $P < 0.05$; **represents $P < 0.01$ compared to the control group; #represents $P < 0.05$; ##represents $P < 0.01$ compared to the cyclopamine group.

Abbreviations: Cyclo, cyclopamine; G1, siRNA-G1 (5'-UGA AUG GAA UGU UUC CGC GAC UGA A-3'); G2, siRNA-G2 (5'-CCA UUG CAU AUG ACU UCC GCC UUA U-3'); siRNA, small interfering RNA.

tumor cells were challenged with different chemotherapeutic drugs for instance.

The synergistic effects of cyclopamine and Gli3 silencing suggest that combined therapy would be more effective in targeting the hedgehog signaling pathway to manage ovarian cancer growth. There are numerous inhibitors for hedgehog signaling pathway being developed and tested in preclinical phases. Cyclopamine mainly acts on smo function.^{2,7} It will be important to find certain agents that target both Gli3 and smo function with a potentially high efficiency.

Other Gli3 proteins such as Gli1 have been found to be associated with ovarian cancer growth, invasion, and induced differentiation.^{12,22} In addition, cyclopamine or Gli1 silencing both suppressed ovarian cancer cell growth significantly.²³ It will be important to investigate the potential functional redundancy between Gli1 and Gli3 in order to explore their interactions when ovarian cells are treated with cyclopamine.

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Disclosure

The authors report no conflicts of interest in this work.

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