

Characterization of sonic hedgehog inhibition in gastric carcinoma cells

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Abstract. Aberrant activation of the sonic hedgehog (Shh) signaling pathway plays an important role in gastric cancer. The exact mechanisms defining how the Shh pathway promotes tumorigenesis or regulates its downstream targets remains elusive. In the present study, the effects of inhibiting the Shh signaling pathway in gastric cancer AGS cells was examined. It was identified that the Shh antagonist, cyclopamine, inhibited cancer proliferation, migration and invasion in a dose- and time-dependent manner. Additionally, it was revealed that several key targets that are activated by the Shh signaling pathway, Gli1 and CXCR4, were downregulated at an RNA and protein level by cyclopamine. The results from the present study may be of benefit in facilitating the development of novel therapeutic strategies to treat gastric cancer in human patients.

Introduction

The hedgehog signaling pathway is critical for its role in normal cell differentiation and embryonic development, as well as in the pathological processes that drive cancer formation (1-3). The ligands of sonic hedgehog (Shh) bind to the transmembrane receptor, Patched (*ptch*) 1 and 2, to relieve the suppression of the transmembrane protein, Smoothed (*Smo*). This subsequently triggers the nuclear translocation of various transcription factors to activate downstream target genes (2,4). In various types of cancer, including ovarian (5), lung (6,7), breast (8), prostate (9), endometrial (10), skin (11) and gastrointestinal (12-14), aberrant activation of *Smo* genes and loss of function mutations in the *ptch* gene relieve the suppression of the *Smo* protein and trigger full-length Gli1 translocation into the nucleus, prompting excessive activation of downstream genes, including c-myc and vascular endothelial growth factor

(VEGF). It has also been demonstrated that inhibition of the Shh pathway by a *Smo* inhibitor, such as cyclopamine, slows or prevents the growth of tumor tissues (15-17).

In the case of gastric cancer cells, excessive Shh signaling activities are well known to affect cancer cell proliferation, migration and invasion, and overexpression of Shh was identified in intestinal metaplasia and stomach adenomas (18). In *in vitro* studies, the Shh pathway and downstream genes/proteins are highly involved in the proliferation and migration of various gastric cancer cell lines, including MKN1/7/45/74, MKN45 and AGS cells (19,20). However, the exact mechanisms defining how the Shh pathway regulates gastric tumorigenesis remains elusive.

In the present study, via the application of cyclopamine, the Shh signaling pathway was inhibited in the human gastric cancer cell line, AGS, and the effect on cell proliferation, migration and invasion was evaluated. Furthermore, it was demonstrated that the molecular and cellular expression of key Shh signaling pathway-associated factors, Gli1 and CXCR4, were markedly downregulated by cyclopamine in AGS cells.

Materials and methods

Cell culture and treatment. Human gastric cancer cell line AGS was obtained from American Type Culture Collection (ATCC CRL-1739) and were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA, USA) and 100 U/ml penicillin/streptomycin. The cells were cultured either with cyclopamine (5-100 μ M; Calbiochem, La Jolla, CA, USA) or without cyclopamine for 24, 48 or 72 h.

Cell proliferation assay. Cells were plated at a concentration of 2.5×10^4 cells/ml of culture medium in 96-well plates for 24 and 72 h. Following the defined culture periods, an MTT assay (Sigma, St. Louis, MO, USA) was applied according to the manufacturer's instructions to calculate the volume of viable cells (21).

Apoptosis assay. Following *in vitro* culture for 24 h, the gastric cancer cells, a total amount of 1×10^6 , were collected in a binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM CaCl_2) after washing with phosphate-buffered saline (PBS; 3×10 min). Fluorescence-activated cell sorting analysis for apoptosis was

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Table I. Cyclopamine induces apoptosis in gastric cancer cells.

Parameter	Control	50 μ M	100 μ M
Rate of apoptosis, 24 h	1.52 \pm 0.51	15.25 \pm 2.11 ^a	22.55 \pm 1.94 ^a
Rate of apoptosis, 48 h	3.15 \pm 0.63	24.32 \pm 2.37 ^a	30.12 \pm 2.33 ^a

^aP<0.05, as compared with the control.

conducted using an Annexin V-FITC/7-AAD kit according to the manufacturer's instructions (Beckman Coulter, Miami, FL, USA). The mixture was incubated for 10 min in a dark room at room temperature and the stained cells were immediately analyzed using a flow cytometer (Cell Lab Quanta SC; Beckman Coulter) to determine the percentage of apoptotic cells.

Invasion assay. Cancer cell migration/invasion was performed by a quantitative cell migration assay (ECM500; Chemicon, Temecula, CA, USA) according to the manufacturer's instructions. Warm Knockout DMEM (Sigma) in the amount of 200 μ l was applied to the extracellular matrix (ECM) layer to hydrate for 2 h at room temperature. AGS cells were then dislodged by trypsinization (0.25% trypsin; Sigma) and dispersed into a homogeneous single-cell suspension at the concentration of 5×10^5 cells/ml, followed by washing and resuspension in Knockout DMEM. Then, cell suspension of 200 μ l was allowed to adhere to the surface at 37°C for 60 min. The migration mediums containing cyclopamine were then put into the bottom chamber. Following 24 h of incubation at 37°C, 5% CO₂ in air, the cells in the upper chamber were stained for 20 min, and dissolved in 10% acetic acid and the optical density (OD) was read at 560 nm on a standard reader.

Quantitative polymerase chain reaction (qPCR). A TRIzol reagent (Roche) was used to isolate total RNA from 5×10^6 cells according to the manufacturer's instructions. First-strand cDNA synthesis and amplification was conducted using an MBI Revert Aid First Strand cDNA Synthesis kit (MBI Fermentas, Amherst NY, USA). The qPCR was performed using an iQ5 Multicolor Real-Time PCR Detection system (Bio-Rad, Hercules, CA, USA). The cycle threshold values were read from the ABI 7000 software. The primers were: Forward, 5'-TCCTTTGGGGTCCAGCCTTG-3' and reverse, 5'-ATGCCTGTGGAGTTGGGGCT-3' for Gli1; forward, 5'-TCAGTCTGGACCGCTACCTG-3' and reverse, 5'-CCACCCACAAGTCATTGGGG-3' for CXCR4; and forward, 5'-AGGTCGGAGTCAACGGATTG-3' and reverse, 5'-GTGATGGCATGGACTGTGGT-3' for GAPDH.

Western blot analysis. RIPA buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate and 1% Na-deoxycholate; pH 7.4) supplemented with protease inhibitor was used to collect the cell suspension for the western blot analysis and a Bio-Rad protein assay (Bio-Rad) was used to calculate the total protein concentrations. Briefly, the protein lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and

transferred onto nitrocellulose membranes (Hybond™-P; Amersham Biosciences, Piscataway, NJ, USA). The membrane was blocked using 0.2% Tween-20 and 5% non-fat dry milk in PBS. The lysates were incubated with a primary antibodies: GLI-1 rabbit polyclonal anti human IgG (H-300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and CXCR-4 rabbit polyclonal IgG anti-human (H-118, Santa Cruz Biotechnology, Inc.) and a horse-radish peroxidase-labeled rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) and detected using X-ray film.

Statistical analysis. Data were calculated in triplicate and expressed as the mean \pm standard error of the mean. Comparisons were made using either student's t-test or one-way analysis of variance post hoc tests. P<0.05 was considered to indicate a statistically significant result.

Results

Inhibition of gastric cancer cell proliferation by cyclopamine. AGS cells were cultured with or without cyclopamine for 24, 48 and 72 h, and the effect of cyclopamine on cell proliferation was measured (Fig. 1). The results demonstrated that when AGS cells were treated with 5 or 10 μ M of cyclopamine for 24, 48 or 72 h, the proliferation densities were unaffected, as compared with the control conditions (P>0.05). This indicated that the application of cyclopamine at lower concentrations did not alter the cell proliferation rate. However, while AGS cells that were treated with 50 or 100 μ M cyclopamine for 48 or 72 h, respectively, cell proliferation was significantly inhibited, indicating that a higher concentration of cyclopamine inhibited the growth of AGS cells in a dose-dependent manner (P<0.05).

Induction of apoptosis in gastric cancer cells by cyclopamine. Secondly, the effects of cyclopamine on the AGS cells were examined. The cells were either untreated (control) or treated with cyclopamine (50 or 100 μ M) for 24 or 48 h, followed by annexin V staining. The results demonstrated that high concentrations of cyclopamine (50 or 100 μ M) induced significant apoptosis in AGS cells (Table I).

Inhibition of cell invasion in gastric cancer cells by cyclopamine. A characteristic feature of gastric cancer cells is their aggressive ability to filtrate and invade a reconstituted basement membrane. The effect of cyclopamine on the cellular invasion of human gastric cancer cells was assessed in the present study. The cancer cells were either untreated (control)

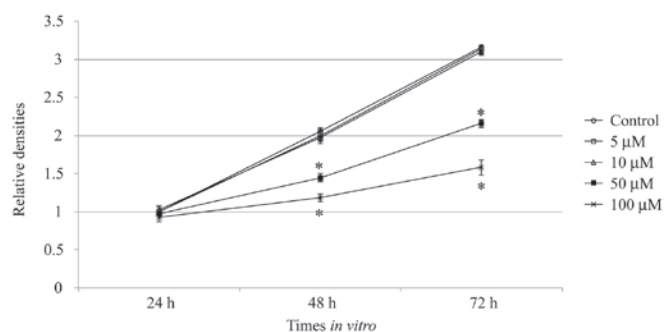


Figure 1. Cyclopamine inhibits AGS cell proliferation. AGS cells were treated without cyclopamine (control) or with varying concentrations of cyclopamine (5, 10, 50 and 100 μM) for 24, 48 and 72 h, and the cell densities were calculated and compared with the density at 24 h in the control condition. One-way ANOVA demonstrated 50 and 100 μM of cyclopamine significantly slowed cell proliferation following 48 or 72 h in culture. * $P < 0.05$ vs. control.

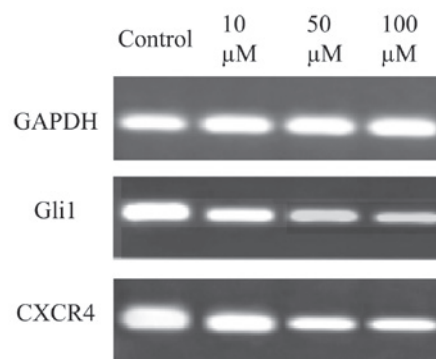
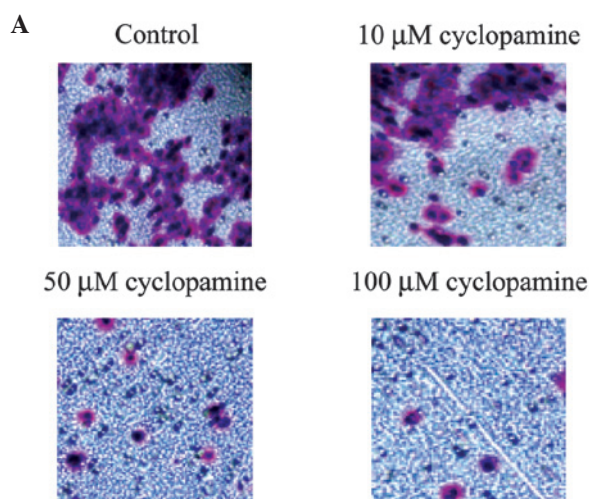


Figure 3. Cyclopamine downregulated Shh-associated gene expression in AGS cells. Following 24 h in culture, cyclopamine downregulated mRNA expression of Gli1 and CXCR4 in gastric cancer cells at concentrations of 10, 50 and 100 μM . Shh, sonic hedgehog.



B

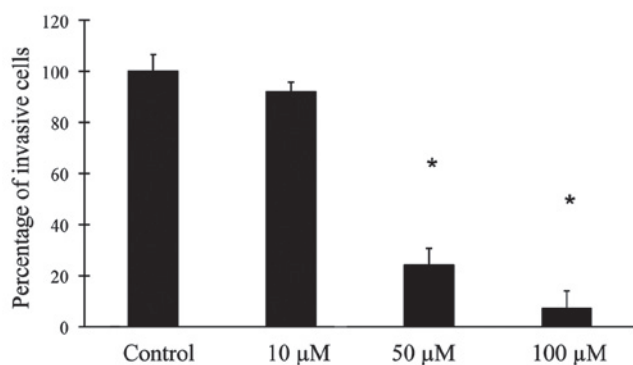


Figure 2. AGS cell invasion was inhibited by cyclopamine. (A) A transwell assay was used to examine the invasion of gastric cancer cells at varying concentrations of cyclopamine (10, 50 and 100 μM). The cells were stained with 0.1% crystal violet. (B) Cyclopamine, at concentrations of 10, 50 and 100 μM , significantly diminished the capability of invasion in gastric cancer cells. * $P < 0.05$ vs. control.

or treated with cyclopamine at concentrations of 10, 50 and 100 μM , and maintained in the culture medium for 24 h (Fig. 2). When treated with 10 μM of cyclopamine, AGS cells demonstrated a similar rate of invasion, as compared with that of the control condition ($P > 0.05$). However, with higher

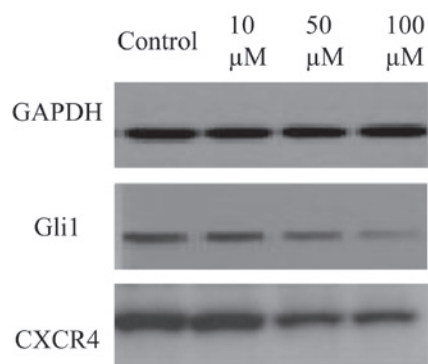


Figure 4. Cyclopamine downregulated Shh-associated protein expression in AGS cells. Western blot analysis revealed that, following 24 h in culture, the protein expression levels of Gli1 and CXCR4 were downregulated in gastric cancer cells by 50 or 100 μM cyclopamine.

concentrations of cyclopamine (50 and 100 μM), the baseline invasions were significantly inhibited. This response was dose-dependent as the greater the concentration of cyclopamine was, the higher the degree of inhibition it induced on cancer cell migration ($P < 0.05$).

Downregulation of Shh-associated factors by cyclopamine in gastric cancer cells. The effects of cyclopamine on gene regulation in AGS cells are demonstrated in Fig. 3. AGS cells were treated with 10, 50 and 100 μM cyclopamine for 24 h. This identified that the higher concentrations of cyclopamine (50 and 100 μM) markedly downregulated the gene expression of Gli1 and CXCR4 in the gastric cancer cells.

Cyclopamine downregulated Shh-associated proteins in AGS cells. The effects of cyclopamine on Shh-related protein expression in AGS cells are presented in Fig. 4. The results were consistent with the gene expression results, as higher concentrations of cyclopamine (50 and 100 μM) downregulated the protein expression of Gli1 and CXCR4 in the gastric cancer cells.

Discussion

The Shh signaling pathway is important in cell differentiation and maturation (1-3,22). However, aberrant activation of the

Shh pathway results in the proliferation of various cancer cell types, including lung, pancreatic and gastric (5,8,23-25).

While the mechanisms of the Shh signaling pathway in promoting gastric tumor formation remain elusive, and the downstream targeting genes continue to be largely unknown, recent studies have indicated that various key factors, including Gill and CXCR4, are closely associated with these pathological processes. These studies identified that the chemokine receptor, CXCR4 and its cognate ligand, CXCL12 were expressed in cancerous tissues and possibly modulated the migration and invasion of tumors in prostate, endometrial and breast cancer (26-29). The *in vivo* and *in vitro* studies have identified that CXCR4 was expressed in gastric carcinoma and gastric cancer cell lines, and correlated with the late developmental stages of lymph node cancer (30).

In the present study, it was demonstrated that, following the inhibition of the Shh pathway through the application of cyclopamine, the proliferation rates and migration capacities in gastric cancer cells were significantly reduced in response to high concentrations of the compound. In addition, it was revealed that the gene and protein expression levels of Gli1 and CXCR4 were consistently downregulated in the gastric cancer cells when high concentrations of cyclopamine were applied. These results were consistent with previous studies that demonstrated that Gli1 and CXCR4 contributed to tumorigenesis in types of cancer other than gastric (23,31,32). In conclusion, the results of the present study provide invaluable insights into the mechanisms of Shh signaling for the regulation of gastric cancer cell growth *in vitro* and these data may ultimately facilitate the development of novel therapeutic targets for the treatment gastric of cancer in human patients.

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