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Cyclopamine Suppresses Human Esophageal Carcinoma Cell Growth by Inhibiting Glioma-Associated Oncogene Protein-1, a Marker of Human Esophageal Carcinoma Progression

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Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Esophageal carcinoma is a common gastrointestinal tumor in humans. Cyclopamine, a Hedgehog (Hh)-pathway-specific inhibitor, is an effective chemotherapeutic drug for suppressing tumor cell differentiation, with unclear mechanisms. We investigated glioma-associated oncogene protein-1 (Gli-1) expression in human esophageal carcinoma tissue and the inhibition of cyclopamine on EC9706 esophageal carcinoma cell growth.

Material/Methods: Gli-1 in tumor tissue was measured by immunohistochemistry (IHC). EC9706 cells were treated with different concentrations of cyclopamine and incubated for different times. MTT method, flow cytometry, and Acridine orange/ethidium bromide (AO/EB) double-fluorescence staining were applied to detect cell proliferation and apoptosis. Western blot (WB) analysis was performed to assess Gli-1 expression.

Results: Gli-1 was associated with patient age, gender, lymphatic metastasis, tumor recurrence, and stage, with significantly ($P<0.05$) positive correlations with age, lymphatic metastasis, tumor recurrence, and stage. At 12 h ($F=214.57$), 24 h ($F=76.832$), 48 h ($F=236.90$), and 72 h ($F=164.55$), the higher the concentration of cyclopamine, the higher the inhibition rate of suppressing EC9706 proliferation, and this effect was significant ($P<0.05$). The number of early-apoptosis cells increased as the concentration of cyclopamine increased. Morphology of EC9706 cells appeared as round with rough edges, karyopyknosis, and karyorrhexis. After 48 h, apoptosis rates of EC9706 cells treated with different concentrations of cyclopamine were (7.73±1.25)% at 2.5 μM, (13.37±1.42)% at 5.0 μM, (22.3±2.92)% at 10.0 μM, and (33.57±1.75)% at 20.0 μM, and the effect was dose-dependent. Gli-1 was obviously reduced after cyclopamine treatment and the effect was dose-dependent.

Conclusions: Gli-1 is highly expressed in human esophageal carcinoma, and could be a marker for use in assessing tumor stage and the deciding on treatment target.

MeSH Keywords: Apoptosis • Cell Proliferation • Esophageal Neoplasms • Glioma • Hedgehogs

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Background

Esophageal cancer arises from the esophagus and is prevalent worldwide [1,2]. In 2012, esophageal cancer was the eighth-most prevalent malignant tumor, with 456 000 new cases per year, causing 400 000 deaths, up from 345 00 in 1990 [3]. Causes of esophageal cancer include tobacco, alcohol, long-term stimulation by hot drinks, poor diet, and chewing betel nut [4]. Treatments of esophageal cancer depend on the cancer stage and the tumor location, as well as general condition and individual preferences of the patient [5]. Small localized tumors can be treated with surgery alone[5], but in other cases, chemotherapy, along with radiotherapy or not, is used as the adjuvant therapy [5]. However, larger tumors can continue to grow slowly during chemotherapy or radiotherapy [6].

Signaling pathways are abnormally activated in esophageal carcinoma. Peng et al. revealed that the MMP-/PAR-1 pathway plays an important role in esophageal tumorigenesis [7]. According to Watanabe et al., the EGF-STAT1 pathway influences the progression of esophageal carcinoma [8]. The Hh signaling pathway plays the critical role in embryo development, and is inactive in normal adults. Hh can be activated in the process of development in many kinds of carcinomas, as well as promoting cells proliferation, survival, and differentiation [9]. Activated Hh in esophageal squamous cell carcinoma is associated with the progression and occurrence of esophageal cancer [10–13]. This association frees Gli in Hh, which can translocate to the nucleus, and then triggers the expression of diverse transcription factors during cell growth. The defect of Hh leads to continuously activating Gli, which is associated with certain types of cancers.

Cyclopamine is a type of steroidal alkaloid extracted from herb species. It can specifically restrain Hh [14]. Recent studies show that cyclopamine has no toxic effect on mammals, which provides good prospects for its application against cancer [15]. Based on this, cyclopamine, as an antitumor drug, is already in phase one clinical trials.

In this study, we observed Gli-1 expression in human esophageal carcinoma tissue, and then investigated the effect of cyclopamine on morphology, proliferation, and apoptosis of EC9706 cells by suppressing Hh, as well as elucidating the basic mechanisms underlying possible treatment of esophageal carcinoma by targeting Hh.

Material and Methods

Patients

Clinical characteristics and tumor samples were collected from 70 patients – 49 males and 21 females – from January of 2011 to December of 2014, who were diagnosed with esophagus cancer according to the criteria of the American Joint Committee on Cancer [16] and the Union Internationale Contre le Cancer [17]. All the patients had received resection and were hospitalized in the First Affiliated Hospital of Shantou University Medical College. All patients were followed for 36 months. Informed consent was obtained from each patient. The study was approved by the First Affiliated Hospital of Shantou University Medical College (the First Affiliated Hospital of SUMC-Scientific Research-No. 2011 008).

Gli-1 detection in human esophagus carcinoma tissue

IHC was used for detecting Gli-1 in human esophagus carcinoma tissues and the adjacent tissues. Tissue slices were made and estimated by 2 pathologists, with consensus. Slices were dried at 68°C for 20 min. Regular de-waxing and gradual ethanol hydration were performed, followed by incubation with 3% H_2O_2 at 37°C for 10 min. Washing with phosphate-buffered solution (PBS) (Solarbio Life Sciences, Inc. Beijing, China) was followed by boiling the slices with citrate buffer solution (0.01 M) (Boster Biological Technology Co., Wuhan, China) at 95°C for 20 min. After cooling to 25°C, we blocked the slices with normal goat serum (Beijing China Ocean Co.) at 37°C for 10 min. Incubation was performed with rabbit anti-Gli-1 polyclonal antibody (1: 500) (ab151796, Abcam, Shanghai, China) at 4°C overnight. After washing with PBS, slices were incubated with goat anti-Rat H&L (1: 200) (Abcam) at 37°C for 30 min. DAB (Beyotime Biotechnology, Shanghai, China) was used for staining at 25°C for 3~30 min until coloring. Hematoxylin-eosin (HE) (Beyotime) was for staining at 25°C for 2 min. Regular dehydration was carried out, and we sealed the slices with neutral resin (Bioway Biotechnology Co., Beijing, China). We observed the results under a microscope (Olympus Corporation, Beijing, China).

EC9706 culture

The human esophagus cancer cell line EC9706 was obtained from the American Type Culture Collection (ATCC). EC9706 cells were cultured in RPMI1640 (Thermo Fisher Scientific, Inc. Shanghai, China) containing 10% fetal bovine serum (FBS) (Thermo), 100 U/ml of penicillin (Sigma), and 100 μg/ml of streptomycin (Sigma), and maintained at 37°C, 5% CO_2, and saturated humidity. EC9706 cells were subcultured (1: 2~1: 3) at 90% confluency after digestion with trypsin (Thermo). EC9706 cells in logarithmic growth phase were used for the following experiments.

EC9706 cell proliferation detection with MTT

EC9706 cells were seeded in 96-well plates (Corning. Shanghai, China) at 6×10^5 cells/ml. Cyclopamine (Sigma) was dissolved

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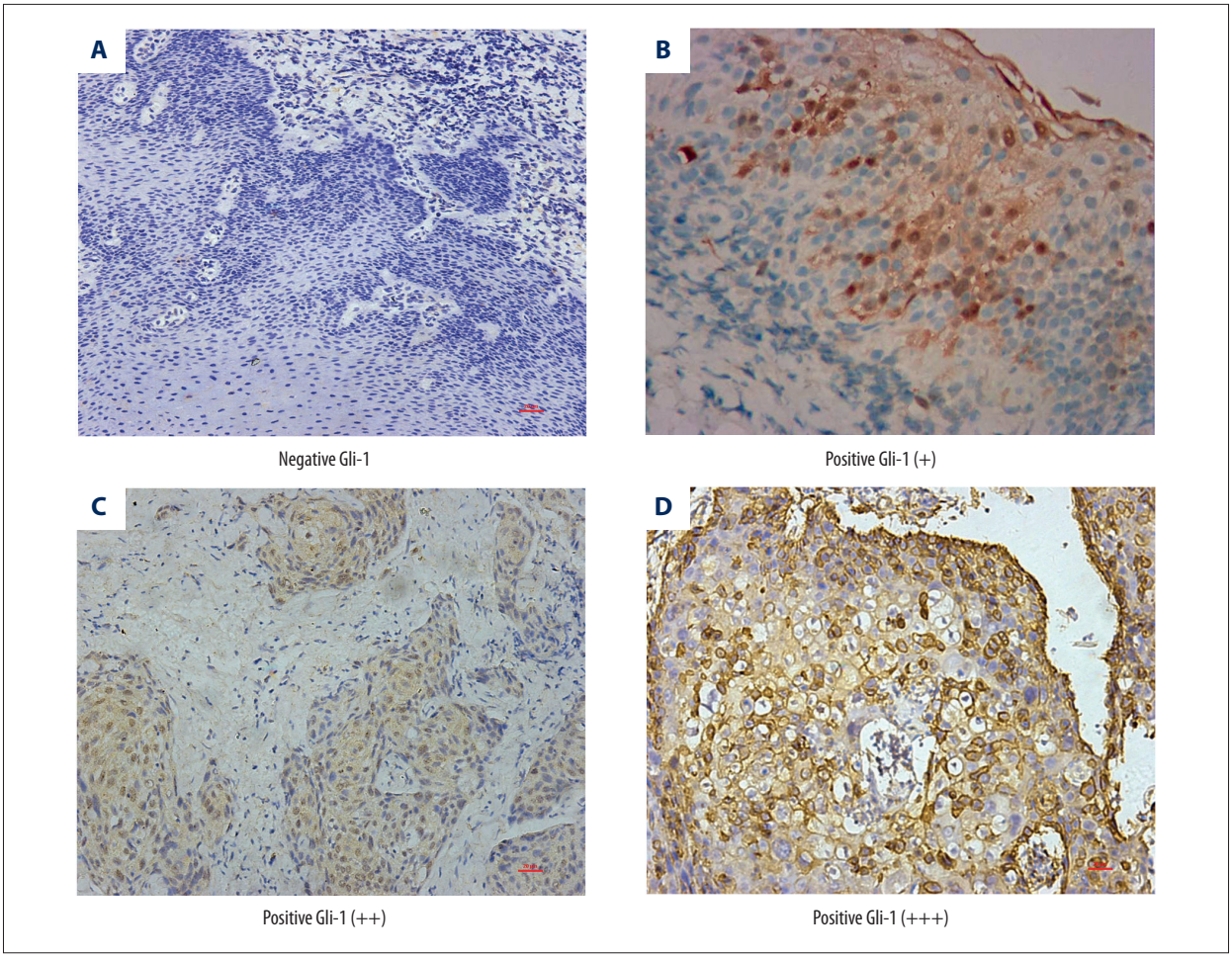

Figure 1. Gli-1 expression in human esophageal carcinoma tissue (**A** Negative Gli-1; **B** Positive Gli-1 (+); **C** Positive Gli-1 (++); **D** Positive Gli-1 (+++).

in dimethyl sulphoxide (DMSO) (Sigma), adjusted to 5 mg/ml, and stored at 4°C. Before using, we adjusted cyclopamine in RPMI1640 (2.5 μM, 5.0 μM, 10.0 μM, 20.0 μM), added it to EC9706 cells, and incubated at 37°C, 5% CO_2, and saturated humidity for 12 h, 24 h, 48 h, and 72h, separately. We added 10 μl MTT solution (5 mg/ml) (Sigma) to each well and incubated at 37°C for 4 h. We discarded the supernatant and added 150 μl DMSO to each well. Cells were incubated with a microplate oscillator (Scientific Industries, Inc., NY, USA) for 20~30 min. Optical density (OD) was read at 492 nm and 630 nm with a microplate reader (Synergy HTX, Biotek Instrument, Inc., Beijing, China). Inhibition rate was calculated as: inhibition rate (%)=(average OD of control group–average OD of dosing group)/(average OD of control group) ×100%. DMSO without cyclopamine was taken as control.

AO/EB double-fluorescence staining

EC9706 cells were seeded in 6-well plates and treated with cyclopamine as mentioned before. We washed the cells with pre-cooled PBS, added 20 μl AO/EB (1: 1) (Solarbio) in 2 ml medium, and incubated the cells at 25°C for 2~5 min. We observed results under a fluorescence microscope (TE2000-U, Nikon, Japan).

Apoptosis detection by flow cytometry

EC9706 cells were seeded in 6-well plates and treated with cyclopamine as mentioned before. Cells were collected by centrifuging at 1000×g for 3 min. We washed the cells twice with pre-cooled PBS and adjusted the concentration to 5×10^5~10^6 cells. An Annexin V-FITC/PI kit (NanJing KeyGen Biotech Co., Nanjing, China) was used for detecting apoptosis. Cells were resuspended with 500 μl binding buffer, mixed with 5 μl Annexin V-FITC, and then mixed with 5 μl propidium iodide (PI), followed by incubation at 25°C in the dark for 5~15 min. Cell apoptosis was measured by flow cytometry (Attune® NxT, Thermo) within 1 h.

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Table 1. Gli-1 expression is associated with human esophagus carcinoma progression.

Age		N	Gli-1				*F*	Adjusted R^2	*P*
			–	+	++	+++			
Age		70	–	–	–	–	8.346	0.242	<0.001*
Gender	Male	49	5	17	12	15	3.055	0.082	0.034*
	Female	21	8	7	2	4			
Lymphatic metastasis	No	26	10	15	1	0	18.575	0.433	<0.001*
	Yes	44	3	9	13	19			
Recurrence	No	19	8	11	0	0	11.706	0.318	<0.001*
	Yes	51	5	13	14	19			
Stage	I	25	12	13	0	0	65.853	0.738	<0.001*
	II	15	1	9	5	0			
	III	23	0	2	9	12			
	IV	7	0	0	0	7			

CI – confidence interval; * $P<0.05$.

Table 2. Effect of cyclopamine on EC9706 proliferation (χ±SDs).

Cyclopamine concentration (μM)	12 h	Inhibition rate (%)	24 h	Inhibition rate (%)	48 h	Inhibition rate (%)	72 h	Inhibition rate (%)
DMSO	0.3222±0.02		0.4612±0.03		0.6322±0.02		0.9427±0.03	
2.5	0.3182±0.05	1.24	0.4448±0.07	3.56	0.5977±0.06	5.46	0.8912±0.06	5.46
5.0	0.3081±0.04	4.38	0.4257±0.04	7.70	0.5558±0.08	12.08	0.7713±0.06	18.18
10.0	0.3033±0.08	5.87	0.4086±0.02	11.41	0.4868±0.03	23.00	0.6023±0.03	36.11
20.0	0.2831±0.03	12.15	0.3677±0.01	20.27	0.4326±0.02	31.57	0.5213±0.01	44.70
F value		214.57		76.832		236.90		164.55
P value		<0.001		<0.001		<0.001		<0.001

WB detecting Gli-1

EC9706 cells were seeded in 6-well plates and treated with cyclopamine as mentioned before. Total protein was extracted from the cells with RIPA buffer (Thermo) and quantified by a BCA kit (Beyotime Biotechnology, Shanghai, China). We analyzed 40 μg proteins by 10% separating gel and 5% stocking gel with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred them to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Shanghai, China). After blocking with 5% skim milk (Millipore) at 25°C for 2 h, protein bands were separately incubated with rabbit anti-Gli-1 polyclonal antibody (1: 2000) (ab151796, Abcam) and β-actin polyclonal antibody (1: 1,000) (Santa Cruz Biotechnology) at 4°C overnight. Then, the bands were incubated with goat anti-rabbit antibody (1: 3000) (Jackson ImmunoResearch Laboratories, Inc. Shanghai, China) at 25°C for 1 h. An electrochemiluminescence (ECL) kit (Millipore) was used for analyzing the proteins.

Statistical analysis

Quantitative data are shown as mean ± standard deviations (χ±SDs) and were analyzed by SPSS 13.0 software. One-Way ANOVA was used for statistical evaluation. Multi-variate analysis was used to determine independent risk factors. $P<0.05$ was considered significant.

Results

Gli-1 is highly expressed in human esophagus carcinoma tissue

As shown in Figure 1, positive Gli-1 expressions were found in human esophagus carcinoma tissues (Figure 1B–1D). Some esophagus carcinoma tissues had negative Gli-1 (Figure 1A).

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Gli-1 is associated with human esophagus carcinoma progression

As shown in Table 1, Gli-1 expression in cancer tissue of human esophagus carcinoma was significantly (P<0.05) associated with patient age, gender, lymphatic metastasis, tumor recurrence, and tumor stage. Males had a higher incidence of human esophagus carcinoma than females. A higher expression of Gli-1 in tumor tissue was associated with greater probability of lymphatic metastasis, as well higher recurrence rate and cancer progression rate.

Multivariate analysis showed that Gli-1 expression in cancer tissue of human esophagus carcinoma patients was positively and significantly (P<0.05) correlated with patient age (R^2=0.242), gender (R^2=0.082), lymphatic metastasis (R^2=0.433), tumor recurrence (R^2=0.318), and tumor stage (R^2=0.738).

Cyclopamine suppresses EC9706 proliferation

As shown in Table 2 and Figure 2, the inhibition rate of cyclopamine suppressing EC9706 proliferation increased as the concentration of cyclopamine increased after 12 h (F=214.57, P<0.001), 24h (F=76.832, P<0.001), 48 h (F=236.90, P<0.001), and 72 h (F=164.55, P<0.001), and this effect was significant. These results indicate that high-concentration cyclopamine can effectively suppress EC9706 proliferation.

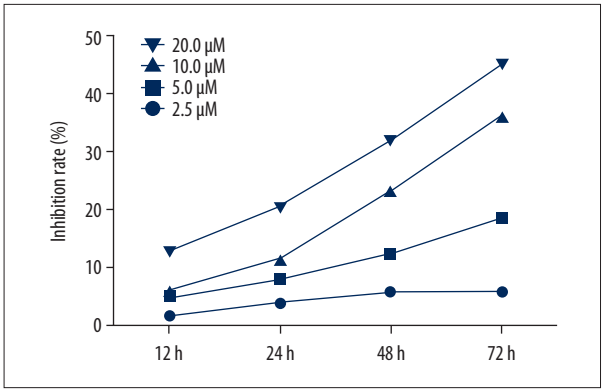

Figure 2. Inhibition rate of cyclopamine (2.5 μM, 5.0 μM, 10.0 μM, 20.0 μM) suppressing EC9706 proliferation at 12 h, 24 h, 48 h, and 72 h.

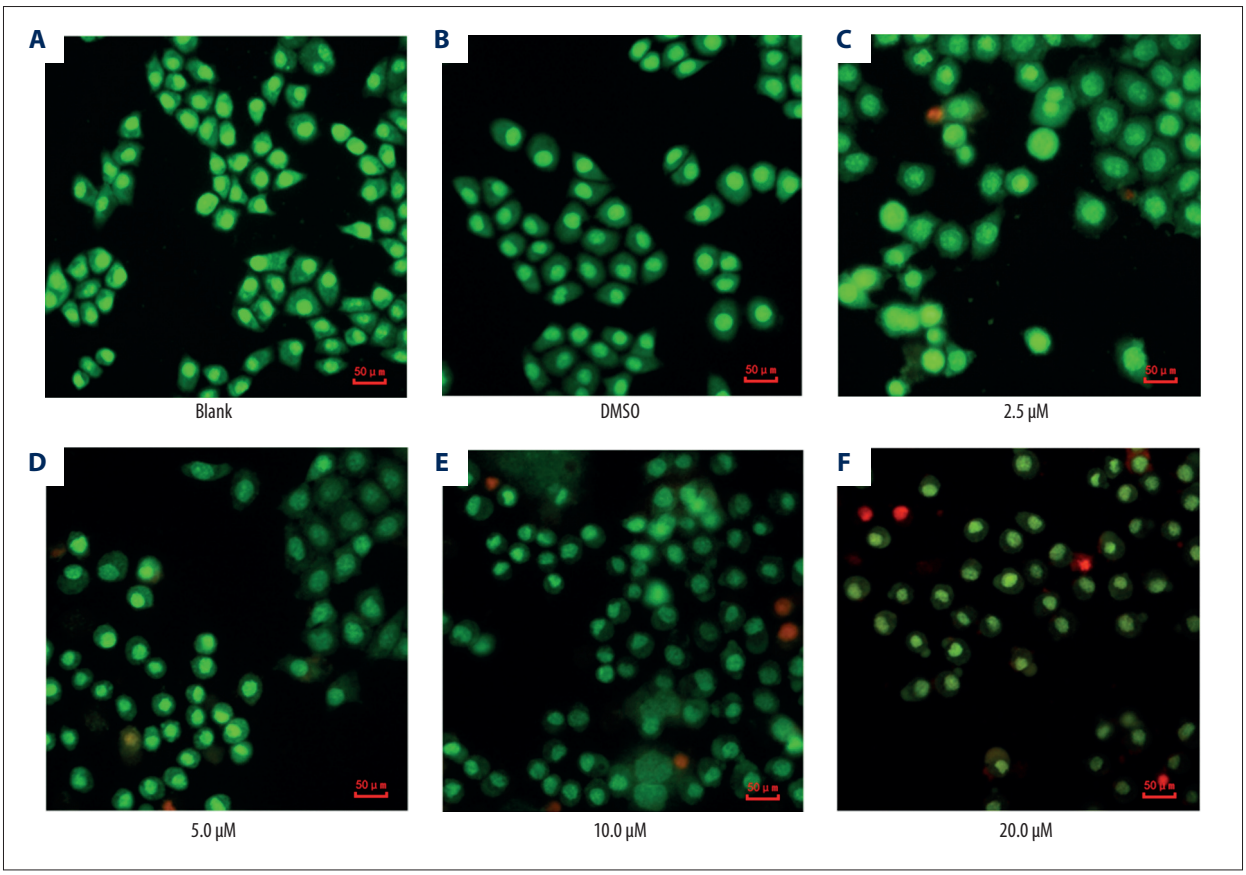

Figure 3. Cyclopamine induced EC9706 apoptosis by AO/EB double-fluorescence staining. (**A** Blank; **B** DMSO; **C** 2.5 μM; **D** 5.0 μM; **E** 10.0 μM; **F** 20.0 μM)

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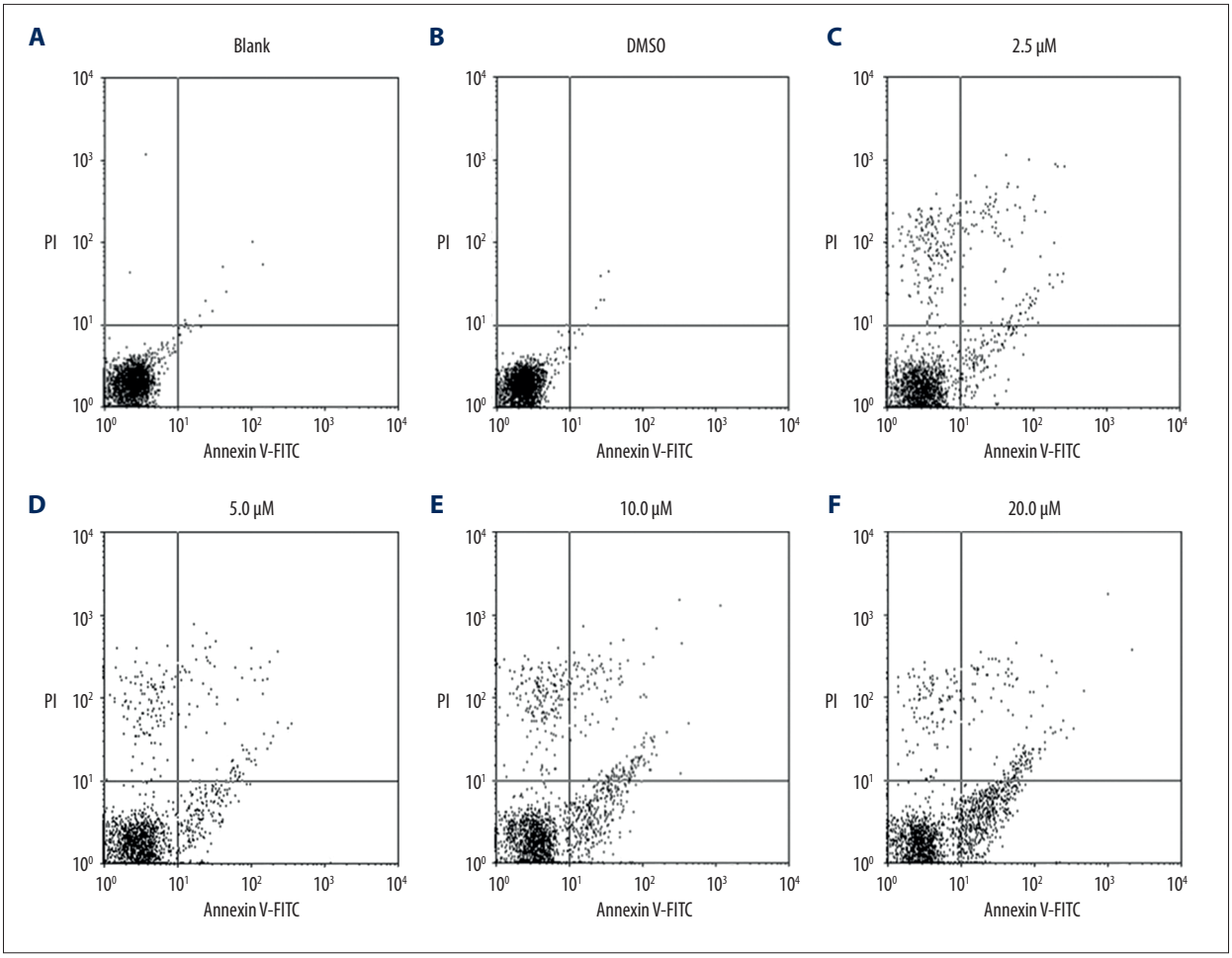

Figure 4. Cyclopamine induced EC9706 apoptosis as shown by flow cytometry. (**A** Blank; **B** DMSO; **C** 2.5 μM; **D** 5.0 μM; **E** 10.0 μM; **F** 20.0 μM).

Cyclopamine promotes EC9706 apoptosis

Fluorescence staining results showed that cyclopamine promoted EC9706 call apoptosis (Figure 3). The treatment time was 48 h. Apoptotic EC9706 cells appeared rounded with rough edges, karyopyknosis, and karyorrhexis. As the concentration of cyclopamine increased, the number of apoptotic cells increased, showing less green fluorescence and more red fluorescence (Figure 3).

Flow cytometry results showed cyclopamine promoted EC9706 cell apoptosis (Figure 4). The treatment time was 48 h. The apoptosis rate of normal EC9706 cells was (0.81±0.07)%, and those with DMSO treating were (1.05±0.13)%. Apoptosis rates of EC9706 cells treated with different concentrations of cyclopamine were (7.73±1.25)% at 2.5 μM, (13.37±1.42)% at 5.0 μM, (22.3±2.92)% at 10.0 μM, and (33.57±1.75)% at 20.0 μM. These results indicate that high-concentration cyclopamine can effectively promote EC9706 cell apoptosis.

Cyclopamine and downregulation of Gli-1 expression

The expression of Gli-1 after exposure to different concentrations of cyclopamine is shown in Figure 5. There was no significant difference in Gli-1 expression between normal EC9706 cells and in EC9706 cells treated with DMSO. With cyclopamine treatment, Gli-1 expression was obviously reduced as the concentration of cyclopamine increased, compared to normal EC9706 cells and EC9706 cells with DMSO treatment. These results indicated that cyclopamine can downregulate Gli-1 expression and suppress the Hh pathway.

Discussion

First, we investigated Gli-1 expression in human esophageal carcinoma tissue. Second, we used cyclopamine to inhibit Hh and downregulate Gli, and then measured the proliferation and apoptosis of EC9706 cells, representing human esophageal carcinoma. Results showed that Gli-1 was highly expressed in

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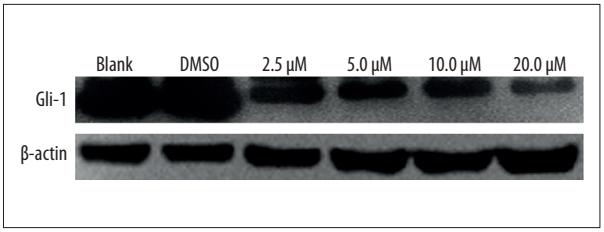

Figure 5. Cyclopamine reduced Gli-1 expression in EC9706.

tumor tissue, which was associated with patient age, gender, lymphatic metastasis, tumor recurrence, and tumor stage. The cellular results showed the effective downregulation of Gli-1 after high-concentration cyclopamine treatment. EC9706 cell proliferation was suppressed but apoptosis was promoted, and the effect was dose-dependent. Our results suggest a potentially therapeutic effect on esophageal carcinoma by targeting the Hh pathway.

The Hh pathway is responsible for regulating and coordinating cellular growth, differentiation, and development of the embryo. Hh has a role in maintaining cell functions, tissue recovery, and tissue regeneration [18]. Some important proteins are involved in the Hh pathway, including patched (Ptc), smoothened (Smo), fused (Fu), suppressor of fused (SuFu), costal-2 (Cos2), and Gli. Hh is regulated by Ptch and Smo as the transmembrane receptors. Gli is the transcription factor of Hh, with multiple functions. In normal cells, Ptc suppresses Smo activity, and then inhibits the downstream protein Gli, which suppresses the transcription of targeted genes. With the combination of Ptc and Hh, the inhibition on Smo is removed. Gli combines with some macromolecular substances, which is helpful for activating the transcription of targeted genes. The loss or mutation of Ptc, or the Smo mutation resulting in inhibition of Ptc, can lead to loss of control of Hh, activating Gli and transcription of targeted genes [19].

The abnormal activation of Hh is common in tumors and is correlated with the pathogenesis and progression of tumors [20]. Numerous scholars found abnormal Hh activation in different kinds of tumors, such as medulloblastoma, basal cell carcinoma, and gastric carcinoma [21,22]. Sui et al. discovered the active Hh in esophageal cancer cells from a rat reflux model [23]. Mori et al. proved the association between Gli-1 expression and lymph node metastasis and tumor progression in esophageal squamous cell carcinoma [10]. More and more related reports confirm the association of Hh and Gli-1 with esophageal carcinoma. In our study, we used EC9706 cells to measure the activity of Hh. WB results showed the abnormal activation of Hh and overexpression of Gli-1 in EC9706 cells, which is in agreement with previous studies.

Cyclopamine is a nonsteroidal alkaloid existing in North American black false hellebore, Indian pyrola, *Veratrum grandiflorum,* and *Fritillariae pallidiflorae*. Cyclopamine has been confirmed as the inhibitor of Hh in many investigations [24–26]. Although some research proved the abnormally active Hh in esophageal carcinoma, there have been few studies focusing on the effect of cyclopamine in suppressing Hh or downregulating Gli-1 in esophageal carcinoma. In our study, we used cyclopamine to inhibit Hh activity as assessed by detecting Gli-1 expression in EC9706 cells. WB results confirmed the successful inhibition of Gli-1 expression, suggesting inhibition of the Hh pathway. Though measuring EC9706 cell proliferation and apoptosis, we found that inhibiting the Hh pathway significantly suppressed EC9706 proliferation but promoted EC9706 apoptosis, in a dose-dependent manner. These results indicate that high-concentration cyclopamine could be used as a drug to prevent esophageal carcinoma development and to promote apoptosis.

Some researchers showed a positive correlation of the expression levels of Smo and Gli-1 with invasion and lymphatic metastasis in esophageal squamous cell carcinoma [10,27,28]. Mori et al. found that silencing Gli-1 obviously suppressed EC9706 proliferation and promoted cell apoptosis, which indicated that the expression of Gli-1 influences the growth of esophageal cells [10]. In our study, we found that downregulation of Gli-1 suppresses EC9706 proliferation and promotes cell apoptosis, which agrees with previous research. However, we did not directly silence Gli-1 or investigate the metastasis of EC9706 cells after downregulating Gli-1, which are limitations of our study. Further investigations should focus on the metastasis of esophageal carcinoma in animal experiments.

Conclusions

Gli-1 expression is a marker of human esophageal carcinoma progression, and cyclopamine might be an effective drug for treating esophageal carcinoma by suppressing the Hh pathway. These results may assist with clinical diagnosis and treatment of esophageal cancer, and elucidate the underlying molecular mechanisms.

Acknowledges

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Conflict in this paper

None.

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