Activation of hedgehog signaling and its association with cisplatin resistance in ovarian epithelial tumors

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Abstract. Few studies have evaluated Hedgehog (Hh) signaling pathway activation in different types of ovarian tumors including benign, borderline and malignant ovarian tumors. The present study investigated the expression of Hh signaling pathway components (SHH, SMO, PTCH, and GLI1) in 193 ovarian epithelial tumor specimens (including 147 malignant epithelial ovarian cancers, 30 borderline ovarian tumors, 16 benign ovarian epithelial tumors) and 11 normal ovarian epithelial tissues by immunohistochemistry. The results demonstrated widespread expression of Hh pathway molecules in ovarian tumors. However, there was no significant difference in the expression intensity of SHH among the four groups (P>0.05). Statistically significant differences were identified in the expression intensity of the SMO, PICH and GLI1 among groups (P<0.001). In addition, significant differences were also revealed in the expression levels of SMO (P=0.013) and GLI1 (P=0.0005) between the platinum drug-sensitive and drug-resistant groups. The overexpression of SMO and GLI1 was further confirmed in the cisplatin-resistant ovarian cancer cell line A2780/DDP by immunofluorescence, flow cytometry and western blotting. The results revealed that the Hh pathway components SMO, PICH and GLI1 are activated in ovarian epithelial tumors. Novel potential associations between cisplatin resistance and the overexpression of SMO and Gli1 in malignant epithelial ovarian cancer were also observed, which may provide an innovative approach to the treatment of drug resistant ovarian epithelial cancer.

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Introduction

Ovarian cancer is one of the most lethal cause of death among gynecological malignancies worldwide. In 2017 there is an estimated 22,440 new ovary cancer cases and 14,080 ovary cancer-related deaths in the United States (1). Data from China showed that an estimated 52,100 new ovary cancer cases and 22,500 ovary cancer-related deaths occurred in China in 2015 (2). Epithelial ovarian cancer accounts for >90% of all ovarian malignancies. Due to the lack of clinically significant symptoms and effective diagnostic markers in the early tumor stages, most paitents will not be diagnosed until the disease reaches an advanced stage (3). Despite advancements in surgery and chemotherapy treatment strategies, the prognosis of ovarian cancer patients remains poor and the 5-year survival rate is only 46% (1). Malignant progression and rapid emergence of drug resistance against conventionally used chemotherapeutic agents are the major problems in the treatment of ovary cancer, one third of newly diagnosed patients and almost all relapse patients become resistant to carboplatin or cisplatin chemotherapy which ultimately results in treatment failure (4). Therefore, appropriate tumor markers and novel therapeutic approaches to achieve early diagnosis and improve patient outcome are urgently needed.

Hedgehog (Hh) signaling pathway is highly conserved from insects to humans and plays an essential role in embryonic development and tissue homeostasis (5). Hh signaling involves a complex network of molecules (6). The pathway can be initiated by three ligands: Desert hedgehog (DHH), Indian hedgehog (IHH) and Sonic hedgehog (SHH). These ligands bind to the transmembrane protein receptor Patched (PTCH), relieving the PTCH-mediated inhibition of transmembrane protein Smoothened (SMO). SMO then initiates an intracellular signaling cascade that leads to the activation and nuclear translocation of the transcription factor family Gli which mediates transcription of genes controlling cell proliferation, differentiation and survival. Aberrant Hh pathway signaling has been implicated in the initiation, promotion, metastases and chemotherapy resistance of a growing number of solid and hematologic malignancies (7). The role of Hh signaling differs in different types of cancer. The initial link between Hh signaling and human cancers was revealed by the discovery that mutations of human PTCH1

are associated with a rare and hereditary form of basal cell carcinoma (BCC), basal cell nevus syndrome (BCNS), which is also named Gorlin syndrome (8,9). Subsequently, activated Hh signaling has been detected in a variety of human cancer types (10-15), either in the tumor or in the stroma.

Aberrant expression of Hh signaling proteins was also detected in ovarian cancer with some conflicting results. Bhattacharya *et al* (16) assessed expression of several Hh pathway components in 19 primary ovarian tumors and the results indicate that overexpression of Gli1 (9/19), SMO (9/19) and SHH (10/19) is a common feature of ovarian cancer while expression of PTCH1 was upregulated in only 1 tumor. However, Yang *et al* (17) indicated that the expression of PTCH1 and GLI1 was not a frequent event in ovarian cancer. Both PTCH1 and GLI1 expression was detected in 9 of 34 (~26%) cancers. Although expression of Hh target genes varies among these research groups, the discrepancies are probably due to the small sample size, the quality of clinical samples and different detection methods. In this study, the expression of Hh pathway components was detected in 193 ovarian epithelial tumor specimens (including 147 malignant epithelial ovarian cancers, 30 borderline ovarian tumors, 16 benign ovarian epithelial tumors) collected in 10 years and 11 normal ovarian epithelial tissues. The associations of Hh components expression level with clinical and pathological features in malignant epithelial ovarian cancers were analyzed to demonstrate the diagnostic and prognostic significances of Hh signaling pathway in ovary cancer. Activation of SMO, PICH and GLI1 was obseved in ovarian epithelial tumors. Results also revealed that the over-expression of SMO and GLI1 proteins might be implicated in cisplatin resistance in ovary cancer patients which was further comfirmed by the observation of SMO and GLI1 overexpression in cisplatin-resistant ovarian cancer cell line.

Patients and methods

Patients. A total of 193 cases with diagnosis of ovarian epithelial tumors, including 147 cases of malignant epithelial ovarian cancer, 30 cases of borderline ovarian tumor, 16 cases of benign ovarian epithelial tumor, were retrieved from the archives of the Obstetrics and Gynecology Department of Peking University Third Hospital (Beijing, China) from 2001 to 2010. A total of 11 cases of normal ovarian epithelial tissue samples were used as control. All patients were surgically treated at the Peking University Third Hospital. Patients with malignant tumors all received cisplatin-based chemotherapy after surgery. Clinical data was collected for each patient through the local hospital database and medical records. Included within the data were demographics, primary and metastatic disease characteristics, relevant laboratory results, surgery, preoperative and postoperative chemotherapy, developed drug-resistance and other informations. Paraffin-embedded specimens of epithelial ovarian tumor obtained during the surgery were retrieved from Pathology Department of Peking University Third Hospital and the expression of Hh pathway componets was detected by immunohistochemistry.

The present study was approved by the Ethics Committee of Peking University Third Hospital and met the standards of the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from all subjects.

Immunohistochemistry. The paraffin blocks from each specimen were cut into 4 μm sections. Tissue sections were dewaxed with xylene and rehydrated through graded ethanol to water. Antigens were retrieved by incubation in natrium citrate buffer for 30 min. The endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide buffer for 10 min. Sections were then incubated in a humidified chamber at 4˚C overnight with primary antibodies. Secondary antibodies were applied for 30 min at room temperature. Sections were counterstained with hematoxylin, dehydrated, cleared and mounted. Primary antibodies used were listed as follows: Rabbit anti human monoclonal antibodies against SHH (1:300; ab-53281), rabbit anti human monoclonal antibodies against SMO (1:200; ab-72130; both Abcam Biochemical, Cambridge, UK), goat anti human polyclonal antibodies against PTCH (1:100; sc-6149), and rabbit anti human polyclonal antibodies against GLI1 (1:50; sc-20687; both Santa Cruz Biotechnology Inc., Dallas, TX, USA).

Positive staining of SHH, SMO, PTCH, GLI1 protein was characterized by apparent membranal or cytoplasmic brown granular. Image collection was performed with a Leica Q550CW image system (Leica, Mannheim, Germany) in three different visual fields of each section. The three fields were chosen according to the following criteria: i) the fields were filled fully with normal tissues or tumor tissues; ii) for the sections derived from tumor samples, no normal tissues should be included in the fileds; iii) there should be no overlap areas in the 3 fields. Collected Images were analyzed with Image-Pro Plus software (Media Cybernetics, Silver Springs, MD). The expression of SHH, SMO, PTCH, GLI1 in each section was represented by the mean integral optical density (IOD) value of three different visual fields. Three sections were analyzed for each specimen and different proteins.

Human ovarian cancer cell lines. Human ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780/DDP were purchased from the Gynecologic Oncology department of GuangXi Medical University (Guangxi, China). The cells were cultured at 37˚C in an atmosphere of 5% CO_2 in RPMI 1640 medium with 10% fetal bovine serum (FBS), 50 IU/ml penicillin, 50 μg/ml streptomycin, and 0.3 μg/ml glutamine. Cells were harvested for immunofluorescent staining, flow cytometry and western blot analysis.

Immunofluorescent staining. A2780 and A2780/DDP cells treated with collagenase type IV were placed into 35 mm dishes containing glass coverslips, next day cells on the coverslip were fixed with 4% paraformaldehyde and incubated at room temperature for 30 min, then those cells were permeabilized with 1% Triton X-100 for 1 h at room temperature, followed by blocking in 1% BSA for 1 h and incubation with rabbit SMO (1:200; ab-72130) antibody and rabbit GLI1 (1:50; sc-20687) antibody diluted in the ratio of 1:100 in blocking solution for 1 h at 37˚C. After washing three times with PBS containing 0.1% Tween-20 and 0.01% Triton X-100 (washing solution), the cells were labeled with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG diluted in the ratio of 1:100 for 1 h at 37˚C and then washed with PBS for 3 times. The samples were counterstained with propidium iodide and coverslip with cells and 50 μl glycerol were mounted to a glass slide.

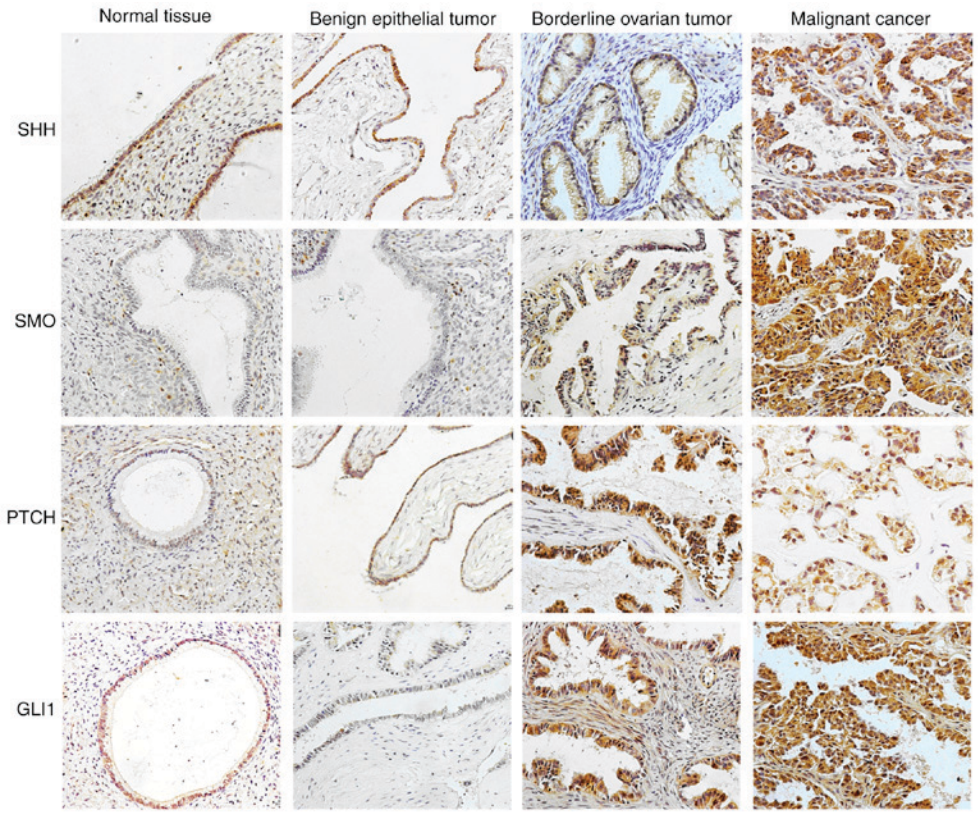


Figure 1. Immunohistochemical staining of SHH, PTCH, SMO and GLI1 proteins in various epithelial ovarian tumors (magnification, x200). SHH, sonic hedgehog; PTCH, Patched; SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

Flow cytometry. The expressions of SMO and GLI1 were also evaluated by Flow cytometry. A2780 and A2780/DDP cells were washed once with PBS and divided to three aliquots with a density of 106 cells/ml. Cells were incubated respectively with PBS, SMO antibody (1:200; ab72130), or GLI1 antibody (1:50; sc-20687) for 30 min at room temperature, and then stained with Goat anti-Rabbit IgG FITC (Sigma-Aldrich, St. Louis, MO, USA) for 20 min in the dark. Incubation was stopped by adding 500 μl phosphate-buffered saline, followed by washing with PBS for 3 times. At least 10,000 cells were assayed by FCM using a FASCalibur flow cytometry (Becton Dickinson, Mountain View, CA, USA). The expressions of SMO and GLI1 were analyzed with CellQuest software (Becton Dickinson).

Protein extraction and western blot. Total protein of cells was extracted using radioimmunoprecipitation (RIPA) lysis buffer completed with proteinase inhibitor from Sigma-Aldrich. Protein concentration was determined using a BCA Protein Assay kit (Beyotime Biotechnology, Shanghai, China). Samples were loaded on 10% sodium dodecyl sulfate polyacrylamide gel and transferred onto polyvinyl difluoride membranes. After transfer, the membranes were blocked in TBST (TBS containing 0.1% Tween-20) containing 5% skimmed milk for 2 h, followed by incubation overnight at 4˚C with rabbit SMO (1:200; ab-72130) antibody and rabbit GLI1 (1:50; sc-20687) antibody. After washing 3 times in TBST, the membranes were incubated with 1:2,000 horseradish peroxidaseconjugated appropriate secondary antibodies for 1 h at 37˚C. Finally, the membranes were processed and visualized using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA).

Statistical analysis. Statistical analyses were performed using SPSS 19.0. Quantitative variables meeting normal distribution were described with mean and standard deviation, differences between groups were analyzed with t-test and variance analysis. The non-normal distribution data were analyzed with rank sum test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of SHH, SMO, PTCH and GLI1 in ovarian epithelial tumors. By immunohistochemistry staining, expression of Hh components SHH, SMO, PTCH and GLI1 in 193 ovarian epithelial tumors (including 147 malignant epithelial ovarian cancers, 30 borderline ovarian tumors, 16 benign ovarian epithelial tumors) and 11 normal ovarian epithelial tissues were evaluated. Positive staining of SHH, SMO, PTCH and GLI1 protein was characterized by apparent membranal or cytoplasmic brown granular (Fig. 1). By reason that positive staining was observed in almost all samples, we used the mean IOD to quantitatively evaluate the expression levels of SHH, SMO, PTCH and GLI1 in each sample, the significant differences betwwen groups were statistically analyzed (Fig. 2). Membranous staining of SHH could be detected in normal tissues and in three types of tumors. IOD values of SHH expression in each group were as follows: Group of normal tissues, 3.45±0.74; group of benign tumors,

4.19±0.95; group of borderline tumors, 3.99±0.69; group of malignant cancers, 4.22±1.46. There was no significant difference in expression intensity among the four groups (P=0.529). Expression of SMO protein was maily detected in the cell membrane and cytoplasm. SMO protein was rarely expressed in the normal ovarian epithelial tissues and benign ovarian epithelial tumors, but was intensively expressed in borderline ovarian tumors and malignant epithelial ovarian cancers. IOD values of SMO expression in each group were as follows: group of normal tissues, 0.71±0.44; group of benign tumors, 0.94±0.12; group of borderline tumors, 4.10±0.15; group of malignant cancers, 4.18±1.47. There were statistically significant differences in the expression intensity of SMO between group of normal tissues groups and group of borderline tumors (P<0.001), between group of normal tissues groups and group of malignant cancers (P<0.001), between group of benign tumors and group of borderline tumors (P<0.001), between group of benign tumors and group of malignant cancers (P<0.001). Expression of PTCH protein was maily observed in the cell membrane and cytoplasm. PTCH protein was rarely expressed in the normal ovarian epithelial tissues, but was intensively expressed in benign ovarian epithelial tumors, borderline ovarian tumors and malignant epithelial ovarian cancers. IOD values of PTCH expression in each group were as follows: group of normal tissues, 0.61±0.12; group of benign tumors, 4.04±0.85; group of borderline tumors, 3.87±0.62; group of malignant cancers, 3.80±1.32. There were statistically significant differences in the expression intensity of PICH between group of normal tissues groups and group of benign tumors (P<0.001), between group of normal tissues groups and group of borderline tumors (P<0.001), between group of normal tissues groups and group of malignant cancers (P<0.001). Expression of GLI1 protein was maily detected in the cell cytoplasm and nucleus. GLI1 protein was rarely expressed in the normal ovarian epithelial tissues and benign ovarian epithelial tumors, but was intensively expressed in borderline ovarian tumors and malignant epithelial ovarian cancers. IOD values of GLI1 expression in each group were as follows: group of normal tissues, 0.68±0.13; group of benign tumors, 0.89±0.11; group of borderline tumors, 4.92±1.14; group of malignant cancers, 5.14±1.77. There were statistically significant differences in the expression intensity of GLI1 between group of normal tissues groups and group of borderline tumors (P<0.001), between group of normal tissues groups and group of malignant cancers (P<0.001), between group of benign tumors and group of borderline tumors (P<0.001), between group of benign tumors and group of malignant cancers (P<0.001).

Expression of SHH, SMO, PTCH and GLI1 in malignant epithelial ovarian cancers with different characteristics. A total of 147 patients with malignant epithelial ovarian cancers were divided into several groups based on different demography characteristics or clinical/pathologic characteristics, expression of SHH, SMO, PTCH and GLI1 in the cancer samples of these groups was analyzed. The median age of the 147 epithelial ovarian cancer patients was 54±10.39 years old. The follow-up period ranged from 4 to 108 months (median 58 months). Of the 147 patients, 54 cases were classified as stage I-II, 93 cases were stage III-IV. Histologically, 65 cases were serous cancer, 15 cases were mucinous cancer, 11 cases were endometrioid cancer, 38 cases were clear cell adenocarcinomas, and 18 cases were other cancer types, such as transitional cell carcinoma and squamous cell carcinoma. The expression intensity of the four proteins represented by IOD values in each group was showed in Table I. No statistically significant correlation was found between the expression of SHH, SMO, PTCH and GLI1 among groups with different ages, or among groups with different histological types, or among groups with different WHO stage, or among groups with different histological grades. The expression levels of Hh components SHH, SMO, PTCH and GLI1 were also independent of whether the patients accepted preoperative chemotherapy or not.

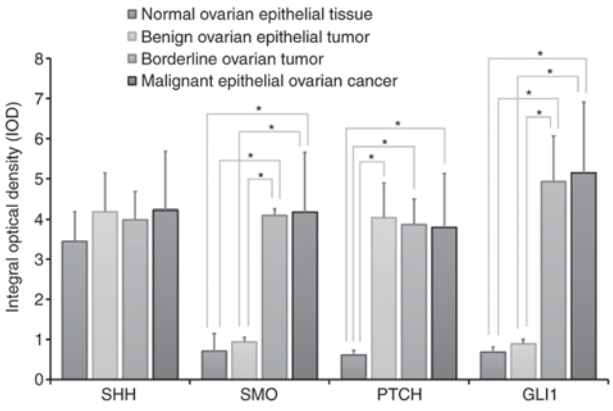


Figure 2. Immunostaining score represented by IOD of Hedgehog pathway molecules in normal ovarian epithelial tissue, benign ovarian epithelial tumor, borderline ovarian tumor and malignant epithelial ovarian cancer. Values represent mean ± SD. *P<0.001. IOD, integral optical density; SHH, sonic hedgehog; PTCH, Patched; SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

However, significant differences were found in the expression of SMO and GLI1 between groups with different chemosensitivity. Among the 147 epithelial ovarian cancer patients, there were 120 patients who were sensitive to platinum-based chemotherapy and 27 patients who were resistant to platinum-based chemotherapy. Compared with the platinum-sensitive group, the staining intensity of SMO and GLI1 protein were obviously stronger in primary platinum-resistant group (Fig. 3). The IOD value of each group was calculated based on the immunohistochemistry results and statistically significant differences of the expressions of SMO and GLI1 were noticed between the platinum-sensitive group and the platinum-resistant group (SMO, P=0.013; GLI1, P=0.0005) (Table I). However, no significant difference in the expression of SHH and PTCH were observed in between the two groups.

Expression of SMO and GLI1 in A2780 cell line and cisplatin-resistant A2780/DDP cell line. The association between the overexpression of SMO and GLI1 proteins and cisplatin resistance was further comfirmed in ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780/DDP by immunofluorescent staining, flow cytometry and western blot. Results from immunofluorescent staining showed that GLI1 protein expressed in the cytoplasm and nucleus of cells,

Table I. Immunohistochemical expression of Hedgehog signal molecules in malignant epithelial ovarian cancer.

Group	Subgroup	Number	SHH	SMO	PTCH	GLI1
Age						
	<40	25	4.01±0.81	3.83±0.83	3.20±0.31	5.35±2.26
	40-70	101	3.81±1.30	3.97±1.01	3.43±0.85	4.88±0.97
	>70	21	4.11±0.42	4.15±0.87	3.51±0.67	4.73±1.02
	P-value		0.080	0.525	0.211	0.690
Histological type						
	Serous	65	3.73±1.36	4.07±1.09	3.24±0.78	5.07±1.65
	Mucinous	15	3.99±0.33	3.83±0.64	3.58±0.29	5.17±0.65
	Clear cell	38	3.94±1.30	4.32±0.85	3.58±0.79	4.67±1.00
	Endomitrioid	11	4.06±0.25	4.39±1.19	3.39±1.22	4.62±1.31
	P-value		0.062	0.343	0.077	0.473
WHO stage						
	I	36	3.95±0.81	5.24±1.08	3.68±1.09	4.59±1.43
	II	18	4.37±1.48	4.61±1.64	4.17±0.66	4.27±0.99
	III	51	4.06±1.02	5.39±1.78	3.55±0.88	4.30±0.06
	IV	12	4.38±1.35	4.59±0.94	3.73±1.08	4.59±2.49
	P-value		0.092	0.099	0.076	0.800
Preoperative chemotherapy						
	Accepted	21	4.96±0.91	3.95±0.77	3.65±1.43	5.69±2.44
	Unaccepted	126	5.58±1.27	4.23±1.55	3.63±0.96	5.27±1.62
	P-value		0.095	0.206	0.947	0.457
Histological grade						
	G1	19	4.96±1.16	4.19±0.92	4.07±1.70	4.55±2.23
	G2	37	4.74±1.51	3.92±0.98	3.40±0.41	6.15±1.43
	G3	35	3.98±1.36	3.40±0.60	3.10±1.05	5.82±1.39
	P-value		0.057	0.061	0.052	0.102
Chemosensitivity						
	Sensitive	120	4.05±1.36	5.31±2.06	3.64±0.98	4.67±1.30
	Resistant	27	4.00±0.77	6.01±1.05	3.61±1.26	6.49±1.42
	P-value		0.792	0.013[a]	0.905	0.0005[b]

[a]P<0.05, [b]P<0.001, sensitive vs. resistant. SHH, sonic hedgehog; PTCH, Patched; SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1; WHO, World Health Organization.

while the SMO protein expressed in the cell membrane and cytoplasm. In the A2780 cells there were sporadic expression of SMO and GLI1 proteins, nevertheless in the cisplatin-resistant A2780/DDP cells there were strong expression of SMO and GLI1 (Fig. 4). The flow cytometry test showed the similar results that the fluorescence intensity of SMO and GLI1 in the A2780/DDP cells was significantly higher than in A2780 cells (Fig. 5). The mean fluorescent intensity of SMO in A2780 and A2780/DDP cells were 26.31 and 70.80 respectively, with the P value of 0.01; The mean fluorescent intensity of GLI1 in A2780 and A2780/DDP cells were 20.74 and 55.72, respectively, with P=0.0004. The expressions of SMO and GLI1 protein were also quantitative analyzed by western blotting. There was no obvious difference of β-actin protein expression between the two cell lines which served as the internal controls. However, GLI1 and SMO proteins both showed stronger expression in A2780/DDP cells compared with A2780 cells (Fig. 6).

Discussion

Activation of the Hh pathway has been observed in several cancer types, including ovarian cancer. However, studies evaluating Hh pathway activation in ovarian cancer show conflicting results. Bhattacharya *et al* (16) assessed expression of several Hh pathway components including SHH, PTCH1, SMO and GLI1 in 19 primary ovarian tumors using real-time PCR, overexpression of Gli1 (9/19), SMO (9/19) and SHH (10/19) was observed while expression of PTCH1 was upregulated in only 1 tumor. Yang *et al* (17) also assessed activation of Hh pathway in ovarian epithelial tumor specimens through analyses of target gene expression by *in situ* hybridization, immunohistochemistry and real-time PCR. Expression of SHH, PTCH1 and Gli1 was detected in 34 ovarian epithelial tumor specimens while SMO expression was detected in 17 ovarian epithelial tumor specimens. Results showed that the positive expression of SHH,

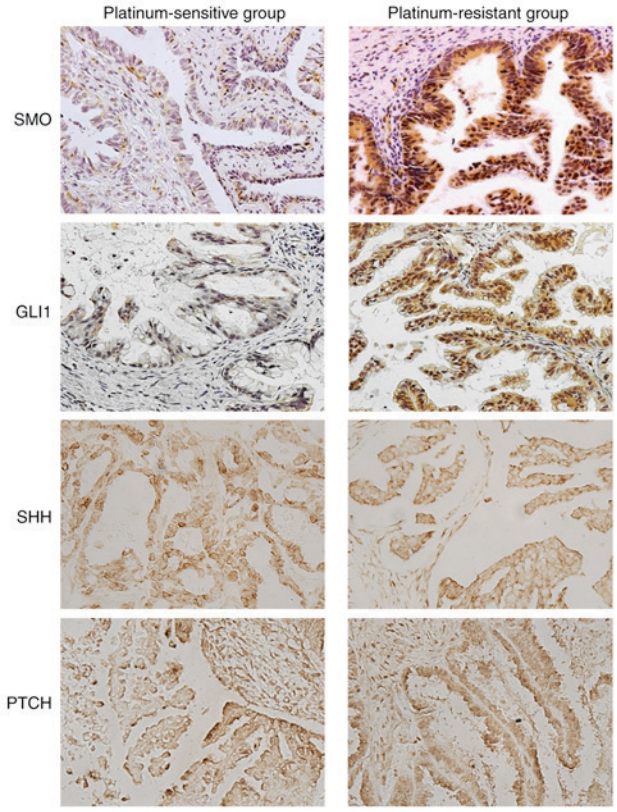


Figure 3. Immunohistochemical staining of SHH, PTCH, SMO and GLI1 proteins in ovarian epithelial cancers of platinum-sensitive group and primary platinum-resistant group (magnification, x200). SHH, sonic hedgehog; PTCH, Patched; SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

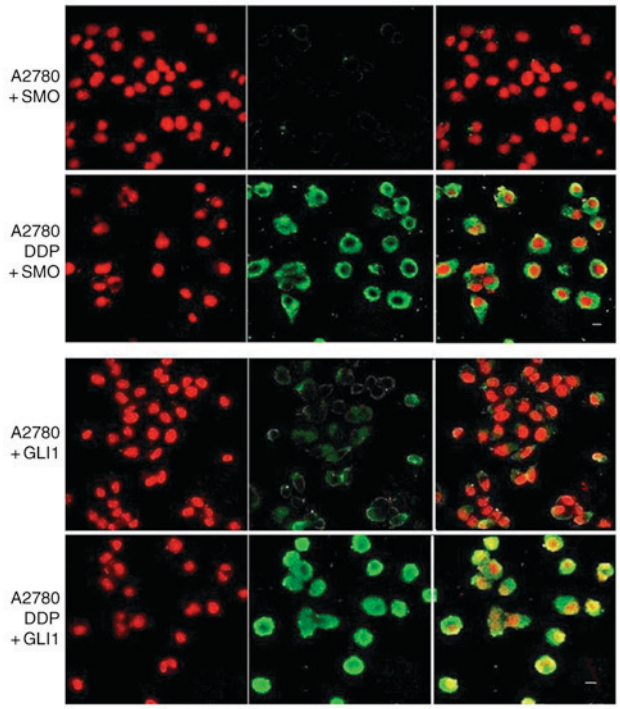
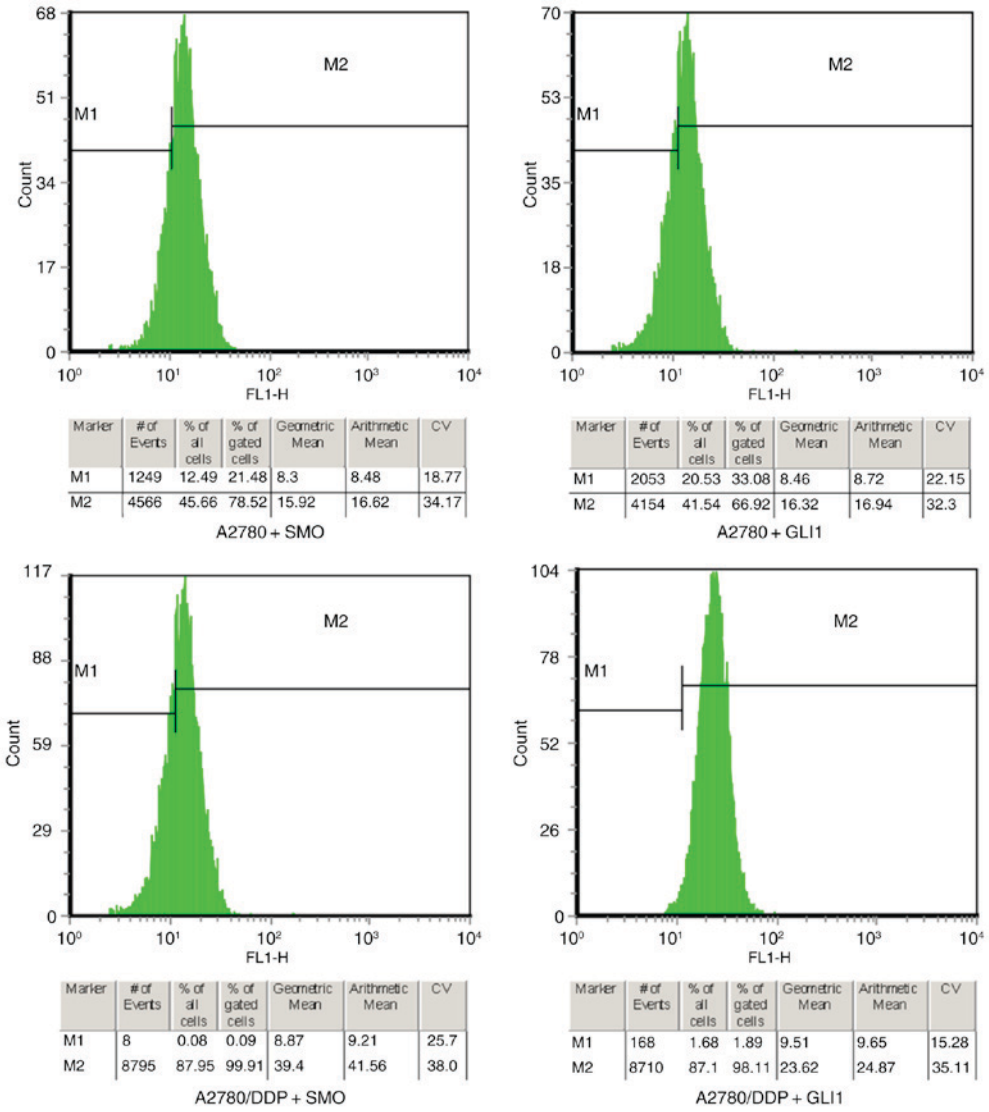


Figure 4. Immunofluorescent staining of SMO and GLI1 proteins in human ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780/DDP (scale bars, 15 μm). SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

PTCH1, SMO and Gli1 was detected in 11 (~32%), 9 (~26%), 4 (~24%), 9 (~26%) cancers correspondingly, indicating that activation of Hh signaling is not a frequent event in ovarian cancers. Chen *et al* (18) investigated Gli1 mRNA levels in 40 ovarian cancer tissues, result showed that expression of Gli1 mRNA in ovarian cancer varied widely, with 7 out of 40 (17.5%) showing high expression. The protein expression of Gli1 was also detected in Chen's work by immunohistochemistry, 8 out of 30 tumors (26.7%) showed strong staining of Gli1. The above studies all focused on the expression of Hh pathway components in malignant ovarian cancers, there are little available data on the expression of Hh signal molecules in various epithelial ovarian tumors of benign, borderline and malignant histology. Chen *et al* (19) investigated the expression of Shh, Ihh, Dhh, Ptch, Smo and Gli1 proteins in benign (14 cases), borderline (15 cases) and malignant (57 cases) ovarian tumors and normal ovarian epithelium immunohistochemically, results showed that the expression of Shh, Dhh, Ptch, Smo and Gli1 in carcinomas were significantly increased compared with those in benign tumors, the expression levels of Hh signal molecules in borderline tumors were intermediate between those of benign and malignant tumors, but the differences were not significant. In our study, the expression of SHH, PTCH, SMO, GLI1 was also tested in benign (16 cases), borderline (30 cases) and malignant (147 cases) ovarian tumors and normal ovarian epithelial tissues. Extensive staining of SHH could be detected in normal tissues and in benign and malignant tumors without significant differences between groups. The result was different from the work of Chen *et al* (19) which showed that there was no SHH expression in normal tissues while there was strong expression of SHH in ovarian tumors. The different results of the SHH expression in normal ovarian epithelial tissues in the two works are hard to explain, more accurate protein quantitative detection technology such as western blot will be needed to further verify the expression of SHH in normal ovarian epithelium. The expression of SMO was observed along the cell membrane and in the cytoplasm, which is consistent with it serving as a membrane receptor and a signal transduction element in the Hh signal pathway. Strong expression of SMO was observed in borderline and malignant tumors while relatively low expresion was observed in normal epithelial tissues and benign tumors, suggesting that activation of Hh signaling might be important in tumor malignant transformation. SMO serves as an important hub in Hh signaling transduction, which may provide a potential target for inhibition of Hh signaling pathway in tumors. The transmembrane protein PTCH is a common Hh receptor which inhibits the activation of SMO in the absence of Hh ligands. In ovarian tumors, we observed an elevated expression in ovarian tumors compared with normal ovarian epithelium, similar with Chen's results. However, there is no obvious trend in PTCH expression level among benign, borderline and malignant tumors. As a transcription factors, when activated by SMO, GLI1 transported from cytoplasm to the nucleus, resulting in the transcription of Hh target genes. Gli1 staining was observed mainly in the cytoplasm in normal ovarian epithelium, nuclear staining was observed in ovarian carcinoma cells, suggesting that it was transported into the nuclei for transcription activation. The expression pattern of



Marker	# of Events	% of all cells	% of gated cells	Geometric Mean	Arithmetic Mean	CV
M1	1249	12.49	21.48	8.3	8.48	18.77
M2	4566	45.66	78.52	15.92	16.62	34.17

Marker	# of Events	% of all cells	% of gated cells	Geometric Mean	Arithmetic Mean	CV
M1	2053	20.53	33.08	8.46	8.72	22.15
M2	4154	41.54	66.92	16.32	16.94	32.3

Marker	# of Events	% of all cells	% of gated cells	Geometric Mean	Arithmetic Mean	CV
M1	8	0.08	0.09	8.87	9.21	25.7
M2	8795	87.95	99.91	39.4	41.56	38.0

Marker	# of Events	% of all cells	% of gated cells	Geometric Mean	Arithmetic Mean	CV
M1	168	1.68	1.89	9.51	9.65	15.28
M2	8710	87.1	98.11	23.62	24.87	35.11

Figure 5. Expression of SMO and GLI1 proteins in human ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780/DDP detected by flow cytometry. SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

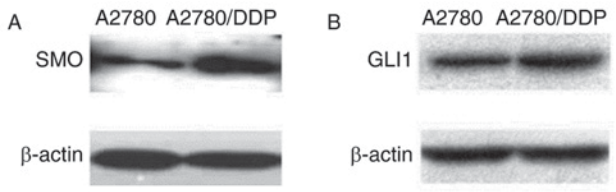


Figure 6. Expression of SMO and GLI1 proteins in human ovarian cancer cell line A2780 and cisplatin-resistant ovarian cancer cell line A2780/DDP detected by western blot. (A) Expression of SMO in A2780 and A2780/DDP cell lines. (B) Expression of GLI1 in A2780 and A2780/DDP cell lines. SMO, Smoothened; GLI1, hedgehog glioma-associated oncogene 1.

GLI1 was similar to the expression pattern of SMO, strong nucleus expression of GLI1 was observed in borderline and malignant tumors while relatively low expresion was observed in normal epithelial tissues and benign tumors, suggesting the overactive Hh signaling in borderline and malignant ovarian tumors. Our study comfirmed the activation of Hh signal pathway in ovarian tumrs, suggesting its role in tumor malignant transformation.

Further we investigated the association between the expression levels of SHH, SMO, PTCH and GLI1 proteins and clinical characteristics of malignant ovarian cancer patients. Results showed that the protein expression levels of these four proteins have no associations with the sex of patient, stage of cancer, pathohistological feature and preoperative chemotherapy. Similarly, Yang *et al* (20) also proved that activation of Hh pathway was not correlated with histological types of tumor in ovarian epithelial cancer. However, studies of Schmid *et al* got different views and they found great variation in the levels of Hh pathway proteins in the studied ovarian cancer samples, even in those with the same staging and histological origins (21). It could be inferred that there is different molecular pathogenesis with variation in the histological origins, stages and classification (22). The activation of certain Hh signal pathway molecule proteins may vary under different conditions.

Many studies suggested the close connection of dysregulation of Hh pathway with chemosensitivity in cancers. Rumjanek *et al* (23) discovered the correlation between multi-resistant leukemia cell strains mediated by abnormally activated Hh pathway and ABC transporter. Hh pathway

inhibitor cyclopamine and GLI1 inhibitor, Vitamin D3 and GANT61, can restore the sensitivity of resistant myeloid leukemic cells to traditional chemotherapy drugs (24). BCL-2 family is also a downstream target of Hh pathway, which is an essential mediator in the apoptosis of mitochondria (25). Loss of mitochondrial apoptosis is one of the causes of multiple drug resistance. Inhibitor of Hh signal pathway can induce apoptosis by downregulating BCL-2. It was also found that GLI1-siRNA could downregulate BCL-2 and restore the sensitivity of pancreatic cancer cells to chemotherapy (26).

However, rare studies have focused on the potential association between Hh signal and chemotherapy resistance in ovarian cancer. Here we observed a significantly increased expression of SMO and GLI1 in patients with primary cisplatin-resistance compared with chemo-sensitive group. With the same measurement parameters, expressions of SMO and GLI1 were significantly higher in cisplatin-resistance cell strain A2780/DDP than in A2780. In Hh pathway, SMO protein is the 'Hub' for Hh pathway while GLI1 is the critical transcriptional regulation factor. Therefore these two proteins constitute the most essential elements in the Hh pathway. Our findings indicated the potential effects of Hh signal pathway in the development of cancer drug resistance which still needs to be further investigated. The close relationship between high expression of SMO and GLI1 in Hh pathway and cancer drug resistance may provide a new target in the reversal of drug resistance in tumor treatment.

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