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Original Article

Dysregulation of hedgehog signaling pathway related components in the evolution of colonic carcinogenesis

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Abstract: Previous studies report controversial role of Hedgehog (HH) signaling in the progression of colon cancer. This study aimed to investigate the expressions of smoothened (SMO) and downstream glioma-associated oncogene homology-1 (GLI1) in colon cancer, colonic adenoma and normal tissues. Colon cancer and normal tissue samples were collected from 49 patients with colon cancer while colonic adenoma tissue samples were obtained from 34 patients with colonic adenoma. Then the expressions of SMO and GLI1 were investigated using immunohistochemistry (IHC). For the detection of SMO and GLI1 expression, IHC staining results indicated that SMO was mainly expressed on the membrane while GLI1 was mainly expressed in the cytoplasm. The positive rates of SMO and GLI1 protein expressions were significantly increased in colon cancer tissue and colonic adenoma tissue when compared with normal colon tissue. In contrast, the significant difference was not found in the positive rates of SMO and GLI1 protein expressions between colon cancer tissue and colonic adenoma tissue. More importantly, it was found that SMO and GLI1 expressions possibly increased gradually from the normal colon to colonic adenoma to the colon cancer. Furthermore, no distinct correlations were detected between the expression levels of SMO and GLI1 and clinicopathological parameters, including age, gender, differentiation and Dukes stage. The present results provided some new information to the possible role of HH signaling in colon cancer progression. SMO and GLI1 maybe suggested asbiomarkers to identify colon cancerous, precancerous and normal tissues as well astherapeutic targets for colon cancer treatment.

Keywords: Colon cancer, hedgehog signaling, SMO, GLI1, evolution

Introduction

Colorectal cancer is ranked as the third most frequent cancer and the third leading cause of cancer-related death both in men and women with an estimated 136,830 newly diagnosed cases and 50,310 deaths in 2014 in the United States [1]. Colorectal adenoma is commonly regarded as a precancerous lesion of colorectal cancer and is recommended as an important opportunity for colon cancer treatment [2]. In clinical practice, laparoscopy-assisted colectomy and open colectomy have been developed for the treatment of non-metastatic colon cancer [3]. Besides, researchers also have recommended some therapeutic drugs as adjuvant chemotherapy for metastatic colon cancer, such as aspirin [4], oxaliplatin and irinotecan [5]. However, colon cancer is traditionally diagnosed as a diverse disease, and its heterogeneity usually makes it difficult to choose appropriate therapeutic approaches to improve the prognosis [6]. Evidence has shown that candidate biomarkers possess great clinical value for their potential use in cancer staging and personalized therapy [7]. Therefore, the identification of biomarkers implicated in colon cancer could benefit the improvement of clinical management.

Hedgehog (HH) signaling pathway has been associated with various types of cancer, including leukemia, brain tumor, lung cancer, and gastrointestinal cancers since the first description of HH as a genetic mutation in Drosophila [8]. In terms of colon cancer, Wang *et al*. demonstrated that HH signaling pathway components were aberrantly expressed [9]. The HH family con-

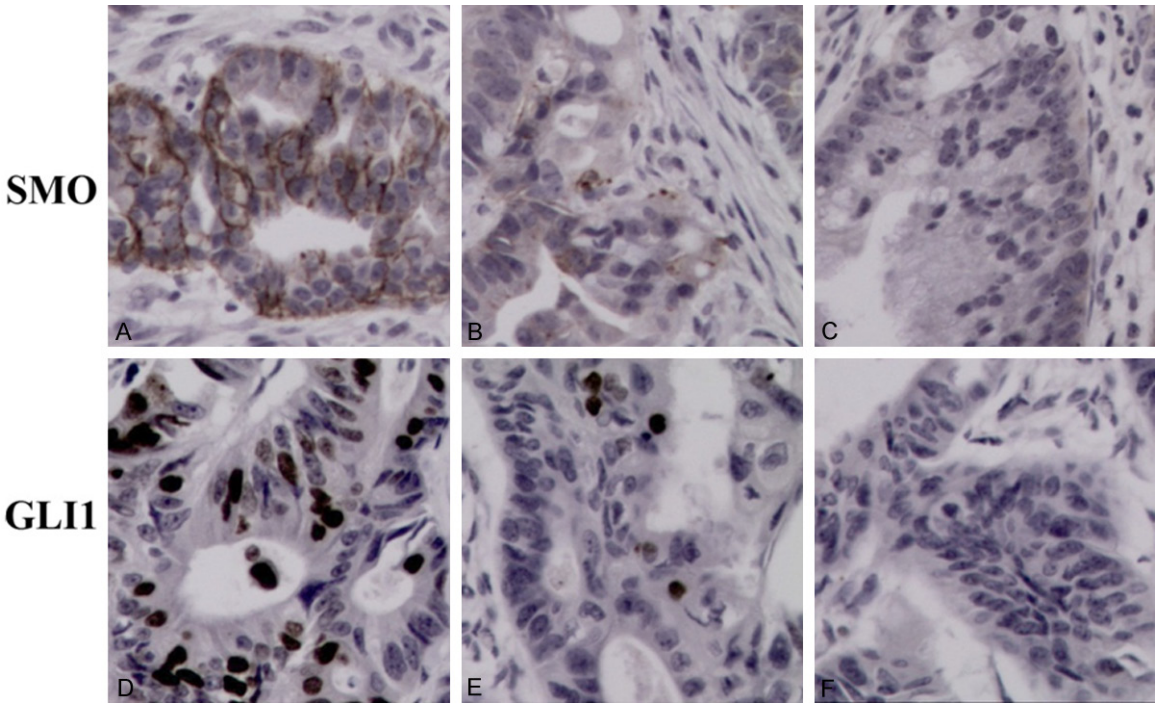

Figure 1. Immunohistochemical expression analysis of SMO and GLI1 proteins in colon tumor, colonic adenoma and normal colon tissues (SP, 200 ×). A and D. Colon cancer tissues, SMO and GLI1 positive +++, the staining is strong. B and E. Colonic adenoma tissues, SMO and GLI1 positive +, the staining is weak. C and F. Normal colon tissues, SMO and GLI1 negative.

sists of three members, i.e. Sonic HH, Desert HH and Indian HH, which can bind to transmembrane receptor, leading to the release of smoothened (SMO) and further activating the downstream glioma-associated oncogene homolog (GLI) [10]. Following that, the activated GLI family members (including GLI1, GLI2 and GLI3) migrate to cells to bind and activate HH target genes involved in various cell functions and cell differentiation [11]. Thus, GLI family members are important for HH signaling activation and function as mediators of the transcriptional regulation in cancer cells. As previously reported, the inhibition of GLI induces DNA damage and extensive cell death in human colon carcinoma cells [12]. Therefore, the HH signaling pathway members may be regarded as important therapeutic targets for the clinical treatment of colon cancer.

Although researchers have demonstrated the aberrant activation of HH signaling in colon cancer, it still needs further confirmation for the disagreements in the roles of SMO and GLI1 in coloniccancer tumorigenesis [13]. As far as we know, the studies of the HH pathway related components (SMO and GLI1) expressed in tissues at three different stages, including the colon cancer, colonic adenoma and normal colon tissues, remain few. In the present study, the colon cancer, colonic adenoma and noncancerous tissue samples were collected and examined for the detection of histopathological changes and expression levels of SMO and GLI1, in an attempt to elucidatetheir possible role in the colon cancer progression.

Materials and methods

Patients and tissue samples collection

Colon cancer and non-canceroustissuesamples were collected from 49 patients diagnosed with colon cancer who underwent surgical operation at the General Surgery department of Huzhou First People's Hospital from September 2012 to September 2013. The colon cancer tissues were taken from the nonnecrosis tissues in the center of colon carcinoma, and the non-cancer normal tissues were taken more than 5 cm away from malignant tumors macroscopically. All the colon cancer

Table 1. Comparisons of the positive rates of SMO and GLI1 protein expression in colon cancer, colonic adenoma and normal colon tissues

Groups	Patients (n)	SMO positive rate	*P*-value	GLI1 positive rate	*P*-value
Colon cancer	49	30 (61.2%)	0.072[a]	21 (42.9%)	0.334[a]
Colonic adenoma	34	14 (41.2%)	0.022[b]	11 (32.4%)	0.012[b]
Normal colon	49	9 (18.3%)	< 0.001[c]	5 (10.2%)	< 0.001[c]

Data are presented as n (%). "a" represents Colon cancer vs. Colonic adenoma, "b" represents Colonic adenoma vs. Normal colon, and "c" represents Colon cancer vs. Normal colon.

Table 2. Relationship of clinicopathological characteristics of patients with colon cancer and positive rates of SMO and GLI1

Items	Patients (n)	SMO positive rate	*P*-value	GLI1 positive rate	*P*-value
Age (years)					
≥ 60	31	20 (64.5%)	0.545	14 (45.1%)	0.427
< 60	18	10 (55.6%)		6 (33.3%)	
Gender					
Male	29	18 (62.1%)	0.887	13 (44.8%)	0.502
Female	20	12 (60.0%)		7 (35.0%)	
Differentiation					
Moderate	40	22 (55.0%)	0.061	17 (42.5%)	0.622
Poor	9	8 (88.9%)		3 (33.3%)	
Dukes stage					
A	7	5 (71.4%)	0.461	3 (42.9%)	0.627
B	13	10 (76.9%)		6 (46.2%)	
C	16	14 (87.5%)		10 (62.5%)	
D	3	2 (66.7%)		1 (33.3%)	

patients received no chemotherapy, radiotherapy and/or immunotherapy before surgical operation. Besides, colonic adenoma tissue samples were obtained from 34 patients diagnosed with colonic adenoma who underwent endoscopic excision at the Digestive Department of Huzhou First People's Hospital from September 2012 to September 2013. Those colon cancer and colonic adenoma tissue specimens were respectively fixed with formalin (10%) and formaldehyde (40 g/L) after separation. The study protocol was approved by the ethics committee of Huzhou First People's Hospital. Written informed consent was obtained from all the participants.

Immunohistochemistry

Envision technique was applied for immunostaining. In brief, 2 μm sections were backed at 65°C for 3 h and then subjected to deparaffinage in xylene for twice and rehydration through graded ethanol. Following, the sections were incubated with 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase and with 0.3% Triton X100 for 30 min to increase cell permeability. Thereafter, sections were incubated with normal serum to block nonspecific binding and then incubated with SMO monoclonal antibody (1:150, Santa Cruz, CA) and GLI1 monoclonal antibody (1:150, Santa Cruz, CA) overnight at 4°C. After washing, the tissue sections were incubated with EnVisionTM secondary antibody (Shanghai Changdao Biotech Corporation, China) for 30 min at room temperature. Finally, the sections were added with diaminobenzidine (DAB) for visualization after washing with PBS. The expression of SMO and GLI1 was evaluated by positive cell ratio and staining intensity using the Immuno-Reactive-Score (IRS) system. The positive cell ratio was graded on a scale of0 -3: 0 = 0-10%; 1 = 10-25%; 2 = 25-50% and 3 = ≥ 50%. The staining intensity was scored accord-

ing to the following criteria: 0 (-, no staining); 1 (+, weak staining); 2 (++, moderate staining) and 3 (+++, strong staining). The total score was calculated according to the sum of the scores for the staining intensity and positive cell ratio. The positive rate was defined as the numbers of cases with IRS score more than 0, divided in each by the total number of cases. The IRS scores were determined by a pathologist blinded to the study program.

Statistical analysis

The data were analyzed using the statistical software package SPSS 15.0 (SPSS Inc., Chicago, IL, USA). The difference among the three groups was calculated and analyzed by Kruskal-Wallis H test. The individual comparison of the positive protein expression rate (SMO and GLI1) between groups was analyzed by Chi square test. Chi square test was also used to investigate the correlation between positive protein expression rate and clinical characteristics of patients. $P < 0.05$ was considered to be significantly different.

Results

Of all the colon cancer patients, the mean age was 61.9 ± 7.3 years old (range: 48-76 years old) and there were 32 males and 17 females. The histopathological results showed that all the colon cancer patients were diagnosed with colon adenocarcinoma, including 40 moderately differentiated and 9 poorly differentiated. In the terms of Dukes stage, there were 6 cases in Dukes A stage, 19 in B stage, 21 in C stage, and 3 in D stage. For the 34 colon adenoma patients, the mean age was 45.9 ± 9.9 years old (range: 25-65 years old) and there were 22 males and 12 females.

Immunohistochemistry staining was employed to observe the protein expression frequency of SMO and GLI1 in colon cancer, colonic adenoma and normal tissues. The SMO protein was mainly expressed on the cell membrane, while GLI1 protein was mainly in the cytoplasm (**Figure 1**). In the normal colon tissues, SMO and GLI1 protein expressions were less abundant than that in the colon cancer and colonic adenoma tissues. In the colon cancer and colonic adenoma tissues, SMO and GLI1 expressions were present in different staining intensities and cell distributions. In the 49 colon cancer samples, SMO and GLI1 positive expressions were detected in 30 cases (61.2%) and 21 cases (42.9%), respectively. In the matched 49 normal samples, SMO and GLI1 positive expressions were found in 9 cases (18.3%) and 5 cases (10.2%), respectively. In the 34 colonic adenoma samples, SMO and GLI1 positive expressions were observed in 14 cases (41.2%) and 5 cases (32.4%), respectively (**Table 1**). Besides, it was found that the positive rates of SMO and GLI1 expression in the colon cancer group was higher than that in the colonic adenoma group, but with no significant difference ($P = 0.072$ and $P = 0.334$, respectively). In contrast, there was statistically significant difference in the positive rates of SMO and GLI1 expression between the colonic adenoma samples and normal colon tissues ($P = 0.022$ and $P = 0.012$, respectively). The same results were also found in the expressions of SMO and GLI1 between the colon can certissues and normal colon tissues (Both $P < 0.001$). Further statistical results showed that there were statistically significant differences in the expressions of SMO and GLI1 among the three groups ($P < 0.001$ and $P = 0.001$, respectively). The results indicated that the SMO and GLI1 expressions possibly increased gradually from the normal colon to colonic adenoma to the colon cancer. Therefore, the above results suggested that SMO and GLI1 may play significant roles in the development of colon cancer.

Furthermore, we also recorded the clinical characteristics of patients with colon cancer and investigated the relationship between positive protein expression (SMO and GLI1) and clinical characteristics of colon cancer patients. As shown in **Table 2**, there were no significant correlations between the expressions of SMO and GLI1 and clinicopathological parameters, such as age ($P = 0.545$ and $P = 0.427$, respectively), gender ($P = 0.887$ and $P = 0.502$, respectively), differentiation ($P = 0.061$ and $P = 0.622$, respectively) and Dukes stage ($P = 0.461$ and $P = 0.627$, respectively).

Discussions

As previously demonstrated, abnormal activation of HH signaling pathway exerts a promotion effect on the tumorigenesis of colorectal cancer [14]. However, some researchers hold the opposite idea that aberrant activation of HH

signaling is not involved in the pathogenesis of colorectal cancer [15]. Thus, controversial views on the possible roles of HH signaling in colorectal tumor genesis have been concluded from various studies [13, 16, 17]. In the present study, we collected colon cancer and non-cancer tissues from 49 patients with colon cancer, and colonic adenoma tissue samples from 34 patients with colonic adenoma. IHC was used to evaluate the protein expressions of SMO and GLI1 in these three different kinds of tissues. It was found that SMO and GLI1 expressions may increase gradually from the normal colon to colonic adenoma to the colon cancer.

The HH signaling pathway consists of three important components, namely, PTCH (patched) and SMO, and GLI transcription factor [18]. The important transmembrane protein SMO can be derepressed and released by the binding of HH ligands to its receptor PTCH, activating the downstream transcription factor GLI and thus leading to HH signaling activation [18]. It has been reported that the protein expression of SMO is dramatically increased in colon cancer tissues relative to the normal colon tissues and its expression is positively associated with lymph node metastasis and cancer stages [16]. Based on the observed results, they concluded that the abnormal activation of SMO makes a contribution to colon cancer progression and SMO expression level could be suggested as an independent biomarker for postoperative metastasis to liver. Consistently, many other studies also confirm the up-regulation of SMO in colon cancer [19]. In accordance with the above results, we observed a dramatic increase in SMO positive expression rate in colon cancer and colonic adenoma comparing with that in normal tissues, which may be derepressed and released from PTCH and thus activate the downstream transcription factor GLI1 to enhance HH signaling pathway in colon cancer, suggesting that SMO may also play a significant role in the evolution of colon cancer and may act as a biomarker to distinguish colon cancer, colonic adenoma and normal tissues.

On the other hand, the expression changes of GLI1 were also determined among colon cancer, colonic adenoma and normal tissues in the present study. GLI protein, as downstream of SMO, acts as important molecular switch to disrupt the HH signaling in colon cancer, whose inhibition can result in terminating HH-dependent survival of cancer cells [20]. Evidence has reported that the GLI1 could particularly modulate the biological behaviors of cancer stem cells, and tumor progression [21, 22]. Ding *et al*. have reported that GLI1 is remarkably up-regulated in colon tissue and closely associated with lymph node metastasis and thus can be used as an indicator of colon cancer progression [23]. The differential expression of GLI1 can lead to HH signaling activation, which exerts anti-inflammatory and anti-apoptotic effects on cancer cells [16]. Targeting GLI using small molecule inhibitor is important to induce colon cancer cell deaths via regulating DNA damage or repair pathways [12]. In line with these findings, our results also showed that GLI1 trended to be expressed in colon cancer tissue than in colonic adenoma and normal tissue, implying that GLI1 also plays a key role in the development of colon cancer.

However, there are several limitations in the present study. We did not follow up the patients to evaluate their prognosis with the expression levels of SMO and GLI1. In addition, the sample size was not large enough. Of note, the present results just provided some new information to the roles of HH signaling in the progression of colon cancer. More clinical studies with large population will be needed in the future to demonstrate the clinical significance of SMO and GLI1 in the evolution of colonic carcinogenesis.

To sum up, our results indicated that the protein expressions of SMO and GLI1 possibly increased gradually from the normal colon to colonic adenoma to the colon cancer. It is therefore could be speculated that the over-expressed SMO and GLI1 may be suggested as diagnostic marker to distinguish colon cancerous, precancerous and normal tissues, as well as the therapeutic targets in the treatment of colon cancer.

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Disclosure of conflict of interest

None.

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