RESEARCH ARTICLE

Expression of Smoothened Protein in Colon Cancer and its Prognostic Value for Postoperative Liver Metastasis

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Abstract

Backgrouds: The hedgehog (Hh) signaling pathway is composed of patched (PTCH) and smoothened (SMO), two transmembrane proteins, and downstream glioma-associated oncogene homolog (Gli) transcription factors. Hh signaling plays a pathological role in the occurrence and development of various cancers. Methods: To investigate the expression of SMO protein in colon cancer and its association with clinicopathological parameters and postoperative liver metastasis, immunohistochemistry was performed with paraffin-embedded specimens of 96 cases. Relationships between SMO protein expression and clinicopathological parameters, postoperative liver metastasis were analyzed. Results: IHC examination showed that SMO protein expression was significantly increased in colon cancer tissues compared to normal colon tissues (P = 0.042), positively related to lymph node metastases (P = 0.018) and higher T stages (P = 0.026). Postoperative live metastasis-free survival was significantly longer in the low SMO expression group than in those with high SMO expression (48.7±8.02 months vs 28.0±6.86 months, P=0.036). Multivariate analysis showed SMO expression level to be an independent prognostic factor for postoperative live metastasis-free survival (95% confidence interval [CI] =1.46-2.82, P = 0.008). Conclusions: Our results suggest that in patients with colon cancer, the SMO expression level is an independent biomarker for postoperative liver metastasis, and SMO might play an important role in colon cancer progression.

Keywords: Colon neoplasm - smoothened gene - postoperative liver metastasis

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Introduction

Colon cancer is one of the most common cancers in the world and has a high propensity for liver metastasis (Ochiai et al., 2008; Jemal et al., 2010; Yassin et al., 2010; Soydinc et al., 2011). The major cause of death from colon cancer is liver metastasis, which is usually resistant to conventional therapies (Fidler, 1990). 35%-55% of patients with colon cancer will develop liver metastases during the course of illness. Survival following hepatic resection of colorectal metastasis now approaches 35%-50%. However, the recurrence rate in 5 years is as high as approximately 65% (Mayo and Pawlik, 2009). Early treatment targeting colon cancer liver metastatic foci might be important for improving patient survival. To date, the precise mechanisms leading to liver metastasis in colon cancer remains unknown, and biomarkers for liver metastasis are still lacking.

The hedgehog (Hh) signaling pathway is known to play essential roles in multiple aspects of embryonic development. Hh protein binds to its receptor human patched 1 homologue (PTCH1), and relieves PTCH1's inhibition on smoothened (SMO), allowing SMO to signal downstream to glioma-associated oncogene homolog (Gli) transcriptional factors, which activates the target genes via specific genomic DNA sequences (TGGGTGGTC) (Kinzler and Vogelstein, 1990; Sasaki et al., 1997). Dysregulation in Hh signaling has been implicated in the pathogenesis of a variety of human tumors including tumors of skin, cerebellum, muscle, lung, digestive tract, pancreas and prostate (Johnson et al., 1996; Cowan et al., 1997; Berman et al., 2003; Watkins et al., 2003; Thayer et al., 2003; Karhadkar et al., 2004). Furthermore, the activation of the Hh signaling pathway is associated with the malignancy of tumors and their progression to metastatic stages (Stecca et al., 2007; Yoo et al., 2008).

We demonstrated previously that expression of glioma-associated oncogene homolog 1 (Gli-1) protein in colon cancer tissues plays a key role in the occurrence and development of colon cancer, and high Gli-1 expression may promote postoperative liver metastasis of colon cancer (Ding et al., 2012). The main aim of the present study was to explore the potential role of SMO protein in colon cancer development. For this purpose, SMO protein expression was detected in colon cancer and normal colon tissues by immunohistochemistry using a tissue microarray approach. Additionally, the correlations of SMO protein expression with the clinicopathological data obtained and prognostic variables examined were explored.

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Materials and Methods

Patients and Tissue samples

Tissue samples were collected from patients with colon cancer who underwent surgical operation in the Second Hospital of Shandong University from July 2002 to December 2007. Formalin-fixed and paraffin-embedded tissue specimens from 96 patients with colon cancer were included. All patients received no chemotherapy and/or other therapies before surgical operation. Table 1 presents the clinicopathological data of the 96 patients. All hematoxylin and eosin (H&E)-stained slides for each patient were reviewed by two pathologists. Clinical and pathological classification and staging were determined according to the 7th American Joint Committee on Cancer criteria. The study protocol was approved by the ethnics committee of Shandong University. Written informed consent was obtained from each participant before data collection.

Tissue microarray (TMA)

The TMA was constructed from tissue cores obtained from formalin-fixed, paraffin embedded tissue blocks. For each H&E-stained slide, two representative areas were selected and the corresponding spots were marked on the surface of the paraffin block. Using a tissue microarray punching instrument, the selected areas were punched out and were placed into the recipient block side by side. Each tissue core was 2 mm in diameter and was assigned with a unique tissue microarray location number that was linked to a database containing other clinicopathological data. TMA sections were then cut from the block in preparation for immunohistochemistry experiments.

Immunohistochemistry (IHC)

The expression of SMO was detected by streptavidin-peroxidase-biotin (SP) immunohistochemical method according to the manufacturer's instructions (Tan et al., 2011; Ding et al., 2012). In brief, paraffin-embedded specimens were cut into 4 μm sections and baked at 60 °C for 60 min. The sections were deparaffinized with xylenes and rehydrated. Then sections were submerged into EDTA antigenic retrieval buffer in a pressure cooker for 10 minutes and then cooled at room temperature for 20 minutes. The sections were treated with 3% hydrogen peroxide in methanol to quench the endogenous peroxidase activity, followed by incubation with normal serum to block nonspecific binding. The sections were incubated with SMO monoclonal antibody (1:50; Santa Cruz; sc-166685, CA) overnight at 4 °C. After washing, the tissue sections were incubated with biotinylated secondary antibody (Maixin Biotechnology Company, Fuzhou, China) for 1h at room temperature, followed by incubation with streptavidin-horseradish peroxidase for 20 minutes. After washing with PBS, diaminobenzidine (DAB) was added for visualization. The sections were counterstained with haematoxylin. For negative controls, the anti-SMO antibody was replaced with PBS.

Evaluation of Immunohistochemical Staining

The stained slides were reviewed and scored independently by two observers blinded to the patients' information. SMO protein expression was evaluated using the Immuno-Reactive-Score (IRS) system (combining positive cell ratio and staining intensity) as suggested by Remmele and Stegner (Remmele and Stegner, 1987; Ding et al., 2012).

Staining intensity was graded according to the following criteria: 0 (-, no staining); 1 (+, weak staining= light yellow); 2 (++, moderate staining = yellow brown) and 3 (+++, strong staining = brown). IRS is the product of staining intensity and the percentage of positively stained cells (graded between 0 and 4, being 1 = <25%, 2 = 25%–50%; 3 = 51%–75%, and 4 = >75%, respectively) (Ding et al., 2012). Cases with discrepancies in IRS score were discussed together with other pathologists until consensus was reached.

Statistical Analysis

Analyses were performed using the statistical software package SPSS 13.0 (SPSS, Chicago, IL, USA). A two-sided Fisher's exact test or X^2 test was performed to analyze possible associations between SMO expression and clinical parameters. The cumulative survival time was computed using the Kaplan-Meier method and compared by the log-rank test. Multivariate analyses were based on the Cox proportional hazards regression model. $P < 0.05$ was considered statistically significant.

Results

SMO protein expression and localization was studied on a large TMA including normal (n = 26) and tumorous colon tissues (n = 96). SMO staining was detectable in both the membrane and the cytoplasm of benign and malignant colon epithelial cells. In the normal colon tissues (Figure 1A), SMO protein expression was less abundant (median IRS =2) than in the colon cancer cells (median IRS = 6) (P = 0.042). In the cells of the colon cancer, SMO expression was present in different intensities and different cell distributions. Following the

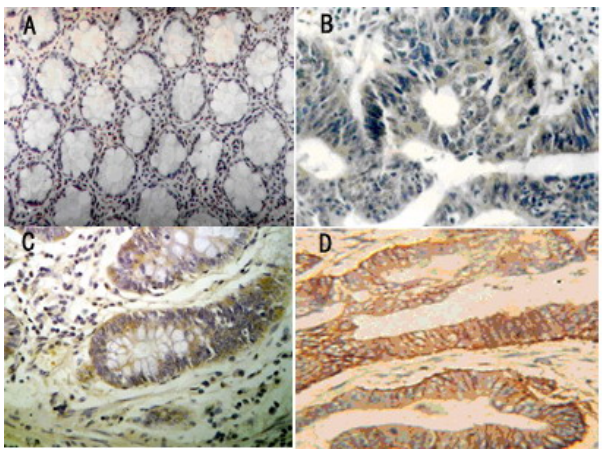

Figure 1. Immunohistochemical Expression Analysis of SMO Protein in Normal Colon Tissue, and Colon Tumors Using A TMA (SP, 200×). (A) normal colon tissue, SMO negative. (B) colon cancer tissue, SMO positive + , the staining is weak. (C) colon cancer tissue, SMO positive ++ , the staining is moderate. (D) Colon cancer tissue, SMO positive +++, the staining is strong

Table 1. Clinicopathological Parameters in Relation to Nuclear SMO Immunoreactivity

Variable	Patients	SMO expression[a]		P value[b]
Categorization	n(%)	high	low	
Gender				
male	60	38	22	
female	36	26	10	0.712
Age				
≥60	50	38	12	
<60	46	26	20	0.37
Tumor size				
≥5cm	42	32	10	
<5cm	54	32	22	0.614
Tumor grade (WHO)				
G1+G2	64	41	23	
G3+G4	32	23	9	0.826
Lymph node status				
N+	51	40	8	
N0	45	24	24	0.018
T stages				
T2	30	15	15	
T3	45	30	15	
T4	21	19	2	0.026

[a]SMO expression, high means IRS≥6 and low means IRS<6; [b]P values were evaluated by chi-square test or the Fisher's exact test

staging criteria of stain intensity, no cases were identified as completely negative, 12 cases (12.5%) were identified as "+" (Figure 1B), 29 cases (30.2%) were identified as "++" (Figure 1C), and 55 cases (57.3%) were identified as "+++" (Figure 1D). We evaluated SMO protein expression in colon cancer cells by determining the IRS, with scores of 1, 2, 3, 4, 6, 8, 9 or 12. The optimal cutoff value for high and low expression level was identified: an IRS score of ≥ 6 was used to define tumors with high expression of SMO, and an IRS score of <6 was used to indicate low expression. The high level (IRS>6), low level (IRSM6) expression of SMO in colon cancer was observed in 64 cases (66.7%) and 32 cases (33.3%), respectively.

There was no significant correlation between the expression level of SMO and biological factors such as patients' age (P=0.370), gender (P=0.712), tumor size (P = 0.614), tumor grade (P = 0.826). In contrast, statistical analyses indicated that SMO expression was positively related to lymph node status, T stages and the correlation was statistically significant (P= 0.018/0.026, respectively). The results of these analyses are summarized in Table 1.

Of the 96 patients with colon cancer, none was lost to follow-up. The median observation period was 38.4 months (ranged, 5.6–60.0 months), with 62 postoperative live metastases. The median postoperative live metastasis-free survival was 30.27±8.99 months. To answer the question whether SMO overexpression might have an impact on patients' clinical outcome. Univariate survival probability curves were calculated based on the immunohistochemical results. Using Kaplan-Meier analysis we found that postoperative live metastasis-free survival in the group of low SMO expression was significantly longer than that of high expression (48.73±2.02 vs 37.98±2.86 months, P=0.036) (Figure 2). Multivariate analysis showed SMO expression status was evaluated as an independent

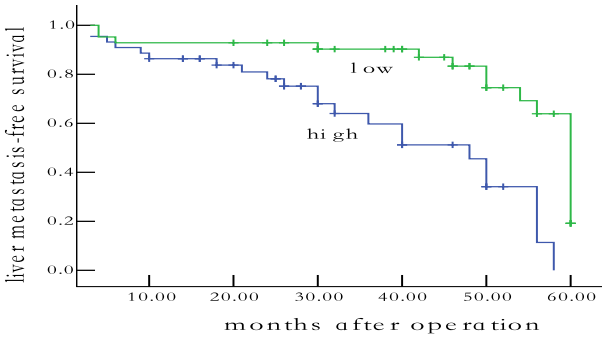

Figure 2. Colon Cancer Patients Expressing High SMO Protein (IRS≥6) Show Unfavourable Prognosis. Univariate Kaplan-Meier analysis was performed on the basis of SMO expression results derived from the TMA. Patients with low SMO expression(IRS<6) displayed longer liver metastasis-free survival estimation (green graph) compared to patients with high SMO expression (blue graph) (P =0.036)

Table 2. Contributing Factors for Postoperative Liver Metastasis

Variable	B	SE	Wald	P value
SMO expression	1.552	0.438	0.216	0.008
T stages	1.55	0.436	0.208	0.01
Lymph node status	1.364	0.384	0.186	0.014

prognostic factor for postoperative live metastasis-free survival (95% confidence interval [CI] =1.46-2.82, P = 0.008, Table 2).

Discussion

The Hh signaling pathway is composed of patched (PTCH) and SMO, two transmembrane proteins, and downstream Gli transcription factors. SMO protein, a key transcriber in Hh signaling pathways, can transport Hh signals to Gli signals, and therefore activate nuclear gene transcription and the Hh signaling pathway (Murone et al., 2000; Ruiz et al., 2002; Oishi and Wang, 2011). Hh signaling pathway is critical to normal mammalian gastrointestinal development. Through epithelial-mesenchymal interactions, Hh signaling ensures appropriate axial patterning of the embryonic gut (Lees et al., 2005). In addition, this pathway has been shown to play a role in homeostasis and differentiation of skin, stomach, and colon in adult tissue (Fukaya et al., 2006). Recent findings have implicated continuous sonic hedgehog signaling activity as playing a pathological role in the growth of various cancers such as oesophageal squamous cell carcinoma, basal cell carcinoma, brain tumor and so on (Ghali et al., 1999; Altaba et al., 2004; Mori et al., 2006).

There has been evidence showing that aberrant expression of SMO protein is associated with the development of some malignant tumors (Yang et al., 2011). For example, the expression of the SMO gene occurs only in prostate cancer tissues but not in benign prostate epithelial cells, targeted inhibition of the SMO gene in controlling activities of the Hh signaling pathway has a critical significance in prostate cancer therapy (Ecke et al., 2008). SMO gene is also over-expressed osteosarcoma cell lines and osteosarcoma biopsy specimens. Inhibition of SMO by cyclopamine, a specific

inhibitor of SMO, slow the growth of osteosarcoma in vitro (Hirotsu et al., 2010). Treatment of malignant pleural mesothelioma-bearing mice with the SMO inhibitor HhAntag leads to a significant inhibition of tumor growth in vivo accompanied by decreased Ki-67 and nuclear YAP immunostaining (Shi et al., 2012). The expression of SMO mRNA is enhanced in the tumor nests of the nodular basal cell carcinoma (BCC), especially at the advancing portions, but is under the detectable level in the superficial BCC cases examined, indicating that smo mRNA expression might be associated with BCC tumor progression (Tojo et al.,1999). Aberrant expression SMO is common in human pancreatic carcinoma tissues and is associated with the low-level differentiation of tumor tissue, which indicat that SMO plays a fundamental role in pancreas tumorigenesis (Shao et al., 2006).The risk of local recurrence of cervical carcinoma after chemoradiation is also associated with the up-regulation of SMO (Chaudary et al., 2012).

In the present study, we examined the SMO expression in paraffin-embedded tumor samples using the IHC method with the monoclonal antibody. Our results showed that SMO is in the membrane and the cytoplasm of cells. High SMO expression (IRS≥6) in the colon cancer tissues was detected in 66.7% of the patients. Furthermore, SMO expression level is correlated with lymph node metastasis and T status, which suggests that SMO may have some correlation with worse biological behavior and clinical aggressiveness of colon cancer.

Cancer metastasis is a complex process involving many genes that function in the tumor cell and at the target organ (Minn et al., 2005; Bos et al., 2009). The molecular mechanism underlying liver metastasis in colon cancer is unclear. Traditional clinicopathological parameters including the depth of invasion, the presence of venous invasion, and lymph node metastasis, have limited prognostic values (Bird et al., 2006). Other variables, such as CD10, CD44, VEGF, TGF-a, P-Cadherin and the density of macrophages in the invasive front have been shown to be correlated with liver metastasis, however the predictive efficacy of these factors remains uncertain (Zhou et al., 2010; Sun et al., 2011; Ding et al., 2012). In the present study, postoperative live metastasis-free survival in the group of low SMO expression was significantly longer than that of high expression. Furthermore, result of multivariate analysis demonstrated that SMO expression was an independent prognostic factor for postoperative live metastasis-free survival. To our knowledge, this is the first report of the relationship between expression of SMO protein and postoperative live metastasis of colon cancer. These findings suggusted that the abberant expression of SMO might contribute to the development of colon cancer and postoperative liver metastasis. Considering together with our previous study on the effect of Gli-1 expression on colon cancer invasion (Ding et al., 2012), we may deduce that the abberant expressed Hh signaling molecules might be targets with the progression of colon carcinoma in advanced stage treated with molecular markers and targeted therapy.

In conclusion, our study demonstrated the expression of SMO protein in normal colon tissues and tumor cells. SMO expression in colon cancer was significantly higher in tumor cells than in normal ones. High SMO protein expression in colon cancer is significantly correlated with lymph node metastasis, T stage, and shorter postoperative liver metastasis-free survival. The results provide evidence that high SMO protein expression may be important in the acquisition of a more invasive phenotype. Furthermore, the expression of SMO protein, as detected by IHC, may be useful as a prognostic biomarker for the poor liver metastasis-free survival of colon cancer patients.

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