

## RESEARCH COMMUNICATION

# Expression and Clinical Significance of Hedgehog Signaling Pathway Related Components in Colorectal Cancer

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### Abstract

**Aim:** To investigate the expression of three components of the Hedgehog (Hh) signaling pathway (SHH, SMO and GLI1) in human colorectal cancer (CRC) tissues and evaluate their association with clinicopathologic characteristics of the patients. **Methods:** Fresh tumor tissues and matched tissues adjacent to the tumor were collected from 43 CRC patients undergoing surgery. Normal colorectal tissues from 20 non-CRC cases were also sampled as normal controls. The expression of SHH, SMO, GLI1 mRNAs was assessed by RT-PCR and proteins were detected by immunohistochemical staining. Associations with clinicopathological characteristics of patients were analyzed. **Results:** SHH mRNA was expressed more frequently in tumor tissues than in normal tissues, but the difference did not reach significance in comparison to that in the adjacent tissues. SMO and GLI1 mRNAs were expressed more frequently in tumor tissues than in both adjacent and normal tissues. The expression intensities of SHH, SMO, GLI1 mRNA in tumor tissues were significantly higher than those in adjacent tissues and normal tissues. Proteins were also detected more frequently in tumors than other tissues. No significant links were apparent with gender, age, location, degree of infiltration or Dukes stage. **Conclusion:** Positive rates and intensities of mRNA and protein expression of Hh signaling pathway related genes SHH, SMO, GLI1 were found to be significantly increased in CRC tissues. However, over-expression did not appear to be associated with particular clinicopathological characteristics.

**Keywords:** Gene - hedgehog signaling pathway - colorectal cancer - clinicopathological variables

*Asian Pacific J Cancer Prev*, 13, 2319-2324

### Introduction

Colorectal cancer (CRC) is a common cancer ranking as the fourth most frequent causes of cancer death in the world (Jemal et al., 2008; Kim et al., 2012; Kimman et al., 2012; Park et al., 2012). In order to predict the prognosis and improve the treatment of CRC, there is a great need to understand the molecular mechanisms leading to tumor formation and progression.

The Hedgehog (Hh) signaling pathway, which regulates proliferation, angiogenesis, matrix remodeling and stem-cell renewal, plays an important role during embryogenesis and in adult life. It is involved in the patterning in a wide variety of the organs including gastrointestinal tract (McMahon et al., 2003; Dessaud et al., 2008). By contrast, deregulation of the Hh pathway is involved in tumor development, as the mutations of several components of this pathway were found in patients with many types of cancers including CRC (Evangelista et al., 2006; Scales et al., 2009). In addition, recent researches have also indicated that tumorigenesis of Hh signaling might be through the molecular pathways of

cancer stem cell (CSC) (Varnat et al., 2009). There are three members in human Hh family i.e. Sonic HH (SHH), Indian HH (IHH), and Desert HH (DHH) (Echelard et al., 1993; Marigo et al., 1995). All these ligands bind to a 12-transmembrane receptor called Patched (PTCH) that restricts Hh signaling by inhibiting a 7-transmembrane receptor called smoothed (SMO) (Bijlsma et al., 2006). SMO releasing leads to stimulation of the downstream Hh signaling cascade, which culminates in the activation of the glioma-associated oncogene homolog (GLI) family of transcription factors. The forms of GLI family, which included GLI1, GLI2 and GLI3, migrate to the cells to bind HH target genes (McMahon et al., 2003; Evangelista et al., 2006; Varjosalo et al., 2008).

On the field of Hh signaling pathway in tumorigenesis, SHH, SMO and GLI1 have been studied frequently. However, most studies focused on the cancers of other organs (Steg et al., 2012; Yang et al., 2012). The data about the roles they play on CRC have not been well documented. Some of them even remained controversial (Berman et al., 2003; Douard et al., 2006; Monzo et al., 2006; Chatel et al., 2007; Varnat et al., 2009;

**Table 1. Sequences of Primer Pairs**

Genes		Primers (5'→3')	Size (bp)	GeneBank
SHH	F	GCTGATGACTCAGAGGTGTAAGGA	280	NM_000193
	R	CCACCGAGTTCTCTGCTTCA		
SMO	F	CTGGTGTGGTTTGGTTTGTG	366	NM_005631
	R	TGGTCTCGTTGATCTTGCTG		
GLI1	F	CGGGTCTCAAACCTGCCAGCTT	387	NM_001160045
	R	GGCTGGGTCCTGACCCTC		
GAPDH	F	TGGTCTCTCTGACTTCAAC	222	NM_002046
	R	GTGAGGGTCTCTCTTCT		

Yoshikawa et al., 2009; Fu et al., 2010; Saqui-Salces and Merchant, 2010; You et al., 2010; Tapati et al., 2011). There were disagreements in the roles of downstream Hh signaling components i.e. SMO and GLI1 play in tumorigenesis of CRC and the possible association of Hh pathway activation with patient's clinicopathological characteristics, i.e. tumor stage and differentiation. In the present study, we simultaneously investigate the mRNA and protein expressions of SHH, SMO, GLI1 related genes in human CRC tissues and evaluated their association with the clinicopathologic characteristics of the patients.

## Materials and Methods

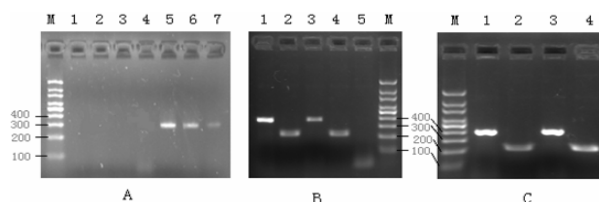
### Patients and specimens

This study was conducted in accordance with the declaration of Helsinki. The study protocols were approved by the ethics committee of Guangzhou First Municipal People's Hospital Affiliated to Guangzhou Medical College. Written consent was obtained from each patient. Consecutive patients (43 cases) with pathologically confirmed colorectal adenocarcinomas, who underwent surgical operations at Department of Gastroenterological Surgery of this hospital between December 1, 2008 and April 30, 2009, were included. None of them received chemotherapy or radiation therapy before surgery. Matched non-CRC patients (20 cases) undergoing surgical operations during the same period of time were also collected as controls.

Fresh tumor tissues and matched mucosa adjacent to the cancer (10 cm beyond the cancer margin) from 43 patients were collected. Normal colorectal tissues from 20 non-CRC patients undergoing surgical operations were also collected as normal controls. Two specimens from each site obtained at surgery resections were immediately frozen in liquid nitrogen and stored at -80 °C until total RNA extraction. An additional sample was fixed in formaldehyde saline and embedded in paraffin for pathological examination. The Dukes pathologic staging system was applied to classify the patients. Dukes' A: lesions contiguous with the bowel wall but not penetrating the muscularis. Dukes' B: lesions penetrating the muscularis into the surrounding fat or adventitia, Dukes' C: lesions with positive lymph node involvement.

### Total RNA extraction and quantitative real-time RT-PCR

Total RNA was extracted by chloroform-isopropanol-ethanol method using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The A260/A280 ratio of RNA determined with colorimetry was between

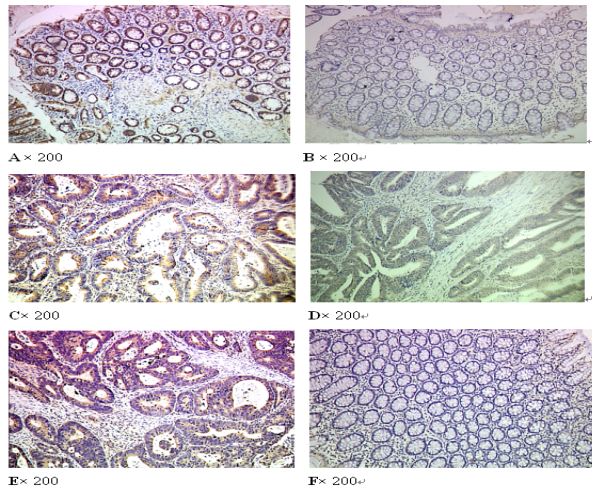


**Figure 1. Gel Electrophoresis of SHH, SMO and GLI1 mRNA Expressions.** A: M: marker; 1-2: bland control; 3-7: SHH (280bp). B: M: marker; 1, 3: SMO (366bp); 2, 4: GAPDH (222bp); 5: bland control. C: M: marker; 1, 3: GLI1 (387bp); 2, 4: GAPDH (222bp)

1.80~2.00. RNA integrity was qualified with formaldehyde degeneration gel electrophoresis at 80V for 15 min. Three electrophoresis bands (5S, 18S, 28S) were visualized. Complementary DNA (cDNA) was synthesized with the instructions on M-MLV reverse transcriptase kit. Briefly, 2 µg of total RNA, 0.5 µg oligo (dT) 15 primer and 12 µl DEPC-treated H<sub>2</sub>O in a final 15 µl reaction volume were incubated at 70 °C for 5 min and subsequently on ice for 5 min to synthesize RNA- primer compound. Then, 5 µl M-MLV buffer, 1.25 µl dNTP, 0.625 µl RNasin, 1 µl M-MLV reverse transcriptase and 2.125 µl DEPC-treated H<sub>2</sub>O constituted RT- reaction mixture. Afterward, 15 µl RNA- primer compound and 10 µl RT- reaction mixture were blended, and incubated at 37 °C for 60 min and then at 70 °C for 15 min to synthesize cDNA.

Three components of the Hedgehog signaling pathway, SHH, SMO and GLI1, were selected for quantitative real-time RT-PCR analysis. The reference sequences for each gene were obtained from Genebank on the NCBI Entrez web site (<http://www.ncbi.nlm.nih.gov/genome/guide/human>). Forward and reverse primer pairs were designed using Primer Premier 5.0 for Windows (PREMIER Biosoft International, Palo Alto, CA, USA) and synthesized by Invitrogen Inc., Shanghai, China, which were listed in Table 1.

Real-time PCR was carried out on a Bio-Rad gradient PCR with electrophoresis apparatus. The reactions included 1 µL cDNA (1:10 dilution), 1ul each primer, 2×PCR master mix 10ul and ddH<sub>2</sub>O 7ul. The final reaction volume was 20 µL. PCR was performed under the following cycling conditions: an initial denaturation step at 95°C for 4 min, followed by 35 cycles of amplification (for SHH and SMO cDNA, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; for GLI1 cDNA, 95 °C for 30 s, 66 °C for 30 s, and 72 °C for 30 s), then elongation at 72 °C for 5 min. Every PCR reaction of each gene and each sample was done in duplicate. PCR products were detected with 2% agarose gel electrophoresis and quantitated using an agarose gel formatter (Figure 1). The mRNA positive rates were the percentage of the numbers appearing electrophoretic straps



**Figure 2. SHH, SMO, GLI1 Protein Expressions (Immunostaining  $\times 200$ ).** A SHH (+) in cancer, B SHH (-) in normal control; C SMO (+) in cancer, D SMO (-) in normal control; E GLI1 (+) in cancer, F GLI1 (-) in normal control

**Table 2. Expressions of SHH mRNA and Protein in CRC, Adjacent Tissues and Normal Colorectal Tissues**

	n	Protein positive			mRNA positive			Intensity
		Rate (%)	-	$\pm$	+	++	Rate (%)	
CRC	43	11(25.6)	12	20	6	5	12(27.9)	0.72 $\pm$ 0.19
adjacent	43	6(15.0)	20	17	5	1	8(18.6)	0.56 $\pm$ 0.11
normal	20	0(0)	17	3	0	0	0(0)	0
P		0.03		0.01		0.03	0.03	0.03

**Table 3. Expressions of SMO mRNA and Protein in CRC, Adjacent Tissues and Normal Colorectal Tissues**

	n	Protein positive			mRNA positive			Intensity
		Rate (%)	-	$\pm$	+	++	Rate (%)	
CRC	43	26(60.5)	5	12	11	15	36(83.7)	0.88 $\pm$ 0.11
adjacent	43	12(27.9)	22	9	6	6	25(58.1)	0.72 $\pm$ 0.22
normal	20	0(0)	18	2	0	0	0(0)	0
P		0.00		0.02		0.00	0.00	0.00

divided by the total numbers of cases.

#### Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were used in the study. Sections were serially cut at 4 $\mu$ m. Envision technique was used for immunostaining. Briefly, Sections were deparaffinized in xylene and rehydrated through graded ethanol, followed by incubation at 60 °C for 50 min in 3% hydrogen peroxide to block endogenous peroxidase. A casein serum-free blocking solution specific for formalin-fixed tissues was applied for 20 min and sections were incubated overnight at 4 °C with primary antibodies.

Primary antibodies were used at a dilution ratio of 1:50 for SHH, 1:50 for SMO, 1:100 for GLI1 (Anti-SHH and Anti-GLI1s were purchased from Santa Cruz Corp. USA, Anti-SMO antibody was purchased from Abcam Corp. UK). Slides were given three 5-min washes in PBS and incubated with secondary antibodies at 37 °C for 30 min in PV-6001 Complex (SMO, GLI1, purchased from Jinqiao Corp. Beijing, China) or in PV-9003 Complex

(SHH, purchased from Jinqiao Corp. Beijing, China). The antibody complex was visualized with diaminobenzidine. Sections were counterstained with HE staining.

Negative controls (absence of primary antibody) were included for each antibody and analyzed for each case (Figure 2). The intensity of staining in the cytoplasm was scored arbitrarily to reflect negative (-), light ( $\pm$ ), moderate (+), and strong, (++) respectively. The scores were determined by a pathologist blinded to the study program. The positive rates were calculated as the numbers of (+) plus (++) cases divided in each by the total numbers of cases.

#### Statistical analysis

The data were examined with the SPSS 13.0 for Windows statistical package (Chicago, IL, USA). Continuous data were expressed as mean  $\pm$ SD and examined by Student's t-test. Categorical variables were expressed as percentage and examined by  $\chi^2$  test. Pearson test (two-tailed) was used to compare the variables among groups.

ANOVA analysis was applied for multiple comparisons. A p value  $\leq 0.05$  was considered statistically significant.

#### Results

Overall 43 patients with CRC (males 24, females 19) with the mean age of 66.4 $\pm$ 10.5 years (range, 36-82 years) were enrolled in study group. In non-CRC control group, there were 20 patients (males 15, females 5, mean age 61.5 $\pm$ 16.4 years). Histopathologically, all of them were adenocarcinomas with 38 being moderately differentiated and 5 poor differentiated. For Dukes staging, Dukes' A included 7 cases, Dukes' B 14 cases, Dukes' C 18 cases and Dukes' C 4 cases.

The positive rate of SHH mRNA expressions in tumor tissues was significantly higher than that in normal colorectal tissues ( $P < 0.05$ ), but did not reach significance in comparison to that in the adjacent tissues ( $P > 0.05$ ) (Table 2). The positive rates of SMO and GLI1 mRNA expressions in tumor tissues were significantly higher than those in the adjacent tissues ( $P < 0.05$ ) and those in normal colorectal tissues ( $P < 0.05$ ). The expression levels of SHH, SMO, GLI1 mRNA in tumor tissues were significantly higher than those in the adjacent tissues ( $P < 0.05$ ) and normal colorectal tissues ( $P < 0.05$ ) (Table 2-4).

Immunohistochemical staining demonstrated that SHH, SMO, GLI1 proteins were mainly expressed in the cytoplasm of tumor cells and normal gland cells. The positive rates and expression intensities of SHH, SMO, GLI1 proteins in tumor tissues were significantly higher than those in the adjacent tissues and those in normal colorectal tissues ( $P < 0.05$ ) (Table 2).

There were no significant relationships among the SHH, SMO and GLI1 mRNA and protein expressions in tumor tissues ( $r = 0.191-0.274$ ,  $P > 0.05$ ) (Tables 5, 6). Furthermore, there were no apparent links found between SHH/SMO/GLI1 expression and any of the clinicopathological characteristics of the cancer patients ( $P > 0.05$ ) (Tables 7, 8).

**Table 4. Expression of GLI1 mRNA and Protein in CRC, Adjacent and Normal Colorectal Tissues**

	n	Protein positive			mRNA positive			Intensity
		Rate (%)	-	±	+	++	Rate (%)	
CRC	43	13(30.2)	21	9	10	3	15(34.9)	0.88±0.14
adjacent	43	5(11.6)	34	4	5	0	7(16.3)	0.64±0.19
normal	20	0(0)	18	2	0	0	0(0)	0
P		0.01		0.01		0.00	0.00	0.00

**Table 5. Relationship among SHH, SMO and GLI1 mRNA Expression in CRC tissues**

	n	Both (+)	Both (-)	r	p
SMO/GLI1	43	14	6	0.191	0.211
SHH /SMO	43	12	7	0.274	0.075
SHH /GLI1	43	6	22	0.197	0.205

**Table 6. Relationship among SHH, SMO and GLI1 Protein expressions in CRC tissues**

	n	Both (+)	Both (-)	r	p
SMO/GLI1	43	9	4	0.191	0.211
SHH /SMO	43	14	2	0.274	0.075
SHH /GLI1	43	4	6	0.197	0.205

**Table 8. Relationship of SHH, SMO, GLI1 Protein Expression Intensities in CRC Tissues to Clinicopathologic Characteristics of Patients**

	n	SHH				P	SMO				P	GLI1				P
		-	±	+	++		-	±	+	++		-	±	+	++	
Gender																
male	24	7	11	5	1	0.67	2	7	6	9	0.6	10	7	5	2	0.5
female	19	5	9	1	4		3	5	5	6		11	2	5	1	
Age (yrs)																
<60																
≥60	10	2	6	1	1	0.89	0	4	4	2	0.76	5	2	3	0	0.84
Location	33	10	14	5	4		5	8	7	13		16	7	7	3	
colon rectum	27	6	16	4	1	0.78	4	9	6	8	0.17	15	5	4	3	0.4
Differentiation	16	6	4	2	4		1	3	5	7		6	4	6	0	
moderate poor																
Infiltration	38	11	16	5	3	0.84	5	11	8	14	0.8	18	8	9	3	0.53
T1+T2	5	1	4	1	2		0	1	3	1		3	1	1	0	
T3+T4																
Dukes stage	21	4	10	3	4	0.17	4	2	6	9	0.38	11	4	4	2	0.82
A	22	8	10	3	1		1	10	5	6		10	5	6	1	
B																
C	7	2	3	0	2	0.3	3	0	1	3	0.38	5	0	2	0	0.5
D	14	2	7	3	2		1	2	5	6		6	4	2	2	
	18	5	10	2	1		1	9	4	4		9	4	5	0	
	4	3	0	1	0		0	1	1	2		1	1	1	1	

**Discussion**

Hh signalling pathway has been shown to be an important promoter of normal colonic cell growth. Mutational activation of Hh signalling pathway has been established to be associated with tumorigenesis in skin, the cerebellum, skeletal muscle, lung, prostate, and some types of tumors in digestive tract including those originating from the oesophagus, stomach, biliary tract and pancreas (Evangelista et al., 2006; Scales et al., 2009; Varnat et al., 2009). However, only a few studies were involved in the pathogenesis of CRC. The exact role of the Hh pathway plays in colorectal tumorigenesis has remained controversial until now, as the data from

**Table 7. Relationship of SHH, SMO, GLI1 mRNA Protein Expressions in CRC Tissues to Clinicopathologic Characteristics of Patients**

	n	SHH (+)	P	SMO (+)	P	GLI1(+)	P
Gender							
male	24	8	0.37	20	1	7	0.38
female	19	4		16		8	
Age (yrs)							
<60	10	4	0.57	10	0.27	3	1
≥60	33	8		26		12	
Location							
colon	27	10	0.17	23	1	10	0.7
rectum	16	2		13		5	
Differentiation							
moderate	38	11	1	31	0.69	13	1
poor	5	1		5		2	
Infiltration							
T1+T2	21	5	0.56	18	1	6	0.4
T3+T4	22	7		18		9	
Dukes stages							
A	7	1	0.57	6	0.68	3	0.56
B	14	4		12		3	
C	18	5		16		8	
D	4	2		2		1	

cell lines, animal models or CRC patients have provided conflicting results.

In experimental studies, Berman detected SHH and IHH mRNA in 37 of 38 (97%) cell lines from oesophageal, stomach, biliary tract, pancreatic and colon carcinomas. The Hh target genes PTCH and GLI1 were co-expressed in most cell lines from oesophageal (4/6), stomach (6/6), pancreatic (5/6) and biliary tract (5/9) tumors. PTCH was not expressed in colon tumor cell lines (0/11), although a few of these cell lines expressed GLI1 in the absence of PTCH. As PTCH and GLI1 were indicators of Hh pathway activity, He concluded that the Hh pathway was not active in these CRC cell lines (Yoshikawa et al., 2009). Chatel analyzed the gene and protein expressions

of the main key members of the Hh pathway (SHH, IHH, PTCH, SMO, GLI1, GLI2, GLI3, SUFU and HHIP) in 7 colon cancer cell lines and found none of them expressed the complete set of Hh pathway members (Chatel et al., 2007). However, by analyzing CRC cell lines as well as human tissues from 40 patients with local or metastatic CRCs, Varnat found consistent expression of PTCH, GLI1 and SHH as well as other Hh-GLI1 pathway components. Expression was detected in epithelial tumor cells in situ in the colon. He did not observe expression of GLI1 protein in the stroma, indicating that the stroma did not have an active Hh-GLI signaling (Varnat et al., 2009). Yoshikawa found that colorectal adenomas expressed SHH more frequently compared with normal mucosa and adenocarcinoma, which suggested that SHH expression might increase during carcinogenesis and then decrease during tumor progression in the adenoma-carcinoma sequence (Yoshikawa et al., 2009). By contrast, in a clinical study for SHH, IHH and GLI1 genes in 36 CRC patients, Fu found the expressions of SHH and GLI1 proteins were increased significantly in tumor tissues compared with those in hyperplastic polyps and colorectal adenomas. IHH was almost lost in both colorectal adenomas and CRC tissues (Fu et al., 2010). In Monzo study, SHH mRNA expression levels were higher in tumor tissue than matched normal tissue from 57 CRC patients, and SHH expression was correlated with patient's clinicopathological characteristics. Higher levels of SHH expression were associated with early stage of the tumor (Monzo et al., 2006). However, the previous study showed that CRC tissues in the high-risk patients for metastasis expressed lower levels of PTCH1 mRNA than those in the low-risk patients. The similar result was found in his experimental study. The mRNA and protein levels of PTCH1 were inversely correlated with the metastatic potential of CRC (LoVo) cell lines. But the expression levels of SHH genes did not differ between the two clinical groups (You et al., 2010). Douard found that SHH mRNA was overexpressed in CRCs in 38 of 44 (86%) patients. Expression levels of GLI1 correlated with SHH expression. SHH overexpression did not appear to correlate with the patient characteristics. Similarly, when he studied HT-29 colorectal cell line, exogenous SHH promoted cell proliferation, while inhibition of SHH expression decreased proliferation. Expression of GLI1 mRNA increased with exogenous exposure to SHH (Douard et al., 2006).

The controversies in the association of Hh pathway activation with tumor stage, differentiation and tumor subtype were also reported in gastric carcinoma. SHH was overexpressed 12.8-fold of tumor tissues as compared with that in the surrounding non-cancerous gastric tissue. There was no correlation between the levels of SHH mRNA expression and the pathologic or clinical features of the tumor, including tumor type, size, location, and stag (Ohta et al., 2005). The elevated expression of Hh target genes PTCH1 or GLI1 occurred in 63 of the 99 primary gastric cancers. However, the activity of the Hh pathway was associated with poorly differentiated and more aggressive tumors (Ma et al., 2005).

To our knowledge, the present study is the first

study to simultaneously investigate the mRNA and protein expressions of SHH, SMO, GLI1 related genes in human CRC tissues and evaluated their correlation with the clinicopathologic characteristics of the patients. Our findings that the expressions of those Hh signalling pathway related genes in CRC tissues were consistent with the results from most reports. Our findings of no association of those gene expressions with the clinicopathological characteristics of the patients provided additional information to the conflicting results mentioned above. This study proved an efficient way for the diagnosis of CRC and a new strategy for CRC treatment by inhibiting Hh signaling and its downstream components. Further prospective studies to determine the potential value of Hh signaling are needed.

There are limitations in this study. Firstly, the comparatively small sample number may be inefficient for a strong conclusion. Secondly, we have not followed-up the patients to evaluate their prognosis. On the whole, this study still added new information to the knowledge already documented.

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