Study of Sonic hedgehog signaling pathway related molecules in gastric carcinoma

Xiao-Li Ma, Hai-Ji Sun, Yun-Shan Wang, Shu-Hong Huang, Jing-Wu Xie, Hong-Wei Zhang

Abstract

AIM: To study the expression of Sonic hedgehog pathway-related molecules, Sonic hedgehog (Shh) and Gli1 in gastric carcinoma.

METHODS: Expression of Shh in 56 gastric specimens including non-cancerous gastric tissues, gastric adenocarcinoma, gastric squamous cell carcinoma was detected by RT-PCR, in situ hybridization and immunohistochemistry. Expression of Gli1 was observed by in situ hybridization.

RESULTS: The positive rate of Shh and Gli1 expression was 0.0%, 0.0% in non-cancerous gastric tissues while it was 66.7%, 57.8% respectively in gastric adenocarcinoma, and 100%, 100% respectively in gastric squamous cell carcinoma. There was a significant difference between the non-cancerous gastric tissues and gastric carcinoma (P < 0.05). Elevated expression of Shh and Gli1 in gastric tubular adenocarcinoma was associated with poorly differentiated tumors while the expression was absent in gastric mucinous adenocarcinoma.

CONCLUSION: The elevated expression of Shh and Gli1 in gastric adenocarcinoma and gastric squamous cell carcinoma shows the involvement of activated Shh signaling in the cellular proliferation of gastric carcinogenesis. It suggests Shh signaling gene may be a new and good target gene for gastric tumor diagnosis and therapy.

Key words: Sonic hedgehog pathway; Sonic hedgehog; Gli1; Gastric carcinoma; Gastric adenocarcinoma; Gastric squamous cell carcinoma

© 2006 The WJG Press. All rights reserved.
Gastric cancer is the second most common cause of cancer-related death in the world. Many Asian countries, including Korea, China, and Japan, have very high rates of gastric cancer[18]. By far, the mechanism of Hedgehog signal in gastric cancer is still unclear. Here we studied the role of Shh signaling pathway-related molecules, Shh and Gli1, in gastric adenocarcinoma and squamous cell carcinoma by RT-PCR, immunohistochemistry and in situ hybridization.

MATERIALS AND METHODS

Samples
A total of 56 specimens of gastric tissues were used in our study. Fifty-six patients, who had undergone curative gastrectomy between 2002 and 2005 from the Department of General Surgery, Shandong Qilu Hospital affiliated to Shandong University, or from Jinan Central Hospital, Shandong Province, China, were enrolled in this study. All of the resected primary tumors were histologically examined by hematoxylin and eosin staining, and confirmed by pathologists. These samples included five resection specimens of non-cancerous gastric tissues, ten well differentiated tubular adenocarcinoma, ten moderately differentiated tubular adenocarcinoma, fifteen poorly differentiated adenocarcinoma, seven papillary adenocarcinoma, three gastric mucinous adenocarcinoma and six gastric squamous cell carcinoma.

Immunohistochemistry
Representative formalin fixed and paraffin embedded tissue sections (6 µm thick) were used for immunohistochemistry with specific antibodies to human Shh (Cat# 9024, Santa Cruz Biotechnology Inc, Santa Cruz, CA). First, tissue sections were deparaffinized, followed by rehydration with serially decreased concentrations of ethanol, and immersed in 3% H2O2 (in distilled water) for 10 min to inhibit endogenous peroxidase activity. Following antigen retrieval in citrate buffer (pH 6.0), the tissue sections were incubated with normal goat serum to block nonspecific antibody binding for 20 min at room temperature. The sections were then incubated with primary antibodies (at 1:200 dilution) at 37°C in humid chambers for 2 h. After washing with PBS 3 times, the sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG) and streptavidin conjugated to horseradish peroxidase for 20 min at 37°C, followed by PBS wash. The sections were incubated with DAB substrate for less than 30 min. Haematoxylin was used for counterstaining. Negative controls were performed in all cases by omitting the first antibodies.

RT-PCR
Total RNA of cells was extracted by an using RNA extraction kit (Promega). Total RNA templates were isolated from 10 µg sections (10 µm) of paraffin-embedded tissue as described elsewhere[16]. Three micrograms of total RNA were reverse transcribed by using M-MLV reverse transcriptase (Promega) with mixture of oligo(dT)15 and random primers (Promega). One-tenth of each RT reaction mixture was then subjected to PCR amplification using Taq DNA polymerase (TAKARA). The PCR primers for detecting specific transcripts are as below: for Shh sense 5′-ACCGAGGGCCTGGAGCAAGA-3′ and antisense 5′-ATTTGGGCCGCCAGGATGTT-3′ respectively. Following denaturation at 94°C for 10 min, 35 PCR cycles were performed at 94°C for 60 s, at 52°C for 50 s, and at 72°C for 60 s. The PCR products were analyzed by 0.7% agarose gel electrophoresis.

In situ hybridization
Shh (L38518) was subcloned in PbluescriptKS+. The linearized pBlueScript-shh was obtained by digestion with Sph I or Xma I restriction endonuclease. Gli1 (X07384) was cloned into pBlueScript M13+KS. The plasmid was digested with Nru I to generate the sense fragment, with Nde I to generate the antisense fragment. Sense and antisense probes were obtained by T3 and T7 in vitro transcription using a kit from Roche (Mannheim, Germany). Tissue sections (6 µm thick) were mounted onto Poly-L-Lysine slides. Following deparaffinization, tissue sections were rehydrated in a series of dilutions of ethanol. To enhance signal and facilitate probe penetration, sections were immersed in 0.3% Triton X-100 solution for 15 min at room temperature and in proteinase K (2 mg/mL) solution for 20 min at 37°C, respectively. The sections were then incubated with 4% (v/v) paraformaldehyde/PBS for 5 min at 4°C. After washing with PBS and 10 × saline citrate, the slides were incubated with prehybridization solution (50% formamide, 50% 4 × SSC) for 2 h at 37°C. The probe was added to each tissue section at a concentration of 1 g/mL and hybridized overnight at 42°C. After high stringency washing (2 × SSC twice, 1 × standard saline citrate twice, 0.5 × SSC twice at 52°C), sections were incubated with an alkaline phosphatase-conjugated sheep anti-digoxigenin antibody, which catalyzed a color reaction with the NBT/BCIP (nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Roche, Mannheim, Germany). Blue indicated strong hybridization. As negative controls, sense probes were used in all hybridization and no positive signal was observed.

Statistical analysis
Analysis was performed by using chi-square test and correlation analysis with SPSS 11.0 software. P < 0.05 was considered statistically significant.

RESULTS

Expression of Shh-mRNA in non-cancerous gastric tissues and gastric carcinoma
Total RNA extracted in paraffin-embedded tissue was pure enough to meet the need for RT-PCR. The length of RT-PCR product was 211 bp. The results of RT-PCR showed that expression of Shh was different in various tissue, including non-cancerous gastric tissues, well differentiated and poorly differentiated adenocarcinoma. Expression of Shh was higher in gastric adenocarcinoma than in non-cancerous gastric tissues (Figure 1), which was confirmed by in situ hybridization. The results of in situ hybridization

www.wjgnet.com
showed that Shh mRNA was expressed in cytoplasm of the fundic glandular epithelium and some stromal cells. Shh was not or lowly expressed in non-cancerous gastric tissues’ glandular epithelium while overexpressed in about 66.7% (30/45) adenocarcinoma patients and in 100% (6/6) gastric squamous cell carcinoma (Figure 2A-F) (Table 1). In tubular and papillary adenocarcinoma glandular epithelium, the expression of Shh showed an increasing trend among well differentiated, moderately differentiated and poorly differentiated adenocarcinomas (Figure 2B-D), while expression of Shh was absent in gastric mucinous adenocarcinoma (Figure 2E). In gastric squamous cell carcinoma, expression of Shh was obvious (Figure 2F). There was a significant difference between the non-cancerous gastric tissues and gastric carcinoma (P < 0.05) (Table 1).

High expression level of Shh protein in gastric carcinoma
The results of immunohistochemistry showed that Shh staining was detected in the fundic glandular epithelium and in the stroma of the stomach (Figure 3A-F). Shh protein was not or lowly expressed in non-cancerous gastric tissues (Figure 3A) and was highly expressed in gastric adenocarcinoma and gastric squamous cell carcinoma (Figure 3B-D, F). The results of Shh expression in various gastric tissue were consistent with results of in situ hybridization. Shh was overexpressed in gastric adenocarcinoma epithelium and squamous cell carcinoma epithelium compared with non-cancerous stomach tissues glandular epithelium. Shh expression was increased with malignant degree aggravation in gastric adenocarcinoma (Figure 3B-D), while there was no or less expression of Shh in gastric mucinous adenocarcinoma (Figure 3E).

Expression of transcriptor Gli1 in non-cancerous gastric tissues and gastric carcinoma
As an Shh signaling transcriptor, Gli1, was detected by in situ hybridization. It showed Gli1 was not or lowly expressed in non-cancerous gastric tissues glands and highly expressed in epithelium cytoplasm, in 57.8% (26/45) gastric adenocarcinoma and 100% (6/6) squamous cell carcinoma (Figure 4A-F) (Table 1). There was a higher expression level of Gli1 in squamous cell carcinoma (Figure 4F). Also, it presented an increasing trend in tubular adenocarcinoma with malignant degree aggravation (Figure 4B-D), while there was no or lower expression of Shh in gastric mucinous adenocarcinoma (Figure 4E). There was a significant difference between the non-cancerous gastric

![Figure 1 Results of Shh RT-PCR](image1)

![Figure 2 Results of Shh expression by in situ hybridization](image2)

![Figure 3 Results of Shh expression by immunohistochemistry](image3)

![Figure 4 Results of Gli1 expression by in situ hybridization](image4)
DISCUSSION

Shh is an important endodermal signal in endodermal-mesenchymal interaction during vertebrate development of the gut tube, especially to gastric gland [17]. Because Shh is expressed consistently in the endodermal epithelium of the gut throughout the period of organogenesis and late embryonic life, it is natural to suppose that its expression is crucial for the differentiation and maintenance of gut tube epithelium. Indeed, the heterozygous mutant mouse for Shh shows various gastrointestinal defects, such as intestinal transformation of stomach, annular pancreas, and duodenal stenosis [18]. Some of these defects may be caused by the absence of Shh effects on the mesenchyme, while others may reflect a direct action of Shh on epithelial cells. Although the expression of Shh is essential for the development of gut tube and the fundic glands of the adult gastrointestinal tract, the role of Shh expression has not been described in the gastric carcinogenesis [14,15].

We examined Sonic hedgehog signaling pathway related molecules expression in non-cancerous gastric tissues and in various types of gastric carcinoma. The results indicated that Shh protein expression is closely correlated with glandular epithelium differentiation in adenocarcinoma and gastric squamous cell carcinoma. The increase of Shh expression in tumor tissue is consistent with the results of Berman et al [14].

Gastric cancer is one of the leading causes of cancer death worldwide. Because of its heterogeneity, gastric cancer has been an interesting model for studying carcinogenesis and tumorigenesis [15]. This study mainly detected the expression of Shh and Gli1 in various types of gastric carcinoma. The elevated expression of Shh and Gli1 in gastric adenocarcinoma and squamous cell carcinoma suggested the involvement of activated Shh signaling in the cellular proliferation of certain type of gastric carcinoma. Interestingly, there was no activation of Shh signaling in mucinous adenocarcinoma. It implies that there may exist different mechanisms in various gastric carcinoma.

In all the tumors with elevated levels of Gli1, expression of Shh was also increased, suggesting that overexpression of Shh may be responsible for elevated expression of Gli1 in gastric adenocarcinoma. However, a high level of sonic hedgehog expression was not always accompanied by elevated Gli1 expression in adenocarcinoma (Table 1), indicating additional regulatory mechanisms for the hedgehog pathway activation. In addition to the hedgehog overexpression, other genetic alterations may be required to activate the hedgehog pathway in gastric cancers, such as Wnt, Notch signal pathway. Further study of the relationship between Sonic Hedgehog and other signal pathways may provide more evidences for a new and good target gene for gastric cancer diagnosis and therapy.

REFERENCES

1. Chiang C, Litington Y, Lee E, Young KE, Corden JL, Westphal H, Beachy PA. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 1996; 383: 407-413

2. Litington Y, Lei L, Westphal H, Chiang C. Sonic hedgehog is essential to foregut development. Nat Genet 1998; 20: 58-61
Bhardwaj G, Murdoch B, Wu D, Baker DP, Williams KP, Chadwick K, Ling LE, Karanu FN, Bhatia M. Sonic hedgehog induces the proliferation of primitive human hematopoietic cells via BMP regulation. *Nat Immunol* 2001; 2: 172-180

Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 2001; 15: 3059-3087

Taipale J, Cooper MK, Maiti T, Beachy PA. Patched acts catalytically to suppress the activity of Smoothened. *Nature* 2002; 418: 892-897

Britto J, Tannahill D, Keynes R. A critical role for sonic hedgehog signaling in the early expansion of the developing brain. *Nat Neurosci* 2002; 5: 103-110

Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. *Neuron* 1999; 22: 103-114

Kenney AM, Rowitch DH. Sonic hedgehog promotes G1 cyclin expression and sustained cell cycle progression in mammalian neuronal precursors. *Mol Cell Biol* 2000; 20: 9055-9067

Katano M. Hedgehog signaling pathway as a therapeutic target in breast cancer. *Cancer Lett* 2005; 227: 99-104

Hahn H, Wojnowski L, Miller G, Zimmer A. The patched signaling pathway in tumorigenesis and development: lessons from animal models. *J Mol Med (Berl)* 1999; 77: 459-468

Berman DM, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA. Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science* 2002; 297: 1559-1561

Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 2003; 422: 313-317

Thayer SP, di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernandez-del Castillo C, Yajnik V, Antoniu B, McMahon M, Warshaw AL, Hebrok M. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 2003; 425: 851-856

Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN, Beachy PA. Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 2003; 425: 846-851

Dimmler A, Brabletz T, Hubek F, Hafner M, Rau T, Kirchner T, Faller G. Transcription of sonic hedgehog, a potential factor for gastric morphogenesis and gastric mucosa maintenance, is up-regulated in acidic conditions. *Lab Invest* 2003; 83: 1829-1837

Ma XL, Huang AH, Wang YS, Xie JW, Zhang HW. Study of RNA extracted from paraffin tissues. *Shandong Daxue Xuebao (Yixue Ban)* 2004; 42: 613-614

van den Brink GR, Hardwick JC, Nielsen C, Xu C, ten Kate FJ, Glickman J, van Deventer SJ, Roberts DJ, Peppelenbosch MP. Sonic hedgehog expression correlates with fundic gland differentiation in the adult gastrointestinal tract. *Gut* 2002; 51: 628-633

Ramilho-Santos M, Melton DA, McMahon AP. Hedgehog signals regulate multiple aspects of gastrointestinal development. *Development* 2000; 127: 2763-2772