Expressions of sonic hedgehog, patched, smoothened and Gli-1 in human intestinal stromal tumors and their correlation with prognosis

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Abstract

AIM: To investigate the role that the hedgehog (Hh) signaling pathway, which includes sonic hedgehog (Shh), Patched (Ptc), Smoothened (Smo) and Gli-1, plays in human gastrointestinal stromal tumors (GISTs).

METHODS: Surgically resected specimens from patients with GISTs, leiomyomas and schwannomas were examined by immunohistochemical staining for aberrant expression of hedgehog signaling components, Shh, Ptc, Smo and Gli-1, respectively.

RESULTS: In GISTs, 58.1% (18 of 31), 77.4% (24 of 31), 80.6% (25 of 31) and 58.1% (18 of 31) of the specimens stained positive for Shh, Ptc, Smo and Gli-1, respectively. In leiomyomas, 92.3% (12 of 13), 92.3% (12 of 13), 69.2% (9 of 13) and 92.3% (12 of 13) stained positive for Shh, Ptc, Smo and Gli-1, respectively. In schwannomas, 83.3% (5 of 6), 83.3% (5 of 6), 83.3% (5 of 6) and 100% (6 of 6) stained positive for Shh, Ptc, Smo and Gli-1, respectively. Immunohistochemistry revealed that the expressions of Shh and Gli-1 were significantly higher in leiomyomas than in GISTs (P < 0.05, respectively). Shh expression strongly correlated with the grade of tumor risk category and with tumor size (P < 0.05, respectively). However, the expressions of Ptc and Smo did not correlate with histopathological differentiation.

CONCLUSION: These results suggest that the Hh signaling pathway may play an important role in myogenic differentiation and the malignant potential of human intestinal stromal tumors.

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Key words: Gastrointestinal stromal tumor; Leiomyoma; Schwannoma; Hedgehog; Immunohistochemistry

Yoshizaki A, Nakayama T, Naito S, Wen CY, Sekine I. Expressions of sonic hedgehog, patched, smoothened and Gli-1 in human intestinal stromal tumors and their correlation with prognosis. World J Gastroenterol 2006; 12(35): 5687-5691

http://www.wjgnet.com/1007-9327/12/5687.asp

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that occur from the esophagus to the anus, including in the omentum, mesentery and retroperitoneum[1]. Small GISTs are often detected during surgery for other conditions, gastroscopy or routine X-ray[1,2]. Some GISTs present with bleeding, perforation, pain, obstruction or a combination of these symptoms[3]. These tumors have a wide clinical spectrum from benign, incidentally detected nodules to malignant tumors[1] categorized into four risk groups: very low, low, intermediate and high[1]. Traditionally, primary mesenchymal spindle cell tumors of the gastrointestinal (GI) tract have been uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas, or leiomyosarcomas). Tumors with epithelioid cytologic features have been designated as leiomyoblastomas or epithelioid leiomyosarcomas[8]. Recently it has been postulated that GISTs originate from Cajal cells in the gastrointestinal tract, which are thought to be pacemaker cells that regulate intestinal motility[6,7]. Thus, GISTs differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Further, GISTs are characterized by the frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[9] and c-kit protooncogene. They also show a remarkable variability in their differentiation, and can be roughly divided into four major categories according to the phenotypic features of the tumors: smooth muscle type, neural type, combined
Tumor classification and selection

A total of 31 GISTs (all cases of stomach), 13 leiomyomas (5 cases of oesophagus, 6 of stomach and 2 of large intestine), and 6 schwannomas (5 cases of stomach and 1 of large intestine) were obtained from patients at Nagasaki University Hospital between 1997 and 2004. The tumor sizes of GISTs were 0.8-8.0 cm in diameter, leiomyomas were 0.1-2.5 cm, and schwannomas were 0.6-4.0 cm. In this study, GISTs were defined and selected as those tumors expressing both c-kit and CD34 surface antigens.

Further, we classified smooth muscle actin expressing tumors into smooth muscle (M) type GISTs, S-100 protein expressing tumors into neurogenic (N) type GISTs, both smooth muscle actin and S-100 protein expressing tumors into committed type GISTs, and those expressing only c-kit and CD34 into uncommitted (UN) type GISTs.[9]

And we classified histomorphologically tumors with epithelioid cell form into epithelioid cell (EP) type GISTs, spindle cell form into spindle cell (SP) type GISTs, both epithelioid and spindle cell form tumors into mixed (MIX) type GISTs.[9]

We also categorized GISTs into four groups according to their malignant potential.[9] The number of mitoses was determined by counting 50 high-power fields (×400) under Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined and selected as tumors expressing smooth muscle actin and not expressing c-kit and CD34. Schwannomas were defined and selected as tumors expressing S-100 protein and not expressing c-kit and CD34. Tumor identification and classification were determined by two independent pathologists (T. Nakayama and I. Sekine), and cases of questionable diagnosis were omitted from this study.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissues were cut into 4-μm sections, deparaffinized in xylene, and rehydrated in PBS. Deparaffinized sections were preincubated with normal bovine serum to prevent non-specific binding and then incubated overnight at 4°C with an optimal dilution (0.1 mg/L) of a primary polyclonal goat antibody against human Shh (N-19), Ptc (C-20), Smo (N-19) and Gli-1 (C-18). Each antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The slides for Shh, Ptc, Smo and Gli-1 were then sequentially incubated with an alkaline phosphatase-conjugated donkey anti-goat immunoglobulin antibody, and the reaction products were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; BRL, Gaithersburg, MD, USA). Primary antibodies preabsorbed with excess antigen peptides or recombinant protein were used as negative controls. Basal cell carcinoma tissue served as the internal positive control for Shh, Ptc, Smo and Gli-1 immunoreactivity. Immunohistochemical analyses were performed independently by two investigators (T. Nakayama and A. Yoshizaki). Shh, Ptc, Smo and Gli-1 expression was classified into two categories depending on the percentage of cells stained: -, 0%-15% positive tumor cells; +, >15% positive tumor cells.

Statistical analysis

The Stat View II program (Abacus Concepts, Inc., Berkeley, CA, USA) was used for statistical analyses. Analyses comparing the levels of Shh, Ptc, Smo and Gli-1 expression were performed using the Mann-Whitney, Kruskal-Wallis and Spearman’s tests. P < 0.05 was taken as significant.

RESULTS

The results from the immunohistochemical analysis of Hh pathway components in human intestinal stromal tumors are summarized in Table 1. Shh expression was heterogeneous and localized to the cytoplasm of cells in GISTs (Figure 1A). Ptc and Smo were localized to the cytoplasm and cell membrane, and Gli-1 was localized to the cytoplasm and nucleus of GIST tumor cells (Figure 1B-D). Immunohistochemical stainings of Hh pathway components in leiomyomas and schwannomas are shown in Figures 2 and 3, respectively. The four proteins of the Hh pathway that we examined showed patterns of expression similar to that observed in GISTs. While only 58.1% (18 of 31) of the GISTs were positive for Shh, nearly all leiomyomas and schwannomas were positive (92.3%, 12 of 13, P < 0.05). The following results were observed for the other proteins: 77.4% (24 of 31), 92.3% (12 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas and schwannomas, respectively.
Table 1  Aberrant expression of Hedgehog pathway signaling proteins in intestinal stromal tumors  n (%)  

|          | Shh | Ptc | Smo | Gli-1 |
|----------|-----|-----|-----|-------|
|          | +   | -   | +   | -     |
| GIST     | n   | +   | -   | +     | -     |
| I        | 31  | 18  | 13  | 24    | 7 (22.6) |
| GIST, M  | 7 (22.6) | 4 (57.1) | 3 (42.9) | 5 (71.4) | 2 (28.6) |
| GIST, N  | 8 (25.8) | 5 (62.5) | 3 (37.5) | 5 (62.5) | 3 (37.5) |
| II       | n   | +   | -   | +     | -     |
| GIST, EP | 5 (16.1) | 3 (60.0) | 2 (40.0) | 4 (80.0) | 1 (20.0) |
| GIST, MIX| 3 (9.7) | 2 (66.7) | 1 (33.3) | 2 (66.7) | 1 (33.3) |
| GIST, SP | 23 (74.2) | 10 (43.4) | 17 (73.9) | 6 (26.1) | 19 (82.6) |
| Leiomoma | 13  | 12  | 7.5 (7.7) | 12 | 92.3 | 17 (73.9) | 6 (26.1) | 19 (82.6) |
| Schwannoma | 6 | 5 (83.3) | 1 (16.7) | 5 (83.3) | 1 (16.7) |

n (%): Tumor cases followed by percentage (%) of total cases. *P < 0.05 between Leiomyoma and GIST in Shh or Gli-1; †P < 0.05 between GIST, UN and GIST, N.

and schwannomas were positive for Ptc, respectively; 80.6% (25 of 31), 69.2% (9 of 13), 83.3% (5 of 6) of the GISTs, leiomyomas and schwannomas were positive for Smo, respectively; and 58.1% (18 of 31), 92.3% (12 of 13), 100.0% (6 of 6) of the GISTs, leiomyomas and schwannomas were positive for Gli-1, respectively. Though schwannomas expressed high levels of each protein examined, they had no statistical correlation with GISTs or leiomyomas.

Immunohistochemical analyses of Hh pathway com-
The results from immunohistochemical analysis of Hh pathway components in GISTs classified histomorphologically by cellular form are shown in Table 1. In all GISTs, 16.1% (5 of 31) of EP cell type, 9.7% (3 of 31) of MIX cell type and 74.2% (23 of 31) of SP cell type were included, respectively. Each Hh pathway component was detected variably in different cellular type of GISTs. However, there was no correlation between the expression of Hh pathway components and cellular subtype of GISTs.

The results from immunohistochemical analysis of Hh pathway components with regard to the malignant potential of GISTs are summarized in Table 2. There were no correlations between mitosis counts and the expression levels of Hh pathway components. In contrast, the results suggested that lower levels of Shh expression correlated with lower risk GIST categories (P < 0.05) and smaller tumor sizes (P < 0.05).

**DISCUSSION**

Recent studies have shown that the Hh pathway plays important roles in cell differentiation, tissue patterning and embryonic development[11,13,21]. However, the role of the Hh pathway in human intestinal stromal tumors is still unclear. We investigated the expression of Shh, Ptc, Smo and Gli-1 in three types of intestinal stromal tumors (GISTs, leiomyomas and schwannomas) using immunohistochemical techniques. Our data demonstrated that Shh and Gli-1 were expressed at higher levels in leiomyomas than in GISTs. It suggested that Shh and Gli-1 expressions were correlated with myogenic differentiation. However, in the subclassification of GISTs, myogenic differentiation did not show expression of Shh or Gli-1. Moreover, the consequence of low expression of Smo in neuronal GISTs is not clear yet. Thus, future studies will address the role of the Hh pathway in the differentiation of intestinal stromal tumors.

Abrogation of the Hh pathway can also lead to tumorigenesis. In this pathway, Gli-1, which is involved in controlling cell proliferation and angiogenesis, is a key target of oncogenic action[3,13,22]. Loss of function Ptc mutations and gain of function Smo mutations are mechanisms of tumorigenesis in many types of tumors such as basal cell carcinomas, medulloblastomas, astrocytomas, small cell lung carcinomas and pancreatic cancers[13,23]. Joensuu
et al.[28] reported a patient in whom Imatinib(STI-571, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST. And Imatinib has been proven to be remarkably efficacious in heavily pretreated GISTs patients with advanced disease in phase III clinical trials[29]. The expression of the Hh pathway is upregulated by the activation of tyrosine kinase through the epidermal growth factor pathway[30], and may be upregulated by the c-kit/tyrosine kinase pathway.

ACKNOWLEDGMENTS

We are grateful to Mr. Toshiyuki Kawada (Nagasaki University Graduate School of Biomedical Sciences, Molecular Pathology) for his excellent immunohistochemical assistance.

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S-Editor Liu Y  L-Editor Zhu LH  E-Editor Ma WH