Identification of signature genes for detecting hedgehog signaling activation in gastric cancer

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Abstract. The aim of this study was to investigate the expression of hedgehog signaling molecules in gastric cancer. *In situ* hybridization, immunohistochemistry and RT-PCR for hedgehog signaling molecules, smoothened (SMO), suppressor of fused [Su(Fu)], and the target genes hedgehog-interacting protein (HIP) and platelet-derived growth factor receptor α (PDGFRα) were performed in 30 gastric cancer and two gastritis specimens. Using *in situ* hybridization, SMO expression was detected in 18/30 cancerous specimens (60%) as well as in 1/2 gastritis specimens (50%). Su(Fu) was expressed in 15/30 (50%), HIP in 14/30 (~47%), and PDGFRα in 6/30 (20%) gastric cancer specimens. Despite the heterogeneous expression pattern, *SMO*, *Su(Fu)* and *PDGFRα* transcripts were highly correlated with the *HIP* transcript in the 30 gastric cancer specimens (p=0.0006, 0.0003 and 0.0441, respectively). Results from the *in situ* hybridization were further confirmed by RT-PCR for the expression of all of the genes and by immunohistochemistry for SMO expression. The findings revealed a set of genes for detecting Hh signaling activation in gastric cancer.

Introduction

The hedgehog (Hh) signaling pathway regulates many processes in tissue development and homeostasis, and the activation of the Hh signaling pathway is associated with many types of human cancer. In the absence of the ligand Hh, hedgehog receptor patched (PTCH) inhibits smoothed (SMO) signaling. When Hh binds to PTCH1, SMO is able to signal, eventually resulting in the formation of activated transcriptional factor Gli molecules and the elevated expression of target genes (e.g., PTCH1, GLI1, HIP and PDGFRα).

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Recent studies have shown that the Hh pathway is involved in gastrointestinal development (1-9). Molecules such as the transcriptional factors GATA-4, GATA-6 (9), FoxF1 and FoxL1 (5), as well as the ERK (7) and epithelial-mesenchymal transition pathways (6), are reported to be associated with Hh signaling in this process. Increasing evidence shows that Hh signaling plays a role in gastric cancer. Expression of sonic Hh is increased in gastric cancer, and gastric lesions are associated with the methylation status of the sonic hedgehog (Shh) promoter (10). Nuclear translocation of Gli1 was found to be higher in undifferentiated-type tumors and to be positively correlated with lymph node metastasis in gastric carcinoma (11). Hh signaling was found to promote gastric cancer cell proliferation (12,13), epithelial-mesenchymal transition (6), mobility and invasiveness (14). We previously demonstrated that overexpression of Hh and its target genes, Gli1 and PTCH1, occurs in gastric tumor tissue. We also showed that the Smo antagonist or Shh neutralizing antibodies inhibit growth and induce apoptosis in gastric cancer cells (15).

It is not known which molecules in the Hh signaling pathway can be used to detect Hh signaling activation in gastric cancer. Elucidation of the Hh signaling activation signature will aid in the clinical diagnosis of gastric cancer and will allow us to understand Hh signaling in gastric cancer in greater detail. In the present study, we analyzed the expression of the *SMO*, *HIP*, *Su(Fu)* and *PDGFRα* genes in 30 gastric cancer and two gastritis specimens using *in situ* hybridization, RT-PCR and immunohistochemistry.

Materials and methods

Tumor specimens. Thirty cases of gastric cancer and two cases of gastritis were received as discarded materials from the Shangdong QiLu Hospital, Jinan, China. Pathology reports and H&E staining of each specimen were reviewed to determine the nature of the disease and the tumor histology. The gastric cancer specimens were categorized into three subtypes according to the WHO guidelines (16) as follows: tubular adenocarcinoma (26 cases), papillary adenocarcinoma (2 cases) and squamous cell carcinoma (2 cases) (Table I).

In situ hybridization. Tissue sections (6-μm) were mounted onto poly-L-lysine slides. Following deparaffinization, the

Table I. Gastric cancer specimens and summary of *Shh*, *Ptch*, *Gli1*, *Smo*, *Hip*, *Su(Fu)* and *PDGFRα* expression from *in situ* hybridization.

No.	Age	Gender	Pathological diagnosis	Stage	SHH[a]	PTCH[a]	GLI1[a]	SMO	SU(FU)	HIP	PDGFRα
1	50	M	Gastritis		-	-	-	±	-	-	-
2	62	M	Gastritis		-	-	-	-	-	-	-
3	69	M	Tubular adenocarcinoma (W)	III	-	-	-	±	-	-	-
4	72	M	Tubular adenocarcinoma (W)	III	+	+	+	+	+	+	±
5	29	F	Tubular adenocarcinoma (W)	III	±	-	-	-	-	±	-
6	54	F	Tubular adenocarcinoma (M)	II	+	+	+	±	+	±	-
7	68	M	Tubular adenocarcinoma (M)	II	+	±	+	±	+	±	-
8	59	F	Tubular adenocarcinoma (M)	III	±	-	-	±	-	-	-
9	73	M	Tubular adenocarcinoma (M)	III	-	-	-	-	-	-	-
10	51	M	Tubular adenocarcinoma (M)	III	±	±	±	+	+	-	-
11	54	M	Tubular adenocarcinoma (P)	III	+	-	-	-	-	-	-
12	49	M	Tubular adenocarcinoma (P)	III	+	+	+	+	+	+	+
13	68	M	Tubular adenocarcinoma (P)	II	-	-	-	±	+	+	-
14	67	M	Tubular adenocarcinoma (P)	I	-	-	-	-	-	-	-
15	59	M	Tubular adenocarcinoma (P)	III	+	±	±	±	+	+	-
16	60	M	Tubular adenocarcinoma (P)	III	+	-	-	-	-	-	-
17	69	M	Tubular adenocarcinoma (P)	III	+	+	+	+	+	+	-
18	70	F	Tubular adenocarcinoma (P)	II	+	+	+	+	+	-	+
19	59	M	Tubular adenocarcinoma (P)	III	+	±	±	±	±	-	-
20	69	M	Tubular adenocarcinoma (P)	III	-	-	-	-	-	-	-
21	56	M	Tubular adenocarcinoma (P)	III	-	-	-	-	-	-	-
22	65	M	Tubular adenocarcinoma (P)	III	-	-	-	-	-	-	-
23	50	F	Tubular adenocarcinoma (P)	II	-	-	-	-	-	-	-
24	77	M	Tubular adenocarcinoma (P)	II	-	-	-	-	-	-	-
25	71	M	Tubular adenocarcinoma (P)	III	-	-	-	-	-	-	-
26	68	M	Tubular adenocarcinoma (P)	III	+	+	+	+	+	±	-
27	57	F	Tubular adenocarcinoma (P)	II	-	-	-	-	-	-	-
28	49	M	Tubular adenocarcinoma (P)	III	±	±	±	+	-	±	-
29	50	M	Papillary adenocarcinoma	I	+	+	+	+	+	+	+
30	67	M	Papillary adenocarcinoma	III	+	±	+	+	+	±	+
31	65	M	Squamous cell carcinoma (W)	II	+	+	+	±	+	+	-
32	65	M	Squamous cell carcinoma (P)	III	+	+	+	+	+	+	+

[a]Results of the *Shh*, *Ptch* and *Gli1 in situ* hybridization are from our previous study (15).

sections were rehydrated in a series of dilutions of ethanol. To enhance the signal and facilitate probe penetration, sections were immersed in 0.3% Triton X-100 solution for 15 min at room temperature, followed by treatment with proteinase K (20 μg/ml) for 20 min at 37˚C. The sections were then incubated with 4% (v/v) paraformaldehyde/PBS for 5 min at 4˚C. After washing with PBS and 0.1 M triethanolamine, the slides were incubated with pre-hybridization solution (50% formamide, 50% 4X standard saline citrate) for 2 h at 37˚C. The probe was added to each tissue section at a concentration of 1 μg/ml and hybridized overnight at 42˚C. After high-stringency washing (2X SSC twice, 1X SSC twice, 0.5X SSC twice at 37˚C), sections were incubated with an alkaline phosphatase-conjugated sheep antidigoxigenin antibody, which catalyzed a color reaction with the NBT/BCIP (nitro-blue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate (Roche). Blue staining indicated strong hybridization. Sense probes were used as negative controls in all hybridizations, and no positive signals were observed.

Immunohistochemistry. The smoothened antibody (ab13118-50; Abcam, Cambridge, UK) was used to perform immunohistochemistry on the tissue sections (6-μm). The procedure of immunohistochemistry was as described elsewhere (15). Negative controls were performed by omitting the first antibody.

RT-PCR. Total RNAs were extracted using an RNA extraction kit according to the manufacturer's instructions (Promega, Madison, WI, USA). PCR was performed using

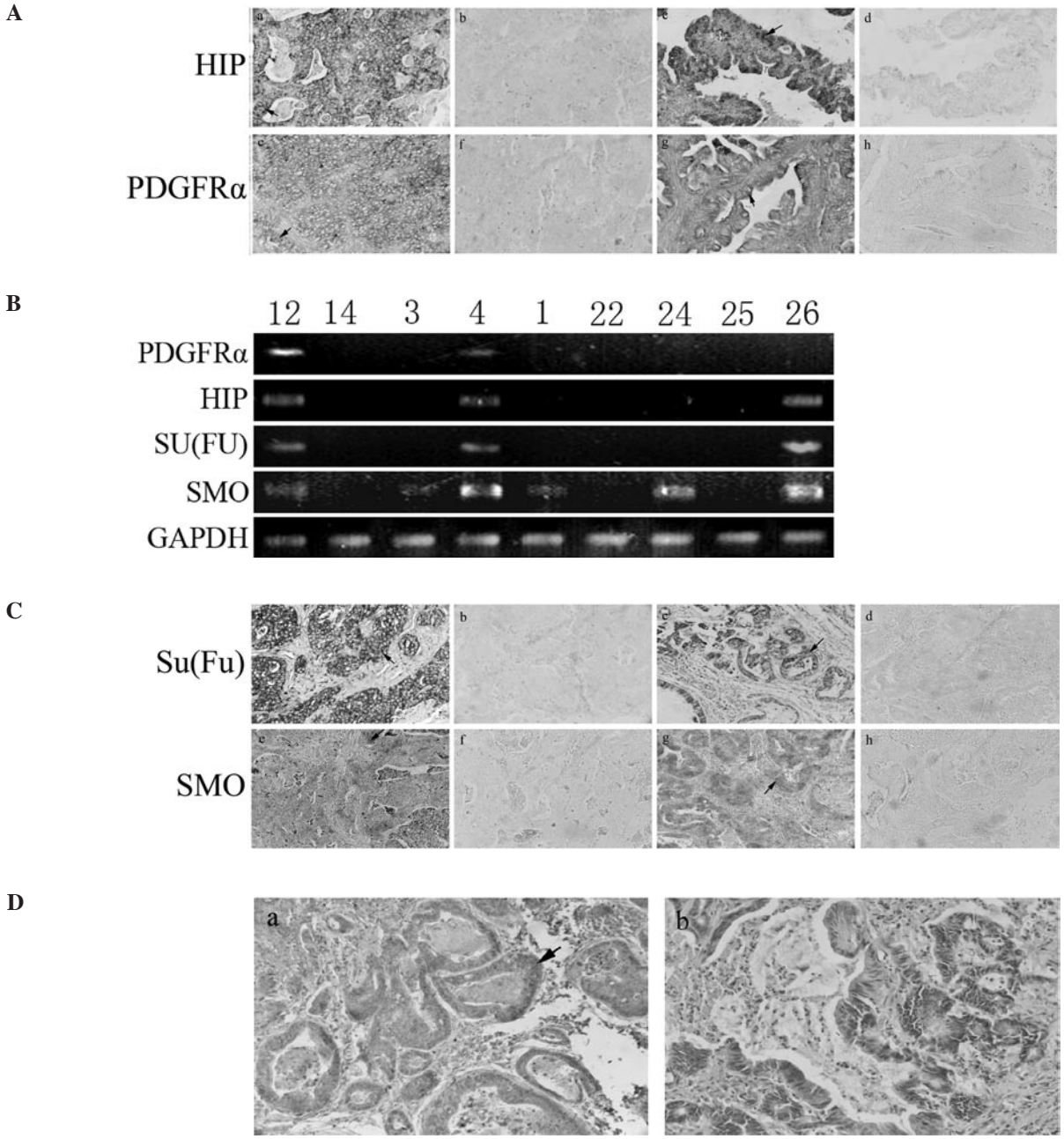

Figure 1. (A) Expression of *HIP* and *PDGFRα* in gastric cancer. The *HIP* and *PDGFRα* transcripts (indicated by arrows) were detected by *in situ* hybridization in poorly differentiated SSC (a and e) and papillary adenocarcinoma (c and g); b, d, f and h were the controls using the respective sense probe (x200). (B) RT-PCR detection of *SMO*, *HIP*, *Su(Fu)* and *PDGFRα* transcripts in gastric cancer. β-actin was used as the endogenous reference. Numbers listed indicate specimen number. (C) Expression of *SMO* and *Su(Fu)* in gastric cancer. The *SMO* and *Su(Fu)* transcripts (indicated by arrows) were detected by *in situ* hybridization. Positive staining was noted in poorly differentiated SSC (a and e) and moderately differentiated tubular adenocarcinoma (c and g); b, d, f and h were the controls using the respective sense probe (x200). (D) Expression of SMO protein in gastric cancer. SMO protein (indicated by arrows) was detected by immunohistochemistry in moderately differentiated tubular adenocarcinoma (a); b is the negative control without the primary antibody (x200).

10 pmol of each primer in a standard 50-ml PCR reaction containing 100 mM dNTPs and cDNA from human tissue cDNA expression libraries as a template. The primer sequences are listed in Table II. DNA was amplified by Taq DNA polymerase for 30 cycles and subsequently run on a 0.8% agarose gel. The bands were visualized under UV light prior to image capture.

Statistical analysis. The two-tailed χ^2 test was used for all statistical analysis.

Results

Expression of Hh target genes in gastric cancer. An increasing number of putative Hh target genes have been identified, but only a few have been evaluated for expression in gastric cancer (15). HIP is a known Hh target gene that encodes a negative regulator of the pathway, forming a negative feedback loop. Several studies have reported that elevated HIP expression indicates activated Hh signaling in human cancer (17,18). PDGFRα expression is elevated in basal cell carci-

Table II. Primers used in RT-PCR.

Gene name	Primers
SMO	F: AAGGCCACGCTGCTCATCTGG R: CATTGAGGTCAAAGGCCAAGC
Su(Fu)	F: AGAGTGCCGCCGCCTTTACC R: ACGGGCTGCATCTGTGGGTC
HIP	F: TTCCATACCAAGGAGCAACC R: TCTTGCCACTGCTTTGTCAC
PGDFRα	F: GCTTTCATTACCCTCTATCCT R: GAATCATCCTCCACGA

nomas, which exhibits activation of the Hh pathway (19). To assess the expression of HIP and PDGFRα in gastric cancer, we first performed *in situ* hybridization analysis. Expression of the HIP transcript was detected in 14/30 gastric cancer specimens (~47%). The majority of the expression was detected in tumor tissue, rather than in the stroma (Table I). While the antisense probe provided a good signal (Fig. 1A, a and c, arrows), the sense probe did not yield any signals (Fig. 1A, b and d), indicating the specificity of *in situ* hybridization. Further analysis indicated that HIP expression was highly correlated with the expression of PTCH1 and Gli1, as determined in a previous study (15) (p=0.0003), indicating that the detection of HIP is as effective as the detection of Gli1 or PTCH1 in gastric cancer.

Expression of PDGFRα was detected in 6/30 gastric cancer specimens (20%). Most samples also expressed Gli1, PTCH1 and HIP (p=0.0062, 0.0062 and 0.0441, respectively). This indicates that, unlike HIP, PDGFRα expression is only detected in a subset of gastric cancer specimens with activated Hh signaling activation (Table I, Fig. 1A, e-h).

To confirm the results from the *in situ* hybridization, we performed RT-PCR in selected specimens in which the tumor content was >70% of the tissue mass. As shown in Fig. 1B, HIP and PDGFRα transcripts were detected in specimens 12 and 4, but not in specimens 14 and 3, which is consistent with the *in situ* hybridization data (Table I). Similarly, specimen 26 had a detectable HIP transcript but not a PDGFRα transcript (Fig. 1B and Table I).

Taken together, we found that the transcripts of HIP, Gli1 and PTCH1 were highly expressed in the gastric cancer specimens, whereas the PDGFRα transcript was detectable only in a subset of cancer exhibiting Gli1, PTCH1 and HIP expression.

Expression of SMO and Su(Fu) in gastric cancer. In addition to Hh target genes, we also investigated the expression of Hh signaling molecules in gastric cancer. SMO is a key signal transducer of the Hh pathway, and deletion of SMO results in the blockage of Hh signaling in mouse embryos (20). A previous study revealed elevated expression of SMO in prostate cancer (21). Su(Fu) is a negative regulator of the Hh pathway, inhibiting the function of Gli molecules through several mechanisms (22). Studies have indicated that reduced expression of Su(Fu) is one mechanism by which Hh signaling is activated (23).

First, SMO expression was examined by *in situ* hybridization. Eighteen gastric cancer specimens and one gastritis tissue specimen had a detectable level of SMO transcript (Table I and Fig. 1C, e and g, arrows). Most of the signal was in the tumor tissue, not in the stroma. Since no signals were detected with the sense probe of SMO, our *in situ* hybridization method was very reliable. RT-PCR was performed using selected specimens to confirm the data from the *in situ* hybridization. SMO expression detected by *in situ* hybridization was confirmed by RT-PCR (Fig. 1B). In one sample (specimen 24), the SMO transcript was detected only by RT-PCR. This was not unexpected, since PCR amplification is more sensitive in detecting gene expression. We also examined SMO protein expression in gastric cancer tissues using SMO-specific antibodies. As shown in Fig. 1D, SMO expression was found in the tissues with a detectable SMO transcript by *in situ* hybridization and RT-PCR. In comparison with HIP and other Hh target genes, the SMO transcript was detected in both the cancerous and gastritis tissues. Furthermore, the SMO transcript was detected in tissues with detectable expression of the Hh target genes HIP, Gli1 and PTCH1 (Table I). These results indicate that SMO expression does not represent Hh target gene activation in gastric cancer.

Next, the expression of Su(Fu) was examined by *in situ* hybridization and RT-PCR. No expression of Su(Fu) was detected in the gastritis samples. The sense probe of Su(Fu) did not detect any signals, while the antisense probe revealed the Su(Fu) transcript in 15 gastric cancer specimens (Fig. 1C, a-d and Table I). In tumors with detectable Hh target genes (indicating activation of Hh signaling), reduced expression of Su(Fu) was not found, suggesting the loss of Su(Fu) is not a common mechanism of Hh signaling activation. The results of RT-PCR confirmed the findings of the *in situ* hybridization.

Taken together, the data indicated that elevated expression of SMO or loss of Su(Fu) expression are not common in gastric cancer.

Discussion

Detection of Hh target gene expression is an important step in the identification of Hh signaling activation in human cancer. However, previous studies have examined only a few Hh target genes. To better understand Hh signaling activation in gastric cancer and to develop methods for its early diagnosis, we investigated the expression of several Hh target genes in gastric cancer. The results of HIP expression are consistent with our previous findings regarding Gli1 and PTCH1 (Table I). By contrast, only a subset of tumors with activated Hh signaling expressed PDGFRα. A high correlation was found between the HIP transcript and the PTCH1 or Gli1 transcript in gastric cancer (p=0.0001). These findings suggest that the expression of HIP, Gli1 and PTCH1 may be used to detect Hh signaling activation in gastric cancer. Although PDGFRα expression can be used to detect tumors with activated Hh signaling, many tumors go undetected due to its insensitivity. It has been reported that transcriptional silencing of the HIP protein is present in gastrointestinal cancer cell lines and a subset of gastric cancer tissues (24). Our studies did not reveal any reduced expression of HIP in tumors with detectable expression of Gli1 and PTCH1, suggesting that

post-transcriptional regulation of *HIP* in gastric cancer is not a major mechanism for Hh signaling activation.

Several reports have indicated that alterations in Hh signaling molecules may be responsible for Hh signaling activation. Su(Fu) is an essential repressor in mammalian Hh signaling (25). Mutations in Su(Fu) have been found in cancer cell lines and tumors (18,26,27), and the SCL/TAL1 interrupting locus depresses GLI1 from negative control of Su(Fu) in pancreatic cancer cells (28). However, we did not observe a significant alteration in the expression of Su(Fu) in the gastric cancer samples, suggesting that Su(Fu) inactivation is not very common in gastric cancer.

SMO expression was found to be elevated in a subset of prostate cancer specimens (21). Our data did not show any increase in SMO expression in gastric cancer. Whether the SMO transcript level can be used to detect Hh signaling activation in other types of tumors remains to be determined.

Expression of PDGFRα has been detected in several types of tumors (29-32), and has been found to be involved in tumor cell growth and metastasis (33-36). It has been reported that Gli1 activates PDGFRα in C3H10T1/2 cells (19); we also found that transcripts of *PDGFRα* were highly co-expressed with Hh signaling. Although the expression of PDGFRα is not as common as that of Gli1 in gastric cancer, identification of the mechanism by which PDGFRα is regulated may further contribute to the understanding of Hh-mediated carcinogenesis. It is known that PDGFRα increases tumor cell proliferation and metastasis. Currently, clinical therapeutics against PDGFRα function are achieved through the administration of STI571 (37). We envision that gastric cancer with detectable expression of PDGFRα may be eligible for treatment with STI571.

In the present study, target gene HIP expression was detected in approximately 47% of the gastric cancer specimens. HIP expression was highly correlated with the expression of PTCH1 and Gli1, indicating that the detection of HIP is as effective as the detection of Gli1 or PTCH1 in gastric cancer. SMO expression was detected in both the cancerous (60%) and gastritis (50%) specimens. Elevated expression of SMO is not common in gastric cancer. Su(Fu) was expressed in 50% of the gastric cancer specimens. Reduced expression of Su(Fu) was not found in the tumors with activated Hh signaling. Despite the heterogeneous expression pattern, the *SMO*, *Su(Fu)* and *PDGFRα* transcripts were highly correlated with the *HIP* transcript in the 30 gastric cancer specimens (p=0.0006, 0.0003 and 0.0441, respectively). The results reveal a set of genes for detecting Hh signaling activation in gastric cancer.

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