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RESEARCH

Identification of Signature Genes for Detecting Hedgehog Pathway Activation in Esophageal Cancer

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Abstract The hedgehog signaling pathway plays an important role in cell growth and differentiation both in normal embryonic development and in tumors. Our previous work shows that hedgehog pathway is frequently activated in esophageal cancers. To further elucidate the role of hedgehog pathway in esophageal cancers we examined the expression of the target genes, hedgehog-interacting protein (HIP) and platelet derived growth factor receptor alpha (PDGFRα) and hedgehog signaling molecules, smoothened (SMO), suppressor of fused (Su(Fu)) in the specimens using in-situ hybridization and RT-PCR. We found that *HIP*, *PDGFRα, SMO* and *Su(Fu)* gene highly expressed in the primary esophageal squamous cell carcinomas but not in normal esophageal tissue. The transcripts of *HIP, PDGFRα* and *SMO* were expressed in 13 of 15 esophageal cancers. *Su(Fu)* expression was missing in 2 esophageal cancers. The results from in-situ hybridization were further confirmed by RT-PCR. Our results revealed a set of genes for detecting hedgehog signaling activation in esophageal cancer.

Keywords Esophageal cancer · Hedgehog · Hedgehog-interacting protein · Platelet derived growth factor receptor alpha · Smoothened · Suppressor of fused

Introduction

Hedgehog (HH) signaling pathway regulates many processes in development, homeostasis in tissues, and ectopic expression of HH signaling pathway components are responsible for tumorigenesis. In the absence of HH, PTCH1 prevents SMO from signaling. When HH binds to PTCH1, SMO is free to pass signal to transcriptional factor GLIs and activate the target genes (e.g. PTCH1, GLI1, HIP and PDGFRα).

Active Smoothened contribute to activating the signaling pathway, result in inappropriate transcription of target genes, which may be important in the pathogenesis of carcinomas. Activated Smoothened mutations have been found in sporadic basal-cell carcinomas [1, 2], over expression of smoothened was found in several tumors [3–5]. HIP is the target gene of HH pathway also is a negative hedgehog signaling regulator by binding to the hedgehog protein. Silence of HIP was found in several cancer cell lines as well as tumors through genetic and/or epigenetic alternations [6–8]. Su(Fu) is an essential repressor in mammalian hedgehog signaling [9]. Mice with both heterozygous PTCH1 and Su(fu) had higher to develop

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tumor [10]. Mutations in Su(Fu) or loss of Su(Fu) expression was reported in cancer cell lines and tumors [11–14]. Expression of PDGFRα was found in several types of tumors [15–18], and involved in tumor cell growth and metastasis [19–22].

Esophageal cancer is one of the most frequent cancers and with high mortality rates in the world. We have demonstrated the over-expression of Sonic hedgehog (*SHH*) and its target genes, *GLI1* and *PTCH1* (patched1) in the tissues of esophageal primary tumors both on mRNA and protein levels. And SMO antagonist or the SHH antibodies treated esophageal cancer cells are inhibited growth and induced apoptosis [23]. But there is short of expression of other genes of this signaling pathway in esophageal primary cancers. To identify if there are activation of other components in this pathway we analyze the expression of *HIP*, *PDGFRα, SMO* and *Su(Fu)* by using in-situ hybridization and RT-PCR.

Material and Methods

Tumor Sample

Specimens from 15 cases of esophageal cancers were received as discarded materials from the Shangdong QiLu Hospital, Jinan, China as our previous report [23]. Pathology reports and H&E staining of each specimen were reviewed to determine the nature of the disease and the tumor histology. Esophageal cancers were identified according to the WHO guideline [24] as squamous cell carcinomas (15 cases) (Table 1). Five specimens of normal esophageal tissues were obtained from five esophageal cancer patients hospitalized in Shandong Provincial Hospital as discarded materials (Table 1).

In-situ Hybridization

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, 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 prehybridization solution (50% formamide, 50% 4×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 (2×SSC twice, 1×SSC twice, 0.5×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 indicated strong hybridization. As negative controls, sense probes were used in all hybridization and no positive signals were observed.

RT-PCR

Total RNAs were extracted using a RNA extraction kit from Promega according to the manufacturer (Promega, Madison, WI). PCR was performed using 10 pmol each of primers in a standard 50 ml PCR reaction containing 100 mM dNTPs and cDNA from human tissue cDNA expression libraries as template. The primer sequences are shown in (Table 2). DNA was amplified by Taq DNA Polymerase for 30 cycles, and then was run on a 0.8% agarose gel mixed with ethidium bromide. The bands were visualized under UV light before taking pictures.

Results

Expression of HIP and PDGFRα in Esophageal Cancers

Several putative HH target genes have been discovered, but only a few of them have been confirmed in esophageal cancer[23]. Several studies indicate that elevated expression of HIP indicates HH signaling activation in human cancer [11, 25]. PDGFRα expression was elevated in basal cell carcinomas, in which HH pathway was activated[26]. To assess expression of HIP and PDGFRα in esophageal cancers, we examined expression of *HIP* and *PDGFRα* in 15 cases of esophageal specimens and 5 normal esophageal tissues using in-situ hybridization. We found that 13 of the 15 (86.7%) tumor specimens expressed *HIP* and *PDGFRα* transcripts (see Table 1 for details). Most of the expression was detected in the tumor tissues (blue in Fig. 1a, f, indicated by arrows), not in the stroma (Fig. 1a, f, indicated by arrow heads). The anti-sense probe showed good signal (Fig. 1a, c, f, h) while the sense probe did not yield any staining (Fig. 1b, d, g, i), indicating specificity of in-situ hybridization. Positive staining of *HIP* and *PDGFRα* did not show in normal tissue (Fig. 1e, j). Further analysis showed that *HIP* and *PDGFRα* co-expressed in all the 13 tissues, and there is consistency of expression of *PTCH1, Gli1, HIP* and *PDGFRα* in 11 of 15 esophageal cancers (Table 1, data obtained from our previous publication[23]), indicating that detection of HIP and PDGFRα is as effective as detection of Gli1 or PTCH1 in esophageal cancer. The result was confirmed by RT-PCR (data not show). Taken together, we found that transcripts of *HIP* and *PDGFRα*

Table 1 Esophageal cancer specimens and summary of *SHH, PTCH1, GLI1, SMO, Su(Fu), HIP* and *PDGFRα* expression from in-situ hybridization

No	Age	Sex	Pathology diagnosis	Stage	[a]SHH/PTCH1/GLI1	SMO	HIP	PDGFRα	Su(Fu)
1	57	F	Squamous cell carcinoma (W)	III	–	±	+	+	+
2	57	M	Squamous cell carcinoma (W)	II	+	+	±	+	+
3	65	M	Squamous cell carcinoma (W)	II	+	+	±	±	+
4	54	M	Squamous cell carcinoma (W)	II	+	±	±	±	+
5	51	M	Squamous cell carcinoma (W)	II	+	±	±	+	+
6	56	F	Squamous cell carcinoma (M)	III	+	+	±	+	N/A
7	64	M	Squamous cell carcinoma (M)	II	–	±	±	+	+
8	52	M	Squamous cell carcinoma (M)	III	+	±	±	±	–
9	47	F	Squamous cell carcinoma (M)	II	–	–	–	–	–
10	64	F	Squamous cell carcinoma (P)	II	+	+	±	+	N/A
11	54	M	Squamous cell carcinoma (P)	III	+	+	±	+	N/A
12	73	M	Squamous cell carcinoma (P)	II	+	–	–	–	–
13	38	F	Squamous cell carcinoma (P)	III	+	+	+	+	N/A
14	55	M	Squamous cell carcinoma (P)	II	–	±	±	±	–
15	45	M	Squamous cell carcinoma (P)	III	+	+	±	±	+
16	60	M	Normal		N/A	–	–	–	–
17	58	M	Normal		N/A	–	–	–	–
18	57	F	Normal		N/A	–	–	–	–
19	64	M	Normal		N/A	–	–	–	–
20	64	M	Normal		N/A	–	–	–	–

[a] The results of *SHH, PTCH1 and GLI1* in-situ hybridization were from our previous study

W well, *M* moderate, *P* poor

were highly expressed in esophageal cancer specimens which contained activation of hedgehog pathway.

Expression of SMO and Su(Fu) in Esophageal Cancers

In addition to HH target genes, we also investigated expression of hedgehog signaling molecules in gastric cancer. We examined expression of *SMO* in 15 cases and *Su(Fu)* in 11 cases of esophageal specimens and 5 normal esophageal tissues using in-situ hybridization. Expressing of *SMO* was found in 13 of the 15 (86.7%) tumor specimens and *Su(Fu)* was expressed in 7 of the 11 (63.6%) tumor tissues (see Table 1 for details). Most of the signal (Blue as indicated by arrows in Fig. 1k, p) was detected in the tumor tissue, not in the stroma (as indicated by arrow heads in Fig. 1k, p). Since the sense probe of *SMO* and *Su(Fu)* did not give any signals, we believe our in-situ hybridization method is very reliable. *SMO* was co-expressed with *HIP* and *PDGFRα* in 13 esophageal cancers. Two cases with expression of *SMO*, *HIP* and *PDGFRα* transcripts lost expression of *Su(Fu)*, indicating that silence of *Su(Fu)* may responsible for HH pathway activating in these two tumors. We did not detect the positive staining of *SMO* and *Su(Fu)* in normal tissues (Fig. 1o, t). We performed RT-PCR to confirm the result of in-situ hybridization (data not show). Taken together, we found elevated expression SMO and Su(Fu) in esophageal cancer which contained activation of hedgehog pathway and there were two specimens showed lost expression of Su (Fu).

Table 2 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

Discussion

It is important to identify activity of hedgehog pathway in cancers to improve diagnosis and treatment. However,

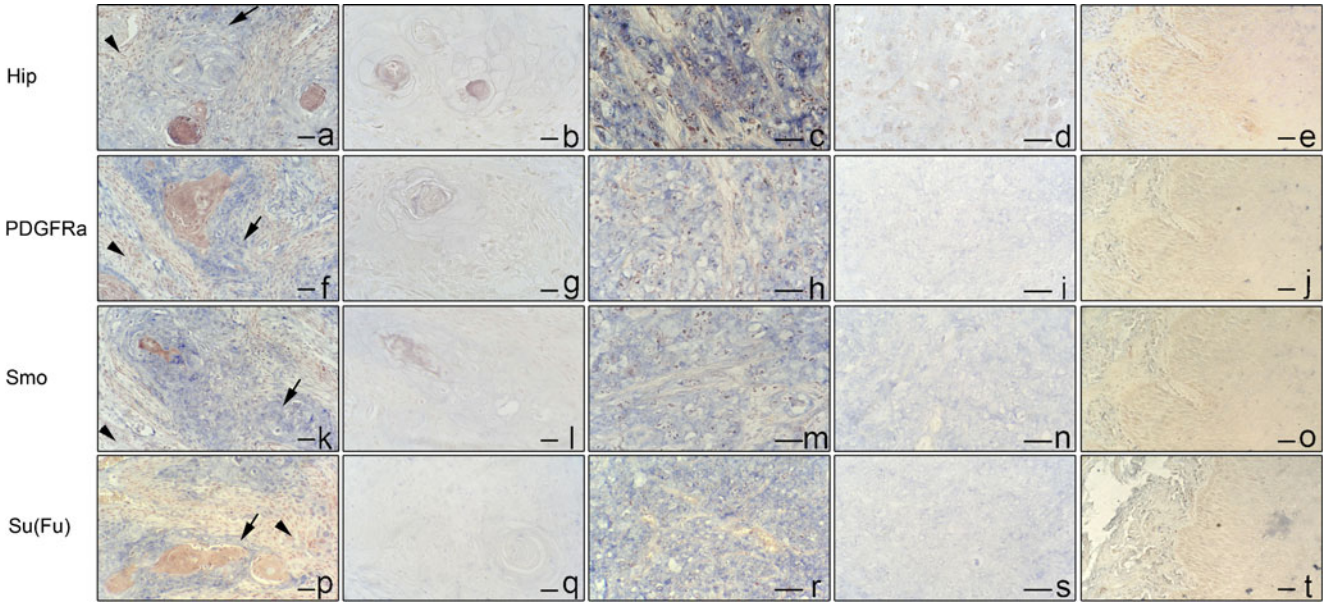

Fig. 1 Expression of *HIP*, *PDGFRα, SMO* and *Su(Fu)* in esophageal tumors. *HIP*, *PDGFRα, SMO* and *Su(Fu)* transcript (blue as positive, indicated by arrows) was detected by in-situ hybridization in normal esophageal tissue (e, j, o, t), well-differentiated Squamous Cell Carcinoma (a, f, k, p) and poorly-differentiated Squamous Cell Carcinoma (c, h, m, r), b, d, g, i, l, n, q and s are their controls with respective sense probe. Bar presents 200 μm

previous studies only detected a few target genes of hedgehog pathway. To better understand expression profile of hedgehog pathway in cancer, we investigate expression of *HIP*, *PDGFRα, SMO* and *Su(Fu)* in esophageal cancer. Our result showed that *HIP* and *PDGFRα* highly expressed in esophageal cancer, which is consistent with expression of *PTCH1* and *Gli1* (Table 1). Our results indicate that HIP and PDGFRα is effective as PTCH1 and Gli1 in identification of activation of hedgehog pathway, which is different from the study we perform in gastric cancer [30]. We found that transcripts of *HIP, Gli1* and *PTCH1* are highly expressed in gastric cancer specimens whereas the *PDGFRα* transcript is detectable only in a subset of cancer with expression of *Gli1, PTCH1* and *HIP.* Further more, the study shows that Gli1 can activate PDGFRα in C3H10T1/2 cells[26]. Various expression of *PDGFRα* in esophageal and gastric cancer suggests that hedgehog pathway functions though different molecule in different type or subtype of cancers. Therefore, identification of mechanism by which PDGFRα is regulated will enrich our understanding in hedgehog-mediate carcinogenesis. Currently, STI571 is used in clinical therapeutics against PDGFRα function[27]. It may shed the light that esophageal cancer with detectable expression of PDGFRα may be eligible for treatment with STI571.

It is reported that transcriptional silencing of HIP protein is found in cancer cell lines and cancer tissues [7]. Our studies did not reveal any reduced expression of *HIP* in tumors with detectable expression of *Gli1* and *PTCH1*, suggesting that silencing of *HIP* in esophageal cancer is not a major mechanism for HH signaling activation.

There are several reports indicating that alterations of HH signaling molecules may be responsible for Hh signaling activation. Su(Fu) is an essential repressor in mammalian hedgehog signaling [9], inhibiting the function of GLI molecules through several mechanisms [28]. Mutations in Su(Fu) have been found in cancer cell lines and tumors [11, 13, 14]. However, we did not find significant alteration in the expression of *Su(Fu)* in esophageal cancer, suggesting that *Su(Fu)* inactivation is not very common in esophageal cancer. SMO expression is elevated in a subset of prostate cancer specimens [29]. Our data showed *SMO* highly expressed in esophageal cancer and expression of *SMO* was consistent with expression of *HIP* and *PDGFRα.* Whether the *SMO* transcript level can be used to detect HH signaling activation in other subtypes of esophageal cancer remains to be determined.

Our study found that *HIP*, *PDGFRα, SMO* and *Su(Fu)* gene are highly expressed in the primary esophageal squamous cell carcinomas. The transcripts of *HIP, PDGFRα* and *SMO* were expressed in 13 of 15 esophageal cancers. *Su(Fu)* expression was missing in 2 esophageal cancer specimens. The results indicate that activation of the hedgehog pathway occurs frequently in esophageal cancers. Our results revealed a set of genes for detecting Hh signaling activation in esophageal cancer.

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