SPANDIDOS PUBLICATIONS

Expression of Sonic hedgehog signaling pathway correlates with the tumorigenesis of intraductal papillary mucinous neoplasm of the pancreas

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Abstract. Sonic hedgehog (*SHH*) is frequently expressed in pre-cancerous lesions and carcinoma of the pancreas. A recent study revealed that its expression was higher in the intraductal papillary mucinous neoplasm (IPMN) of the pancreas than in the pancreatic carcinoma. However, the correlation between its signaling pathway and tumorigenesis of IPMN has not yet been well documented. We investigated the expression of mRNA and protein of *SHH* as well as its downstream transcription factor *Gli1* in 19 microdissected lesions from 15 cases and in 75 lesions from 33 cases of the IPMN by one-step quantitative real-time reverse transcription-polymerase chain reaction and immunohistochemistry, respectively. *SHH* and *Gli1* mRNAs were detected in all the examined lesions and 8 out of 19 lesions in IPMNs, respectively. *SHH* and *Gli1* mRNAs were likely to be up-regulated from the adenoma and from borderline to carcinoma cells, respectively. Immunohistochemical analysis also reported that *SHH* and *Gli1* expression was correlated with the grade of cell atypia. These findings suggested that *HH* signaling was activated in IPMNs and contributed to tumorigenesis in these types of neoplasms.

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Abbreviations: IPMN, intraductal papillary-mucinous tumor of the pancreas; PanIN, pancreatic intraepithelial neoplasm; SHH, sonic hedgehog; QRT-PCR, quantitative real-time reverse transcription-polymerase chain reaction; IPM-A, intraductal papillary-mucinous adenoma; IPM-B, borderline intraductal papillary-mucinous neoplasm; IPM-C, intraductal papillary-mucinous carcinoma; IPMB-C, borderline intraductal papillary-mucinous neoplasm to intraductal papillary-mucinous carcinoma; SMOH, smoothened; PTCH, patched; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; PBS, phosphate-buffered saline

Key words: sonic hedgehog, Gli1, intraductal papillary mucinous neoplasm of the pancreas

Introduction

Intraductal papillary-mucinous neoplasia (IPMN) of the pancreas is a unique neoplasm that is considered to be a pre-cancerous lesion analogous to adenomatous polyps of the colon (1). The basic favorable prognosis of the patients with IPMN suggests that the dysplastic component may remain *in situ* for a long time. We previously demonstrated, however, the activation of oncogenes such as K-*ras* (2,3) and c-*erb* B-2 (4), accumulation of p53 (3) or the expression of a member of the inhibitor of the apoptosis family, survivin (5) and loss of chromosome 18q (6) in IPMN, suggesting the malignant potential of this neoplasm. In addition, stromal infiltration and distal metastasis have been reported even in this type of tumor (7,8). However, it is still difficult to differentiate the malignant from the benign ones in spite of the establishment of various diagnostic methods for IPMNs.

Sonic hedgehog (*SHH*) expression was frequently seen in pancreatic cancer tissues and in pancreatic intraepithelial neoplasia (PanIN) (9), which is considered to be a precursor lesion of pancreatic cancer, whereas it was undetectable in the islets, acini or ductal epithelium from a normal adult pancreas (10). Pancreata from transgenic mice in which *SHH* overexpression was driven by the pancreatic-specific *Pdx-1* promoter showed abnormal tubular structures, a phenotype of human PanIN-1 and -2 (10). In addition, the Hedgehog (*HH*) pathway activation induced by the transfection of immortalized human pancreatic ductal epithelial cells with *Gli*, a downstream mediator of *HH* signaling, up-regulated the majority of foregut markers seen in PanIN lesions (11). These findings indicate that *SHH* mediates pancreatic carcinogenesis from an early to a late stage. On the other hand, a recent study revealed that IPMNs expressed a significantly higher level of

SHH mRNA than pancreatic cancer did and that over-expression of *SHH* was an early event in the development of IPMN (12). However, to our knowledge, the association of *SHH* expression with histological malignancy of IPMN and/or involvement of *Gli* in this type of neoplasm has not yet been well studied. In this study, we investigated the expression of *SHH* and *Gli1* in IPMN tissues in order to assess the role of the hedgehog signaling pathway in tumorigenesis of the IPMN.

Materials and methods

Tissues. The subjects were a total of 41 cases of IPMN who underwent surgery at the department of gastroenterological surgery at Tohoku University Hospital from January 1995 to March 2007. Informed consent was obtained from all patients before surgery. The tissues collected at the time of surgery were immediately snap-frozen in liquid nitrogen and stored at -80˚C or fixed in 10% paraformaldehyde overnight and embedded in paraffin wax. Nineteen lesions from 15 IPMN samples and 33 IPMN tissues were used for quantitative real-time reverse transcription-polymerase chain reaction (QRT-PCR) and immunohistochemistry, respectively. The ductal lesions from IPMNs were histopathologically classified according to the WHO classification with slight modification (2-4): hyperplasia (nonpapillary hyperplasia); intraductal papillary-mucinous adenoma (IPM-A, papillary hyperplasia); borderline intraductal papillary-mucinous neoplasm (IPM-B, atypical hyperplasia) and intraductal papillary-mucinous carcinoma (IPM-C, carcinoma).

Microdissection and one-step quantitative real-time RT-PCR. The frozen tissues embedded in the Tissue-Tek O.C.T. compound medium (Sakura, Tokyo, Japan) were cut into 8 μm sections using a cryostat (Jung CM3000, Lica, Nussloch, Germany) and then fixed in cold methanol and stained with toluidine blue. Histologically hyperplastic (including normal duct), adenoma, borderline and cancerous ductal cells from IPMNs, respectively were dissected using LaserScissors Pro300 (Cell Robotics Inc., Albuquerque, NM) according to the manufacturer's protocols (Fig. 1). These microdissected samples were collected in 350 μl of RLT lysis buffer and extracted using an RNeasy micro kit (Qiagen, Hilden, Germany) with DNase I treatment in accordance with the manufacturer's recommendation. One-step quantitative real-time RT-PCR was performed with a QuantiTect SYBR-Green RT-PCR kit (Qiagen) using a LightCycler (Roche diagnostics, Basel, Switzerland). The primer pairs used were *SHH* (13), forward 5'GAAAGCAGAGAACTCGGTGG3' and reverse 5'GGAAAGTGAGGAAGTCGCTG3'; *Gli1* (14), forward 5'CTCCCGAAGGACAGGTATGTAAC3' and reverse 5'CCCTACTCTTTAGGCACTAGAGTTG3'; GAPDH, forward 5'GGCGTCTTCACCACCATGGAG3' and reverse 5'AAGTTGTCATGGATGACCTTGGC3'. All reactions were performed according to the manufacturer's protocol. The specificity of each PCR reaction was confirmed by melting curve analyses. The level of the target gene expression in each sample was normalized to the respective GAPDH expression level. The normalization was done according to the methods previously reported (15).

Immunohistochemistry. Localization of *SHH* and *Gli1* in IPMNs was investigated by immunohistochemistry. The tissue sections were deparaffinized and antigens were retrieved by boiling the sections in Target retrieval solution (Dako, Carpinteria, CA) in the microwave oven. Then, the sections were incubated in methanol with 0.3% hydrogen peroxide for 30 min in order to block the endogenous peroxidase activity. Thereafter, a histofine kit (Nichirei, Tokyo, Japan) was used. After treatment with 10% serum block solution for 30 min at room temperature, the slides were incubated with the polyclonal goat anti-human *SHH* antibody (Santa Cruz Biotechnology, Inc. Santa Cruz, CA) or the polyclonal rabbit anti-human *Gli1* antibody (Chemicon, Temecula, CA) overnight at 4˚C. After being treated with biotinylated anti-goat or anti-rabbit IgG for 30 min at room temperature, the sections were incubated with peroxidase-conjugated streptavidin for 30 min at room temperature. Between the incubations, the specimens were washed in phosphate-buffered saline (PBS) three times. Visualization of the immunoreaction was carried out in 0.06 mM 3,3'-diamino-benzidine tetrahydrochloride (Dojin, Kumamoto, Japan) containing 2 mM hydrogen peroxide in PBS for several min at room temperature. For the negative control, the immunostaining processes were performed by replacing the primary antibody with PBS. The negative control sections showed no specific immunoreactivity.

The immunostaining of *SHH* was evaluated as positive when >20% of the cells showed immunoreactivity. *Gli1* staining was judged as positive when nuclear expression was detected. The evaluation of immunostaining was done independently by two observers (K.S. and A.K.) who had not been informed of the histological diagnosis.

Statistical analysis. Differences between the mRNA expression of *SHH* or *Gli1* and histological grade of IPMN was analysed by Bonferroni analysis and a p-value <0.017 was considered to be statistically significant. Differences between immunoreactivity of *SHH* or *Gli1* and histological grade of IPMN were analysed by the Chi-square test. P<0.05 was considered to be statistically significant.

Results

As shown in Fig. 1, target lesions were carefully micro-dissected without contamination of different histological lesions and then RNAs were extracted. Thus, this enabled us to compare the accurate levels of RNA among various lesions. Using these samples, we investigated the expression levels of mRNA of *SHH* and *Gli1* in 19 lesions from 15 IPMNs by one-step QRT-PCR. The reliability of this method for evaluating clinical samples was previously reported (12). *SHH* mRNA was detected in all samples examined (Table I). The expression levels of *SHH* mRNA were increased stepwise in hyperplasia, IPM-A and IPM-B to IPM-C (IPMB-C). IPMNB-C lesions expressed significantly higher *SHH* mRNA than those with hyperplastic cells (P=0.01, Fig. 2A), while the mean expression level was higher in IPM-A than in hyperplasia but a significant difference could not be found between these lesions (P=0.095, Fig. 2A). *SHH* mRNA levels did not differ between IPM-A and IPMB-C (P=0.229).

xpression levels of *SHH* and *Gli1* mRNA in microdissected lesions from IPMNs.

Patient No.	Expression level		
	Histological grade	*SHH*	*Gli-1*
1	Hyperplasia	1.33	0
2	Hyperplasia	1.12	0
	IPM-A	7.41	2.18
	IPM-A	3.29	1.32
	IPM-A	2.64	0
3	IPM-B	21.12	1.43
4	IPM-A	18.25	0
5	IPM-A	7.26	0.35
6	IPM-C	12.20	1.49
7	IPM-C	11.08	3.16
8	IPM-A	6.14	0
9	IPM-A	10.24	0
	IPM-C	16.11	2.49
10	IPM-C	13.15	0
11	IPM-C	4.38	3.11
12	IPM-B	1.85	0
13	Hyperplasia	6.04	0
14	Hyperplasia	2.80	0
15	Hyperplasia	0.77	0

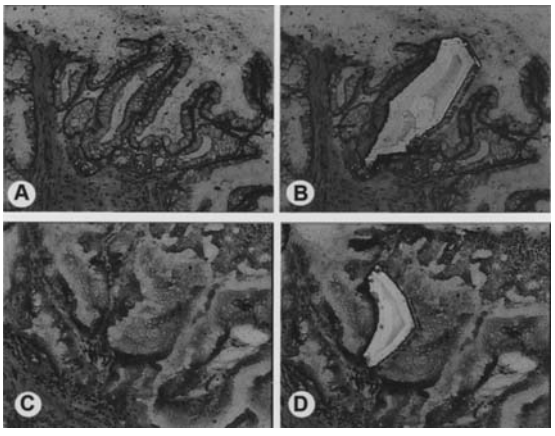

Figure 1. RNA extraction was performed from the microdissected lesions. Toluidine blue stained adenoma (A) and border line lesions (C) were selectively cut by laser, blown off by the large-capacity laser (B and D) and recovered in lysis buffer.

On the other hand, *Gli1* mRNA was detected in less than half of the lesions (8/19, 42.1%) (Table I). The expression of *Gli1* mRNA was frequently detected in IPMB-C (5/7) while less frequently in IPM-A (3/7) or hyperplastic lesions (0/5). The measurement of *Gli1* mRNA expression levels showed that it was not different between hyperplasia and IPM-A (P=0.348, Fig. 2B). IPMNs border line to carcinoma lesions expressed higher *Gli1* mRNA levels than those with adenoma lesions, although this did not reach a statistical significance (P=0.047, Fig. 2B). Significantly higher *Gli1* mRNA expression levels were seen in IPMB-C compared to hyperplastic cells (P=0.009, Fig. 2B).

Since numbers of frozen samples were limited, we employed immunohistochemistry using specific anti-*SHH* and *Gli1* antibody in order to assess the correlation between tumorigenesis of IPMNs and *HH* signaling pathway in a large series of samples. Immunolocalization of *SHH* was basically found in the cytoplasm of vessels and tumor cells, while no or faint immunorectivity was found in non-tumorous ducts, acinar cells or inflammatory cells neighboring tumor cells (Figs. 3A and B). Consistent with the results of QRT-PCR, immunoreactivity of *SHH* was frequently found in 59 out of 75 IMPN lesions (78.7%) and the number of cells expressing *SHH* was increased as the histological grade of malignancy progressed (P=0.00002, Table III). *Gli1* expression was seen occasionally in the cytoplasm of normal duct cells and the cytoplasm and nuclei of tumor cells (Figs. 3C and D). Since transcriptional activity of *Gli1* was increased when localized in the nuclei but not in the cytoplasm (16,17), we regarded nuclear immunoreactivity of *Gli1* as the *HH*-signal activated cells. Immunoreactivity of *Gli1* in the nuclei was found in 30 out of 75 lesions (40%). Nuclear expression of *Gli1* was

Table II. Correlation between the histological grade of intraductal papillary-mucinous neoplasms of the pancreas and *SHH* or *Gli-1* expression.

	Histology							
	Hyperplasia (n=12)		IPM-A (n=29)		IPM-B (n=20)		IPM-C (n=14)	
Expression	*SHH*	*Gli-1*	*SHH*	*Gli-1*	*SHH*	*Gli-1*	*SHH*	*Gli-1*
Negative	8	12	7	21	1	9	0	3
Positive	4	0	22	8	19	11	14	11

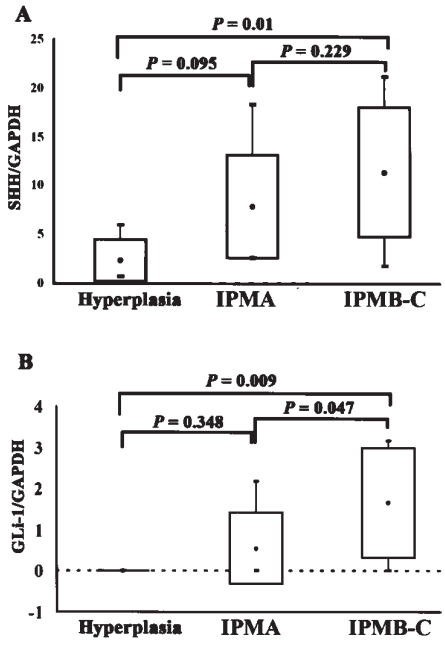

Figure 2. The levels of *SHH* (A) and *Gli* (B) mRNA in microdissected hyperplastic, adenoma (IPM-A) and border line carcinoma (IPMB-C) lesions. Nineteen lesions from 15 frozen samples were microdissected and RNAs were extracted. RNAs were subjected to one-step quantitative real-time RT-PCR and target genes were normalized to GAPDH expression. *SHH* and *Gli1* mRNA expressions were significantly higher in border line carcinoma IPMN (IPMB-C) than hyperplastic lesions (hyperplasia) (P=0.01 and P=0.009, respectively, Bonferroni analysis).

Table III. Relationship between *SHH* or *Gli1* expression and histological grade in intraductal papillary-mucinous neoplasms of the pancreas. P-value was evaluated by the χ^2 test.

	Negative	Positive	P-value
SHH expression			
IPM-hyperplasia	8	4	0.00002
IPM-A	7	22	
IPMB-C	1	33	
Gli1 expression			
IPM-hyperplasia	12	0	0.0001
IPM-A	21	8	
IPMB-C	12	22	

Table IV. Correlation between expression of *Gli1* and *SHH*.

	Gli1		
			P-value[a]
SHH	Negative	Positive	
Negative	15	1	0.00189
Positive	30	29	

[a]Analysed by the χ^2 test.

associated with the histological grade of IPMNs (P=0.0001, Table III). Consistent with the results of QRT-PCR, *Gli1* expression was frequently found in IPMB-C (22/34) but less frequently in IPM-A (8/29) or in hyperplasia (0/12) (Table III). In addition, *Gli1* expression was observed in all but one of the IPM-C lesions where *SHH* was expressed, and was significantly correlated with *SHH* expression (Table IV, P=0.00189).

Discussion

IPMN is distinct from pancreatic cancer because of its intraductal growth in the main pancreatic duct or secondary branches. This type of tumor usually shows better prognosis compared to pancreatic cancer because of its slow growing and rare invasion of the parenchyma (1). However, recent evidence indicates that once IPMN show stromal invasion, it progresses like pancreatic cancer (7,8). Therefore, it is important to know how IPMN obtains an aggressive phenotype. Recently, IPMNs have been demonstrated to express significantly higher *SHH* mRNA levels than pancreatic carcinoma and normal pancreatic epithelial cells, suggesting that *SHH* plays an important role in the development of IPMNs. Therefore, we investigated *Gli1* expression in various IPMN lesions in addition to *SHH* expression in various lesions of IPMNs in order to clarify the role of this pathway in tumorigenesis or the development of these types of tumors. We clearly demonstrated in the current study that *Gli1* was also expressed in IPMNs, suggesting that

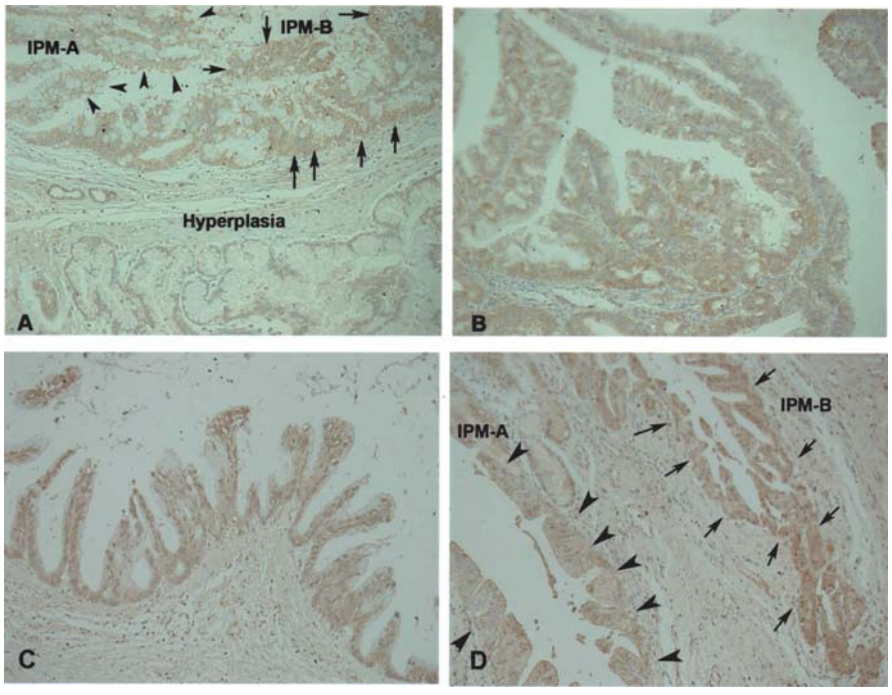

Figure 3. Expression of *SHH* and *Gli1* in intraductal papillary-mucinous tumors (IPMNs). Intense immunoreactivity was observed in cytoplasms of adenoma (IPM-A, arrow head) and border line (IPM-B, arrow) lesions, while no staining was found in hyperplasitic lesions (hyperplasia) (A). Intense immunoreactivity was observed in carcinoma lesions (B). (C), *Gli1* was observed in adenoma lesions. (D), Borderline lesions (IPM-B) show intense nuclear staining and diffuse cytoplasmic staining (arrow) while adenoma lesions (IPM-A) demonstrate only weak cytoplasmic staining (arrow head). *Gli1* was judged as positive when nuclear staining was evident, thus the border line lesion was positive however it was negative in the adenoma lesion.

the *HH* signaling pathway was activated in IPMNs. However, the expression of *Gli1* was less frequently detected than that of *SHH* in IPMNs by both QRT-PCR and immunohistochemistry, indicating that the *SHH* signaling pathway was not always activated in the lesions where the ligand was already expressed. For example, QRT-PCR and immunohistochemical analysis reported that *Gli1* was not expressed in hyperplasia while mRNA expression or immunoreactivity of *SHH* was detected. Similarly, more than half of IPM-A expressed *SHH* alone. It is likely that mRNA of *SHH* and *Gli1* was up-regulated during the progression from hyperplasia to IPMA (P=0.095) and from IPMA to IPMB-C (P=0.047), respectively, suggesting that overexpression of *SHH* is an earlier event than that of *Gli1* in tumorigenesis of IPMN. Taken together, these findings suggest that stimulation of the signaling pathway by the ligand expression may occur during the progression from hyperplasia to adenoma and consequently, activation of the downstream pathway may facilitate the transition from benign to malignant IPMNs.

The hedgehog pathway plays a pivotal role in the development of embryonic organs, including pancreas and tissue polarity (18-20). Hedgehog proteins act thorough the transmembrane proteins *PTCH* and *SMOH*, which regulate the transcriptional activity of *Gli* zinc-finger transcription factors (21,22). Abnormal activation of the hedgehog pathway is involved in a number of tumors, such as basal skin cell carcinomas, medulloblastomas, lung cancers, prostate cancers and pancreatic cancers (23-25). Activation of Hedgehog signaling, through loss-of-function mutations of *PTCH* or activated mutations of *SMOH*, occurs frequently in human basal carcinoma and medulloblastomas (26,27). On the other hand, expression of *PTCH* and *Gli* within cells from several types of digestive tract tumors, including pancreatic cancer that also expresses hedgehog ligands suggests the autonomous operation of an active signaling process (28). In addition, recent studies revealed that the *HH* signaling pathway co-operated with oncogenic *ras* signaling in order to contribute to pancreatic carcinogenesis (29-31) and that this oncogenic *ras* activated the *HH* pathway in a ligand-independent manner (31). In this context, the *HH* signaling pathway seems to be dominantly activated in an autocrine or paracraine manner in IPMNs since *Gli1* was expressed in the lesions where *SHH* expression was observed. However, an alternative mechanism for the activation of the *HH* pathway by oncogenic K-*ras* (31) may also be a possible mechanism for the activation of the *HH* pathway in IPMNs because we found one IPM-C lesion expressing *Gli1* but not *SHH* whereas K-*ras* mutation was frequently detected (2,3).

In summary, we demonstrated that the *HH* pathway was frequently activated in malignant IPMNs, suggesting that the activation of the *HH* signaling pathway contributes to the transition from a benign to a malignant phenotype of IPMNs. In addition, the measurement of *SHH* or *Gli1* mRNA levels may be a useful tool in distinguishing tumorous lesions from hyperplastic lesions in IPMNs.

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