Polish Journal of Pathology (2016.6) 67(2):136-144.

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Original paper

Gli2 protein expression level is a feasible marker of ligand-dependent hedgehog activation in pancreatic neoplasms

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The hedgehog pathway is known to promote proliferation of pancreatic ductal adenocarcinoma (PDA) and has been shown to restrain tumor progression. To understand how hedgehog causes these effects, we sought to carefully examine protein expression of hedgehog signaling components during different tumor stages. Genetically engineered mice, *Pdx1-Cre;LSL-Kras^{G12D}* and *Pdx1-Cre;LSL-Kras^{G12D};p53$^{lox/+}$*, were utilized to model distinct phases of tumorigenesis, pancreatic intraepithelial neoplasm (PanIN) and PDA. Human pancreatic specimens of intraductal papillary mucinous neoplasm (IPMN) and PDA were also employed. PanIN and IPMN lesions highly express Sonic Hedgehog, at a level that is slightly higher than that observed in PDA. GLI2 protein is also expressed in both PanIN/IPMN and PDA. Although there was no difference in the nuclear staining, the cytoplasmic GLI2 level in PDA was modest in comparison to that in PanIN/IPMN. Hedgehog interacting protein was strongly expressed in the precursors, whereas the level in PDA was significantly attenuated. There were no differences in expression of Patched1 at early and late stages. Finally, a strong correlation between Sonic Hedgehog and GLI2 staining was found in both human and murine pancreatic tumors. The results indicate that the GLI2 protein level could serve as a feasible marker of ligand-dependent hedgehog activation in pancreatic neoplasms.

Key words: hedgehog, Gli2 protein, carcinoma, pancreatic ductal, pancreatic neoplasms, carcinogenesis.

Introduction

Pancreatic ductal adenocarcinoma (PDA) is the fourth and fifth leading cause of cancer-related death in the United States and Japan, respectively. At present, its 5-year overall survival rate is less than 10% [1, 2]. Since the majority of PDA cases are diagnosed at the advanced or metastatic stages of disease, PDA is generally treated with chemotherapy rather than surgical intervention. Gemcitabine, one of the stan-

dard treatments for PDA, is the first anticancer drug to effectively reduce the clinical symptoms associated with the disease and to achieve a modest survival benefit [3, 4]. Some options, such as tegafur/gimeracil/oteracil [5] and capecitabine [6], have offered consistent improvement in patient outcomes. More recently, regimens such as FOLFIRINOX [7] and albumin-bound paclitaxel plus gemcitabine [8] represent a 'new era' of treatment for PDA patients. These new options improve survival, and serve as neoadjuvant chemotherapy for locally advanced and borderline resectable disease [9, 10, 11]. The identification of rational therapeutic strategies based on the diversity of the molecular signature is therefore warranted.

Hedgehog (Hh) protein is a morphogen that is required for proper pattern formation during embryonic development, including the organogenesis of the pancreas [12, 13]. The aberrant activation of the Hh signal is one of the core pathways in pancreatic cancer, and it has been considered to be a promising therapeutic target [13]. Sonic hedgehog (SHH) is highly expressed in tumor cells [14], and overexpression of the ligand in mice was found to induce pancreatic intraductal neoplasm (PanIN), a PDA precursor lesion [15]. The authors and other researchers have demonstrated that inhibition of the Hh signaling pathway reduces or suppresses growth of PDA and that SHH primarily acts on the stromal compartment of the tumor [16, 17, 18, 19]. However, despite being demonstrated to have significant anti-tumor functions in pre-clinical animal models, in clinical trials, the Hh inhibitor vismodegib in PDA was not found to improve the response of an unselected cohort [20]. The discrepancy between the expectations that were based on the pre-clinical model and the results of the clinical trial calls for a revision of our theoretical views on the signaling pathways at different stages of tumorigenesis.

SHH, one of three homologs of Hh, is aberrantly expressed, not only in PDA, but also in precursor lesions including PanIN [15] and intraductal papillary mucinous neoplasm (IPMN) of the pancreas [21]. However, the difference in the expression levels of the Hh ligand and other molecules associated with the pathway in the earlier and later stages of pancreatic tumorigenesis have remained to be elucidated. We employed two genetically engineered mouse models and human resected specimens to study the expression of these proteins.

Material and methods

Strains of genetically engineered mice: PanIN and PDA models

All of the animal experiment protocols were approved by the Institutional Animal Care and Use Committee of Asahikawa Medical University. We used two genetically engineered mouse models, which were created with the modification of several oncogenic genes [22]. Briefly, the mouse strains used in this study included the following alleles: *Pdx1-Cre*, *LSL-Kras^{G12D}*, and *p53^{lox}*. The *Pdx1-Cre* mice intercrossed with *LSL-Kras^{G12D}* (*Pdx1-Cre;LSL-Kras^{G12D}*) developed non-invasive PanIN precursor lesions, which provide a model of early pancreatic tumorigenesis. The further introduction of *p53^{lox}* (*Pdx1-Cre;LSL-Kras^{G12D};p53$^{lox/+}$*) leads to predominantly ductal adenocarcinomas, which are defined by the presence of neoplastic glandular/ductal cells in a dense fibrous stroma, providing a model of invasive PDA. All experiments were performed on an FVB/n background. The mice in the PanIN and PDA models were sacrificed at 15-24 weeks and 12 weeks of age, respectively.

Human pancreatic tissue samples

Surgically resected pancreatic specimens were collected from 9 patients with benign and non-invasive IPMN (male, n = 8; female, n = 1; mean age, 68.3 years [range: 53-80 years]), and 17 patients with invasive PDA (male, n = 8; female, n = 9; mean age, 68.6 years [range: 42-83 years]) after obtaining approval from the research ethics committee of Asahikawa Medical University. The patient classifications and histological grade of the tumors are summarized in Table I.

Immunohistochemical analysis

Formalin-fixed and paraffin-embedded tumor specimens from human samples were utilized for the histological assessments. Tumor specimens from mice were fixed in zinc fixative solution (IHC ZINC fixative, BD Biosciences, San Jose, CA, USA) for 24-36 hours at room temperature, and subsequently embedded in paraffin.

Four-μm-thick specimens were deparaffinized and rehydrated in progressively decreasing concentrations of ethanol. The antigens of the formalin-fixed human tumor specimens were retrieved by boiling the tissue sections in Target Retrieval Solution (DAKO, Glostrup, Denmark) for 10-20 min in a microwave oven. No pretreatment was performed for zinc-fixed mouse tumor specimens. The sections were then incubated with phosphate buffered saline supplemented with 5% bovine serum albumin (Wako Pure Chemical Industries Ltd., Tokyo, Japan) and 0.1% Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) for 1 hour at room temperature to block the non-specific binding sites. A VECTOR M. O. M. Immunodetection Kit (Vector Laboratories, Inc., Burlingame, CA) was also used for the analysis of mouse specimens.

Table I. Patient characteristics

	Pancreatic tumor	
	IPMN (n = 9)	**PDA** (n = 17)
Mean age (range)	68.3 (53-80)	68.6 (42-83)
Male : Female	8 : 1	8 : 9
Location of tumor	Pancreatic head: 6	Pancreatic head: 8
	Pancreatic head and body: 1	Pancreatic body: 5
	Pancreatic body and tail: 1	Pancreatic tail: 2
	Whole pancreas: 1	Pancreatic body and tail: 2
Operative procedure	Pancreaticoduodenectomy: 6	Pancreaticoduodenectomy: 8
	Distal pancreatectomy: 2	Distal pancreatectomy: 9
	Segmental pancreatectomy: 1	
Pathological findings	Adenoma: 6	Moderately differentiated PDA: 14
	Borderline malignancy: 1	Poorly differentiated PDA: 2
	Non-invasive carcinoma: 2	Undifferentiated carcinoma: 1

IPMN – intraductal papillary mucinous neoplasm; PDA – pancreatic ductal adenocarcinoma

Immunohistochemical staining was performed using rabbit anti-SHH (1 : 50 for mouse specimens, 1 : 200 for human specimens, Santa Cruz Biotechnology, Inc, Santa Cruz, CA), rabbit anti-Gli2 (for mouse specimens: 1 : 200 [Abcam, Cambridge, UK]; for human specimens: 1 : 200 [NOVUS Biologicals, LLC, Littleton, Co]), rabbit anti-PTCH1 (1:200 [NOVUS Biologicals, LLC]), rabbit anti-Hip (for mouse specimens: 1 : 200 [Abcam]; for human specimens: 1 : 200 [Thermo Fisher Scientific Inc., Waltham, MA]) and mouse anti-PCK26 (1 : 200 or 400 [Sigma Aldrich, St. Louis, MO]) antibodies. For the secondary antibody, highly cross-absorbed Alexa Flour 594-conjugated goat anti-rabbit IgG, or Alexa Flour 488-conjugated goat anti-mouse IgG (Life Technologies, Carlsbad, CA) was utilized. Nuclei were counterstained with 100 ng/ml DAPI (Sigma Aldrich). The sections were mounted in an aqueous mounting medium.

The images were examined with a fluorescence microscope (BV9000, Keyence, Osaka, Japan). The ImageJ software program (version 1.38) was used to quantify the mean intensity of staining in 4 to 10 random viable fields (20× objective).

Statistical analysis

All results are expressed as the means ± SEM unless otherwise noted. The statistical significance of differences was determined using the Mann-Whitney U test or Kruskal-Wallis test followed by Dunn's multiple comparison test. The correlation between SHH and Hh signaling was analyzed with Pearson's correlation coefficient. All statistical analyses were performed using the Prism software package (version 5.0). P values of less than 0.05 were considered to be statistically significant.

Results

Aberrant expression of SHH was observed in both the earlier and later stages of murine and human pancreatic neoplasms

In order to determine the stage of PDA in which Hh signaling is activated, we used pancreatic tissues from two strains of genetically engineered mice: *Pdx1-Cre;LSL-Kras^{G12D}*, which spontaneously develop non-invasive PanIN precursor lesions; and *Pdx1-Cre;LSL-Kras^{G12D};p53$^{lox/+}$*, which develop invasive PDA with a dense fibrous stroma [22]. SHH expression was observed in the PanIN and PDA lesions but not in normal acinar cells as reported previously [15] (Fig. 1A). There was no significant difference in the intensity of SHH staining in the PanIN and PDA mouse models (Fig. 1E).

We next sought to evaluate whether aberrant SHH expression was observed in human tissue. Since it is generally difficult to obtain PanIN specimens, IPMN tissues were employed as a human model for the early stage of pancreatic tumorigenesis. Benign or non-invasive, branched-type IPMN specimens (n = 9) were selected and immunostaining patterns were compared with classic PDA with abundant desmoplasia (n = 17) (Table I). The aberrant expression of SHH was observed in both IPMN and PDA lesions (Fig. 2A); there was no significant difference in the intensity of SHH staining (Fig. 2E).

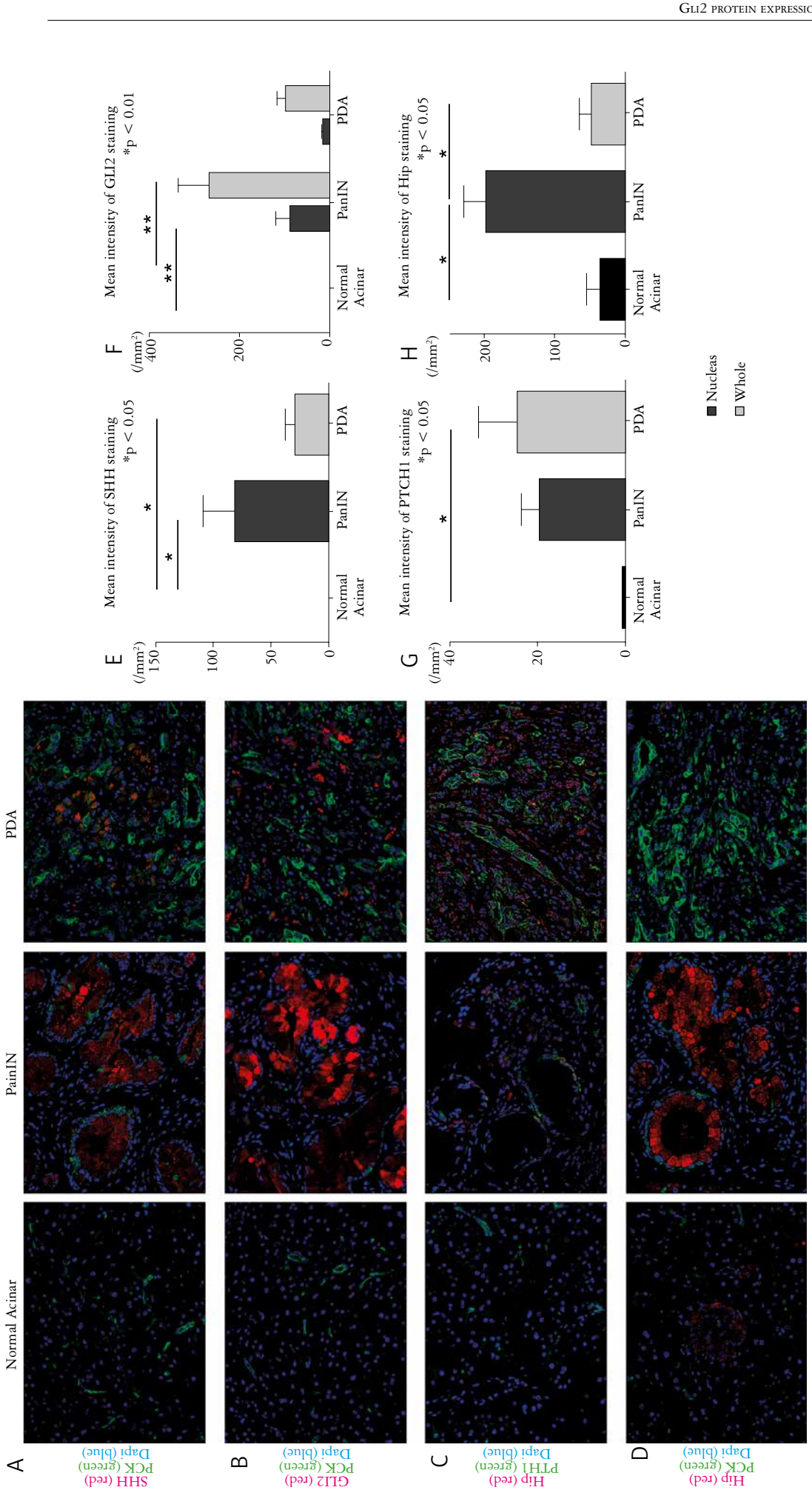

Fig. 1. Immunohistochemical analysis of Sonic hedgehog and key Hedgehog pathway components using genetically engineered mice. A–D) Immunohistochemical staining was performed on tissue sections of normal acinar cells from a wild-type mouse (left column, n = 4), PanIN lesions from a Pdx1-Cre;LSL-Kras^{G12D} mouse (center column, n = 7 for SHH, Gli2 and Hip staining, n = 5 for PTCH1 staining) and PDA lesions from a Pdx1-Cre;LSL-Kras^{G12D};p53$^{lox/+}$ mouse (right column, n = 6 for SHH, Gli2 and Hip staining, n = 5 for PTCH1 staining). Representative images obtained using anti-SHH (A, red), anti-Gli2 (B, red), anti-PTCH1 (C, red), anti-Hip (D, red) and anti-PCK26 (green) counterstained with DAPI (blue: counterstaining) are shown. E–H) Mean intensity of SHH and key Hh pathway components was quantified using the ImageJ software program. E) There was no significant difference in the intensity of SHH staining in the PanIN and PDA mouse models. F) Intensity of Gli2 staining was weaker in PDA lesions than in PanIN lesions; however, the difference was not significant. G) There was no significant difference in the intensity of PTCH1 staining in PanIN and PDA lesions. H) The intensity of Hip staining was significantly stronger in PanIN lesions than in PDA lesions

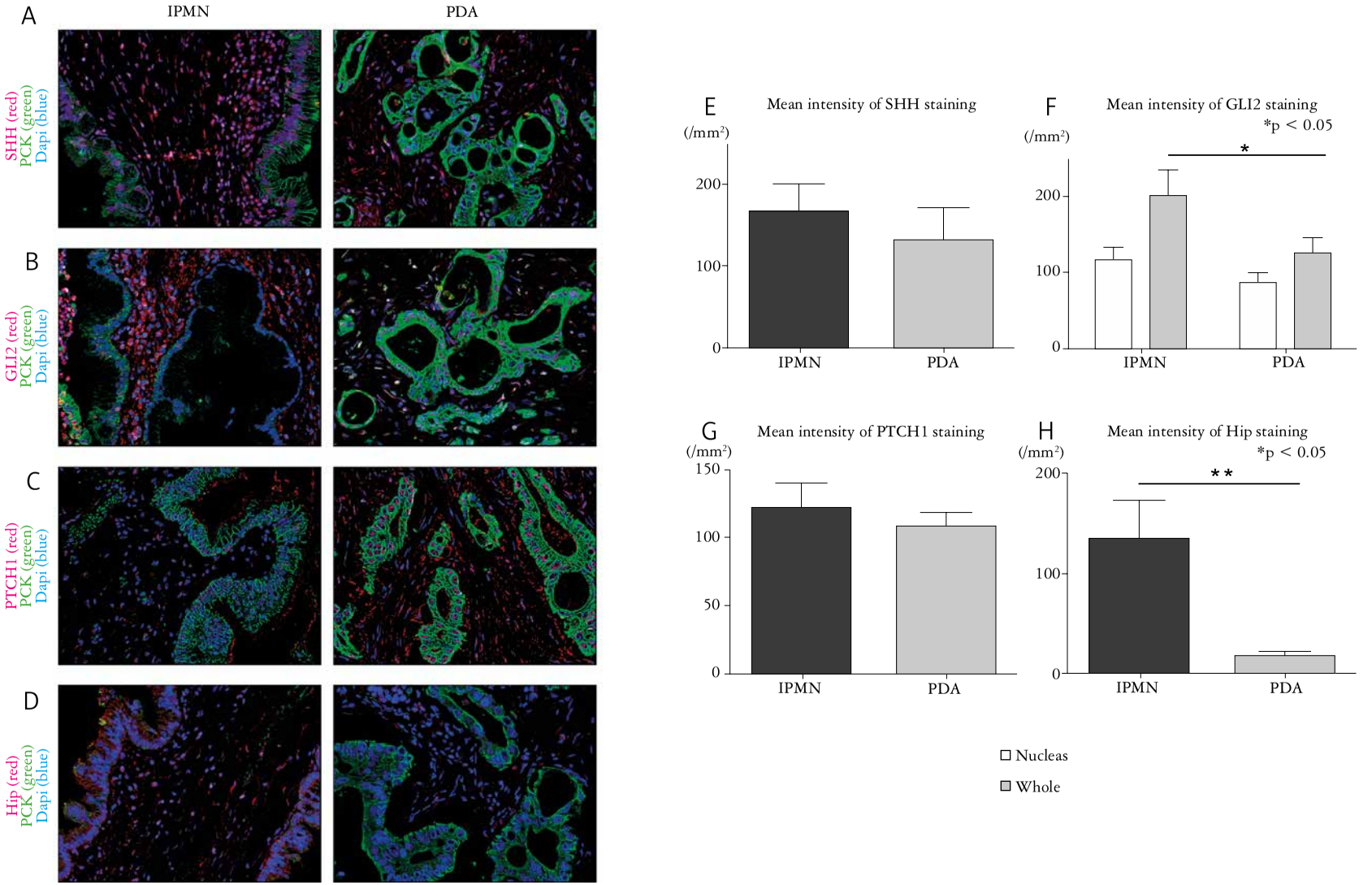

Fig. 2. Immunohistochemical analysis of Sonic hedgehog and key Hedgehog pathway components using human pancreatic tissue samples. A–D) Immunohistochemical staining was performed on surgically resected human pancreatic tissue sections of benign or non-invasive IPMN (n = 9, left column) and invasive PDA (n = 17, right column). Representative images obtained using anti-SHH (A, red), anti-Gli2 (B, red), anti-PTCH1 (C, red) and anti-Hip (D, red) and anti-PCK26 (green) counterstained with DAPI (blue: counterstaining) are shown. E–H) Mean intensity of SHH and key Hh pathway components was quantified using the ImageJ software program. E) Aberrant expression of SHH was observed in both IPMN and PDA lesions. F) Expression of GLI2 was significantly weaker in PDA lesions than in IPMN lesions. However, there was no marked difference in the nuclear translocation of GKI2. G) No difference was observed in the expression of PTCH1. H) The expression of Hip was significantly reduced in PDA in comparison to IPMN

Hh signaling activation was observed in the earlier stage but downregulated in the later stage of pancreatic tumorigenesis

Since Gli transcription factors are major mediators of the Hh pathway [23, 24, 25], we next performed an immunohistochemical analysis of Gli expression using genetically engineered mouse specimens. Aberrant expression of Gli2 was observed in PanIN and PDA lesions but not in the normal pancreas (Fig. 1B). The intensity of Gli2 staining was weaker in PDA lesions than in PanIN lesions; however, the difference was not significant (Fig. 1F). There was no difference in Gli2 nuclear staining.

PTCH1 and Hip, which affect Hh signaling through negative feedback [26, 27], are also considered to be indicators of the activation of Hh signaling [15, 28]. We therefore evaluated the protein expression of PTCH1 and Hip in genetically engineered mouse specimens. The expression of PTCH1 was observed in PanIN and PDA lesions but not in the normal pancreas (Fig. 1C). On the other hand, the expression of Hip was weak in the islet cells of the normal pancreas and strong in PanIN lesions (Fig. 1D), which is consistent with the results of a previous study [29]. Curiously, the intensity of Hip staining was significantly stronger in PanIN lesions than in PDA lesions. There was no significant difference in the intensity of PTCH1 staining in PanIN and PDA lesions (Fig. 1G, H).

In human specimens, the expression of GLI2 was significantly weaker in PDA lesions than in IPMN lesions (Fig. 2B). However there was no marked difference in the nuclear translocation of GKI2 (Fig. 2F), suggesting higher expression of GLI2 in the cytosol of IPMN lesions in comparison to PDA lesions. Similar to the murine samples, the expression of Hip was significantly reduced in PDA in comparison to IPMN (Fig. 2D, H). No difference was observed in the expression of PTCH1 in the two types of tumors (Fig. 2C, G).

Gli2 protein expression was a relevant indicator of the ligand-dependent activation of Hh signaling

The expression of key components of the Hh pathway were altered in PDA in comparison to precursor lesions, suggesting that the ligand-dependent activation of Hh signaling may be differentially regulated in the early and late stages of pancreatic tumorigenesis. We therefore examined the correlation between

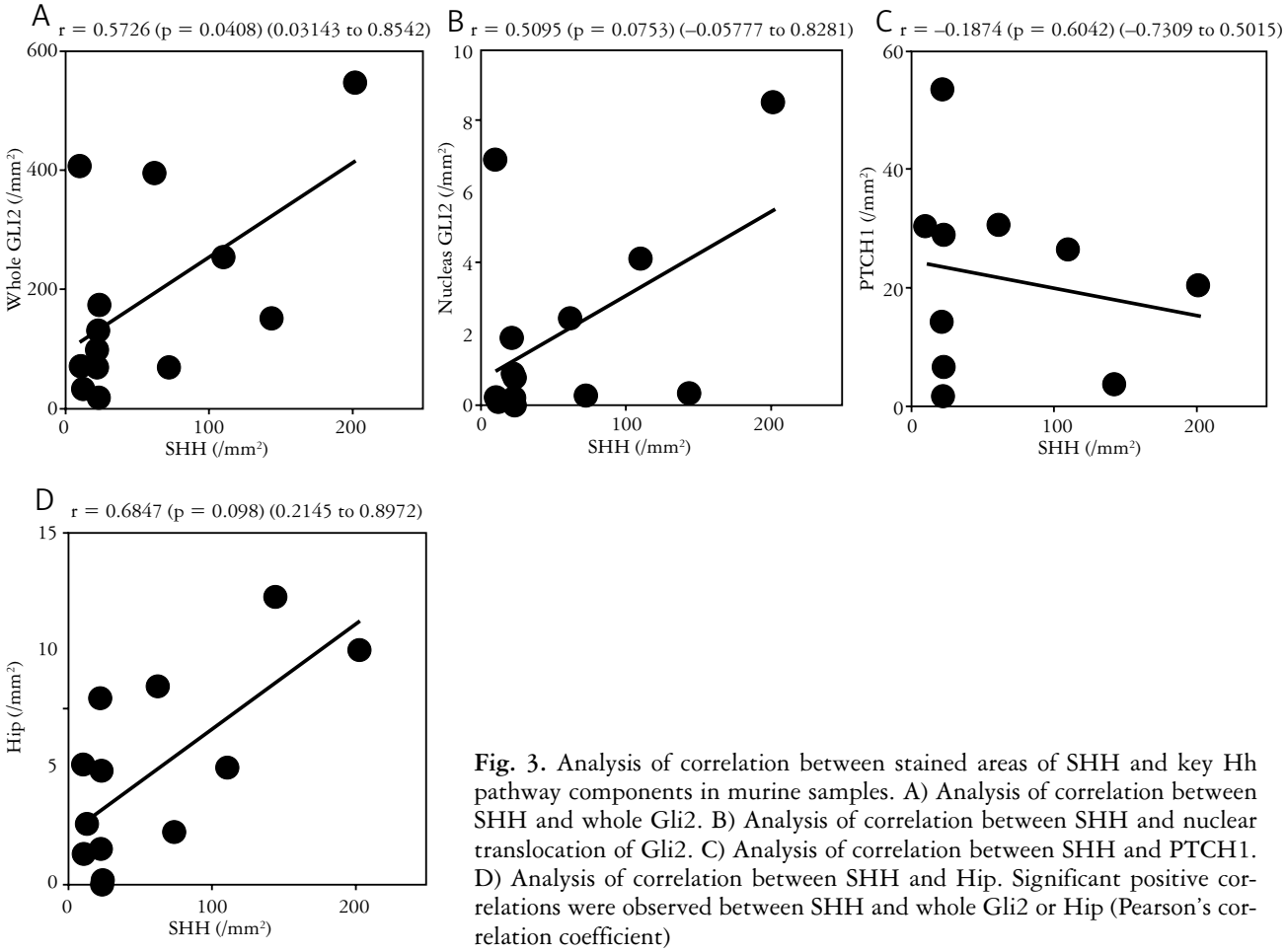

Fig. 3. Analysis of correlation between stained areas of SHH and key Hh pathway components in murine samples. A) Analysis of correlation between SHH and whole Gli2. B) Analysis of correlation between SHH and nuclear translocation of Gli2. C) Analysis of correlation between SHH and PTCH1. D) Analysis of correlation between SHH and Hip. Significant positive correlations were observed between SHH and whole Gli2 or Hip (Pearson's correlation coefficient)

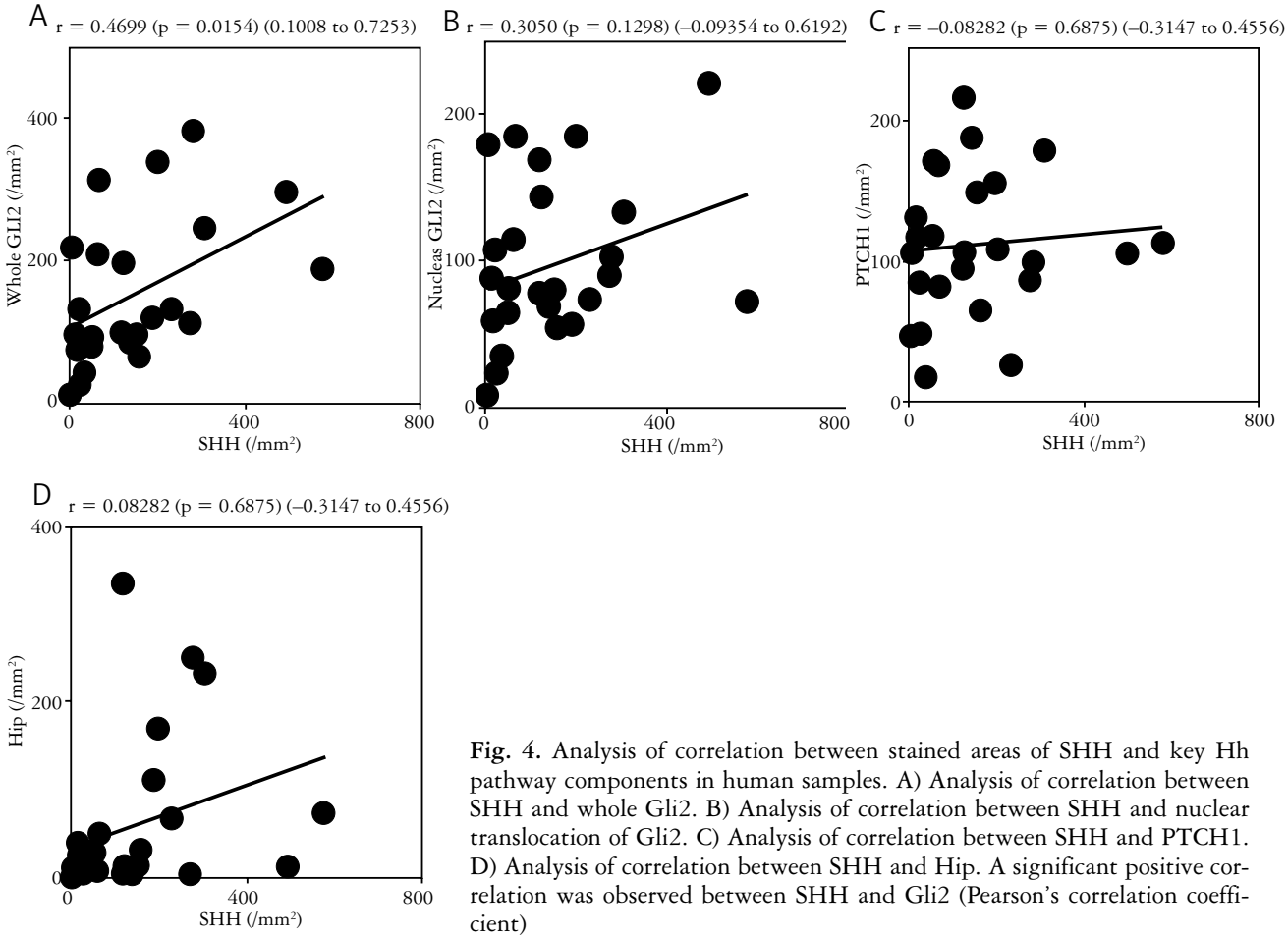

Fig. 4. Analysis of correlation between stained areas of SHH and key Hh pathway components in human samples. A) Analysis of correlation between SHH and whole Gli2. B) Analysis of correlation between SHH and nuclear translocation of Gli2. C) Analysis of correlation between SHH and PTCH1. D) Analysis of correlation between SHH and Hip. A significant positive correlation was observed between SHH and Gli2 (Pearson's correlation coefficient)

the SHH ligand and Hh signaling. Because there was almost no expression of SHH or Hh signaling in the normal pancreas (both in acinar cells and in ductal compartments), this analysis was performed in mouse PanIN and PDA specimens. Significant positive correlations were found between SHH and Gli2 (Pearson's R = 0.5726) and between SHH and Hip (R = 0.6847). No correlation was found between SHH and PTCH1 (Fig. 3). In human specimens, a significant positive correlation was also observed between SHH and GLI2 (R = 0.4699), while neither Hip nor PTCH1 was correlated with SHH expression (Fig. 4). These results indicated that the Gli2 protein level was a relevant indicator of the ligand-dependent activation of Hh signaling.

Discussion

We performed immunohistochemical analyses of the Hh pathway in pancreatic pre-cancerous lesions and invasive PDAs and compared the results. The intensity of Hip staining was significantly weaker in PDA lesions in comparison to precursor lesions. There were no significant differences in the intensity of the SHH and PTCH1 staining. The intensity of Gli2 staining was significantly reduced in human PDA in comparison to IPMN specimens, whereas there was no difference in the nuclear staining of the two histotypes.

Numerous PanIN-like lesions developed in the pancreas of transgenic mice in which the aberrant expression of SHH was driven by the Pdx1 promoter, suggesting a tumor-initiating function of the Hh pathway in pancreatic progenitor cells [15]. However, invasive PDAs were not observed in the Pdx1-SHH transgenic mice due to their short lifespan; thus the model is not feasible for proving the role of SHH during progression to the formation of invasive tumors [30]. Another transgenic model in which Gli2 is conditionally activated together with oncogenic *Kras*, specifically in the pancreatic epithelium, develops undifferentiated carcinoma [30]. However, in these models, it is not evident whether the Gli signal is mediated through the SHH-dependent canonical pathway. In the current study, the Gli2 protein in PDA was not upregulated relative to PanIN/IPMN. Instead, cytoplasmic Gli2 staining was observed to be more abundant in the precursor lesions, indicating that ligand-dependent Hh signaling may be active in the early phase of pancreatic tumorigenesis, whereas alternative ligand-independent signals mainly regulate Gli signatures in the advanced stages. Further

studies are required to differentiate the role of the ligand-dependent Hh signals in precursor lesions from the role in the later stages.

Hip is a negative regulator of the Hh pathway [27]. It can bind to all three mammalian Hedgehog ligands (SHH, Indian Hedgehog, Desert Hedgehog) with an affinity similar to that of PTCH1 [27]. Consistent with a previous report, which showed the localization of human Hip in the normal and diseased pancreas [29], we observed weak staining of Hip in normal islet cells in our present study. Although Hip-positive staining was observed in PDA lesions [29], another study demonstrated that Hip expression was downregulated in several human cancers of the liver, lung, stomach, and colon [31]. Gene silencing, mediated through methylation, was hypothesized to be responsible for Hip downregulation [32, 33], potentially resulting in activation of the ligand-dependent pathway. Our data clearly support the hypothesis that Hip is expressed in benign/pre-cancerous tumors (PanIN and IPMN) but not in invasive PDA lesions. In addition, a recent study demonstrated that Hip can block the paracrine but not cell-autonomous (ligand-dependent) activation of the Hh signaling pathway [34]. The downregulation of Hip in the advanced stage of PDA may induce the canonical Hh signaling pathway in the stromal components.

We found a positive correlation between SHH and Gli2 protein expression in pancreatic neoplasms. This was particularly obvious in human tumors, in which a closer relationship was observed between SHH and cytoplasmic Gli2 staining. It should be noted that the Gli2 transcriptional program is associated with an undifferentiated phenotype of PDA, and that there was no clear difference in the nuclear GLI2 staining of benign IPMNs and invasive PDAs. Oncogenic *Kras* [35] and TGF-β-Smad3 signaling [36] have been shown to induce the Gli1 transcriptional machinery independently of the Hh ligand. Furthermore, Gli2 protein is subject to other forms of complex post-translational regulation, such as phosphorylation [37]. The cytoplasmic expression of Gli2 in SHH-overexpressing neoplasms may reflect the protein stability associated with the Hh ligand-dependent pathway. Our data indicate that the Gli2 protein is a precise marker of canonical Hh signaling in pancreatic tumors.

Although clinical trials targeting Hh signaling in pancreatic cancer patients have failed, the impact of the pharmacological inhibition of Hh signaling on tumor cells may therefore be dependent on stage and histotype. The expression of Gli2 protein, particularly in the cytoplasm, may be a feasible marker for predicting the effect of vismodegib. Further *ex vivo* analysis, which utilizes patient-derived tumor cells, will be required to clarify the SHH-dependent signatures.

In conclusion, our data demonstrated a significant positive correlation between SHH and Gli2, both in human and murine pancreatic neoplasms. The results suggest that Gli2 expression level may be a feasible marker of ligand-dependent Hh activation.

Acknowledgements

We thank Norihiko Shimizu (Asahikawa Medical University) for his support in maintaining the genetically engineered mice. The authors are also thankful to Rushika Perera (University of California, San Francisco) and Nabeel Bardeesy (Massachusetts General Hospital Cancer Center) for critical reading of the manuscript and helpful discussions.

The authors declare no conflict of interest.

This work was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (J.S.; 23701042, Y.M.; 22590754, K.K; 23790759). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Matsuno S, Egawa S, Fukuyama S, et al. Pancreatic Cancer Registry in Japan: 20 years of experience. Pancreas 2004; 28: 219-230.
2. Jemal A, Siegel R, Ward E, et al. Cancer statistics, 2008. CA Cancer J Clin 2008; 58: 71-96.
3. Burris HA 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 1997; 15: 2403-2413.
4. Moore MJ, Goldstein D, Hamm J, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. J Clin Oncol 2007; 25: 1960-1966.
5. Ueno H, Ioka T, Ikeda M, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. J Clin Oncol 2013; 31: 1640-1648.
6. Cunningham D, Chau I, Stocken DD, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. J Clin Oncol 2009; 27: 5513-5518.
7. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. N Engl J Med 2011; 364: 1817-1825.
8. Goldstein D, El-Maraghi RH, Hammel P, et al. nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. J Natl Cancer Inst 2015; 107: pii: dju413.
9. Motoi F, Ishida K, Fujishima F, et al. Neoadjuvant chemotherapy with gemcitabine and S-1 for resectable and borderline pancreatic ductal adenocarcinoma: results from a prospective multi-institutional phase 2 trial. Ann Surg Oncol 2013; 20: 3794-3801.
10. Kim EJ, Ben-Josef E, Herman JM, et al. A multi-institutional phase 2 study of neoadjuvant gemcitabine and oxaliplatin with

radiation therapy in patients with pancreatic cancer. Cancer 2013; 119: 2692-700.
11. Pipas JM, Zaki BI, McGowan MM, et al. Neoadjuvant cetuximab, twice-weekly gemcitabine, and intensity-modulated radiotherapy (IMRT) in patients with pancreatic adenocarcinoma. Ann Oncol 2012; 23: 2820-2827.
12. Apelqvist A, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Curr Biol 1997; 7: 801-804.
13. Jones S, Zhang X, Parsons DW, et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 2008; 321: 1801-1806.
14. Hidalgo M, Maitra A. The hedgehog pathway and pancreatic cancer. N Eng J Med 2009; 361: 2094-2096.
15. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. Nature 2003; 425: 851-856.
16. Nakamura K, Sasajima J, Mizukami Y, et al. Hedgehog promotes neovascularization in pancreatic cancers by regulating Ang-1 and IGF-1 expression in bone-marrow derived pro-angiogenic cells. PloS one 2010; 5: e8824.
17. Olive KP, Jacobetz MA, Davidson CJ, et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 2009; 324: 1457-1461.
18. Tian H, Callahan CA, DuPree KJ, et al. Hedgehog signaling is restricted to the stromal compartment during pancreatic carcinogenesis. Proc Natl Acad Sci USA 2009; 106: 4254-4259.
19. Bailey JM, Swanson BJ, Hamada T, et al. Sonic hedgehog promotes desmoplasia in pancreatic cancer. Clin Cancer Res 2008; 14: 5995-6004.
20. Catenacci DV, Bahary N, Nattam SR, et al. Final analysis of a phase IB/randomized phase 2 study of gemcitabine (G) plus placebo (P) or vismodegib (V), a hedgehog (Hh) pathway inhibitor, in patients (pts) with metastatic pancreatic cancer (PC): A University of Chicago phase 2 consortium study. 2013 ASCO Annual Meeting 2013. J Clin Oncol 2013; 31 (suppl; abstr 4012).
21. Ohuchida K, Mizumoto K, Fujita H, et al. Sonic hedgehog is an early developmental marker of intraductal papillary mucinous neoplasms: clinical implications of mRNA levels in pancreatic juice. J Pathol 2006; 210: 42-48.
22. Bardeesy N, Aguirre AJ, Chu GC, et al. Both p16(Ink4a) and the p19(Arf)-p53 pathway constrain progression of pancreatic adenocarcinoma in the mouse. Proc Natl Acad Sci U S A 2006; 103: 5947-5952.
23. Haycraft CJ, Banizs B, Aydin-Son Y et al. Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS genetics 2005; 1: e53.
24. Bai CB, Auerbach W, Lee JS, et al. Gli2, but not Gli1, is required for initial Shh signaling and ectopic activation of the Shh pathway. Development 2002; 129: 4753-4761.
25. Park HL, Bai C, Platt KA, et al. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 2000; 127: 1593-1605.
26. Holtz AM, Peterson KA, Nishi Y, et al. Essential role for ligand-dependent feedback antagonism of vertebrate hedgehog signaling by PTCH1, PTCH2 and HHIP1 during neural patterning. Development 2013; 140: 3423-3434.
27. Chuang PT, McMahon AP. Vertebrate Hedgehog signalling modulated by induction of a Hedgehog-binding protein. Nature 1999; 397: 617-621.
28. Jia Y, Wang Y, Xie J. The Hedgehog pathway: role in cell differentiation, polarity and proliferation. Arch Toxicol 2015; 89: 179-191.
29. Kayed H, Kleeff J, Esposito I, et al. Localization of the human hedgehog-interacting protein (Hip) in the normal and diseased pancreas. Mol Carcinog 2005; 42: 183-192.
30. Pasca di Magliano M, Sekine S, Ermilov A, et al. Hedgehog/Ras interactions regulate early stages of pancreatic cancer. Genes Dev 2006; 20: 3161-3173.
31. Olsen CL, Hsu PP, Glienke J et al. Hedgehog-interacting protein is highly expressed in endothelial cells but down-regulated during angiogenesis and in several human tumors. BMC Cancer 2004; 4: 43.
32. Martin ST, Sato N, Dhara S, et al. Aberrant methylation of the Human Hedgehog interacting protein (HHIP) gene in pancreatic neoplasms. Cancer Biol Ther 2005; 4: 728-733.
33. Tada M, Kanai F, Tanaka Y, et al. Down-regulation of hedgehog-interacting protein through genetic and epigenetic alterations in human hepatocellular carcinoma. Clin Cancer Res 2008; 14: 3768-3776.
34. Kwong L, Bijlsma MF, Roelink H. Shh-mediated degradation of Hhip allows cell autonomous and non-cell autonomous Shh signalling. Nat Commun 2014; 5: 4849.
35. Mills LD, Zhang Y, Marler RJ, et al. Loss of the transcription factor GLI1 identifies a signaling network in the tumor microenvironment mediating KRAS oncogene-induced transformation. J Biol Chem 2013; 288: 11786-11794.
36. Dennler S, Andre J, Alexaki I, et al. Induction of sonic hedgehog mediators by transforming growth factor-beta: Smad3-dependent activation of Gli2 and Gli1 expression in vitro and in vivo. Cancer Res 2007; 67: 6981-6986.
37. Pan Y, Wang C, Wang B. Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. Dev Biol 2009; 326: 177-189.

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