EDITORIAL

Rationale for Inhibition of the Hedgehog Pathway Paracrine Loop in Pancreatic Adenocarcinoma

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Summary

The role of hedgehog pathway in the biology of pancreatic adenocarcinoma is an emerging area of investigation and provides a novel field for treatment intervention. Recent studies have shown the activation of the hedgehog pathway in pancreatic cancer. Despite the initial assumption of an autocrine mechanism, it seems that the hedgehog pathway contributes to the molecular conversation between tumor and its microenvironment through a paracrine loop. Furthermore, members of the hedgehog pathway crosstalk with other pathways; they regulate tumor angiogenesis and are associated with cancer stem cells. In addition, there is preclinical evidence about the efficiency of hedgehog inhibitors both *in vitro* and *in vivo* and the first clinical trials with those compounds in the treatment of patients with pancreatic adenocarcinoma, are already under way.

Introduction

Pancreatic Adenocarcinoma

Ductal adenocarcinoma of the pancreas, which defines the vast majority of pancreatic neoplastic diseases, is the fourth most common cause of cancer related death in USA [1]. Most of the patients are diagnosed at a time when distant metastases are present and usually the tumors are resistant to treatment. Gemcitabine and erlotinib are the only compounds that have proven to marginally improve prognosis for some of the patients. Even the patients with localized disease that undergo surgery have a five year survival of only 20% [1].

Pancreatic adenocarcinoma is derived from its precursor lesions, the pancreatic cancer precursor lesions (PanINs) which progress to pancreatic adenocarcinoma through accumulation of a series of genetic alterations in several genes including KRAS, $p16^{INK4A}$, Trp53 and smad4 [2]. The spectrum of pancreatic carcinogenesis starts with the low grade PanIN that gives rise to PanIN lesions of grade 2 and 3 (in situ adenocarcinoma of the pancreas) in a stepwise fashion [2]. A lot of genetic alterations that are found in invasive pancreatic adenocarcinomas are present in PanIN lesions at lower frequencies [2].

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Abbreviations DHH: desert hedgehog; GDC-0449: HhAntag691; HEPM: human embryonic palatal mesenchymal; IHH: Indian hedgehog; SAHA: suberoylanilide hydroxamic acid; SHH: sonic hedgehog; SIL: SCL/TAL1 interrupting locus; SMO: smoothened homolog; SUFU: suppressor of fused homolog

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Hedgehog Pathway

The hedgehog pathway has an important role during embryonic development [3, 4]. Recently, aberrant activation of the pathway has been described in human neoplastic diseases like basal cell carcinoma [5], medulloblastoma [6], small cell carcinoma [7] and others. The canonical hedgehog pathway includes three hedgehog ligands, sonic (SHH), Indian (IHH) and desert (DHH) hedgehog that bind to patched, a 12 pass transmembrane protein which releases smoothened homolog (SMO). SMO subsequently allows Gli family transcription factors to locate in the nucleus and affect the expression of a variety of genes [8].

This is a review of the literature concerning the role of the hedgehog pathway in pancreatic adenocarcinoma and the rationale of inhibiting this pathway in the context of clinical trials.

Hedgehog Pathway Activation in Pancreatic Adenocarcinoma Cells

The initial reports about the role of the hedgehog pathway in pancreatic adenocarcinoma suggested an autocrine loop that leads to the pathway activation through overexpression of the hedgehog ligands like SHH [9]. This concept was based on the aberrant

expression of SHH in pancreatic cancer and its precursor lesions PanIN 1-3 whereas it was absent in normal pancreatic tissue [9]. In addition, inhibition of the hedgehog pathway at the level of SMO with cyclopamine resulted in blockage of cell proliferation and induction of apoptosis in many pancreatic cell lines *in vitro*, depending on their SMO expression level [9]. Cyclopamine was able to reduce tumor volume and promote apoptosis in mouse xenografts demonstrating *in vivo* activity [9]. Besides, activation of the hedgehog pathway in human pancreatic epithelial cells through Gli1 transfection lead to up regulation of a series of genes that are over-expressing in early PanIN lesions in comparison to normal pancreatic ducts [10]. Finally, SHH was reported to enhance proliferation and invasiveness by increasing matrix metalloproteinase 9 (MMP9) [11], cathepsin B [12] or loss of E-cadherin [13] in pancreatic duct adenocarcinoma cells.

However, cyclopamine concentration needed to inhibit proliferation in pancreatic duct adenocarcinoma cells was high and even higher for inducing apoptosis in all those first studies [14]. Furthermore, there was lack of correlation between growth inhibition and hedgehog pathway target gene activity in various tumor types including pancreatic adenocarcinoma [14]. Recombinant SHH failed to increase the endogenous Gli mRNA levels in two pancreatic adenocarcinoma cell lines and inhibition of the hedgehog pathway with specific antagonists did not have any effect either [14]. Those data taken together indicate that SHH presence in pancreatic adenocarcinoma cells is not linked with Gli activation in the same cells and make the autocrine loop assumption less likely. It was suggested that the effects of cyclopamine at high concentrations on proliferation and apoptosis of the pancreatic duct adenocarcinoma cells had been the result of altering non-specific targets. Xu *et al.* [15] has provided evidence that this is the case in apoptosis where a SMO specific activator, purmorphamine was not able to reduce the apoptosis caused by cyclopamine at basal levels. Nevertheless, cell proliferation was fully restored implying that the role of the hedgehog pathway might be different between cell proliferation and cell survival. An open question is how Gli gets activated in pancreatic duct adenocarcinoma cells supposing that this does not happen via SHH.

Nolan-Stevaux *et al.* [16] have sought the possibility of Gli1 regulation independently of SMO and showed that TGFbeta was able to induce expression of Gli1 and Gli3 in pancreatic adenocarcinoma cell lines regardless of the SMO status. Furthermore, KRAS inhibition with small interfering RNA (siRNA) resulted in a marked reduction of Gli1 expression and *vice versa* implying a feedback loop. Cooperation between the hedgehog pathway and KRAS has been shown before in transgenic mice [17]: over expression of Gli2 only gave rise to tumors that did not resemble pancreatic adenocarcinoma and did not progress through PanINs. On the other hand, when both Gli2 and KRAS were over expressed, PanIN lesions and later on pancreatic adenocarcinoma tumors were formed. Interestingly, this was the case when only KRAS was over expressed, but the addition of Gli2 over expression accelerated the process of carcinogenesis. The same study shows that Gli2 can activate Akt pathway but cannot induce KRAS mutations. Negative regulation of Gli2 from suppressor of fused homolog (SUFU) has been shown to be a possible connection between KRAS and Gli1 given that the cytoplasmic protein SCL/TAL1 interrupting locus (SIL) was able to abolish this negative regulation and that it was KRAS rather than SHH that enhanced the interaction of SIL with SUFU [18]. When SUFU interacts with SIL, Gli1 is free to translocate in the nucleus and act as a transcription factor. This interaction between KRAS and Gli1 was shown to be SIL dependent. Activated KRAS is believed to promote Gli1 via the RAS/RAF/MEK/ERK pathway in cell lines [19]. However, in a recent study [20] mutated KRAS was shown to suppress the hedgehog pathway and specifically Gli2 in pancreatic duct adenocarcinoma cells while increasing SHH release at the same time. Given the high frequency of KRAS mutations in pancreatic adenocarcinoma, this piece of data provides a mechanism through which an autocrine hedgehog loop is blocked in favor of a paracrine one. The discrepancy with previous studies [16, 19] was attributed partially to different experimental conditions.

Hedgehog Pathway Activation in the Stroma of Pancreatic Adenocarcinomas

Yauch *et al.* [14] showed that the concentration of cyclopamine needed to inhibit cell growth in a human embryonic palatal mesenchymal (HEPM) cell line is significantly lower than in the pancreatic cancer cell lines and this inhibition correlated with the level of the inhibitor. In addition, recombinant SHH was able to activate the hedgehog pathway in the HEPM cell line but not in pancreatic cancer cell lines [14]. They finally showed that the hedgehog pathway was activated in the stroma of xenografts and that this activation was necessary for the growth of the xenografts. These data favor a paracrine role of Hedgehog family ligands like IHH and SHH which are produced in the pancreatic cancer epithelial component and act in the tumor stroma. The paracrine activity of hedgehog ligands was validated in a series of mouse pancreatic adenocarcinoma models [21, 22].

SHH signaling pathway has been found to be activated in fibroblasts in the stroma of pancreatic adenocarcinomas. Specifically, gene expression profiling showed that SMO was upregulated in cancer associated fibroblasts but not in normal pancreatic fibroblasts [23]. Besides, overexpressing SHH was shown to induce desmoplasia and to promote motility of fibroblasts from the tumor stroma in a pancreatic cancer cell line (Capan 2) [24]. Interestingly, it was shown that stromal hedgehog signaling had a role in tumor angiogenesis and lymphangiogenesis and that hypoxia inducible factor-1 alpha (HIF1 alpha) and

vascular endothelial growth factor (VEGF) were expressed in tumor fibroblasts under the influence of SHH [21].

The ability of SHH to induce angiogenesis in pancreatic adenocarcinoma was confirmed by two additional studies. SHH increased VEGF production in endothelial progenitor cells [25] and regulated migration of bone-marrow derived pro-angiogenic cells as well as tumor vasculature formation by increasing angiopoietin-1 (Ang-1) and insulin growth factor-1 (IGF-1) in those cells in pancreatic ductal adenocarcinoma xenografts [26].

An alternative mechanism of SHH paracrine activity was suggested by Yamasaki *et al*. [27]. In this study, it was shown that inflammation activated monocytes were able to produce SHH and increase cell proliferation in pancreatic cancer cell lines. This is different from the rest of the literature, since paracrine activity is focused on tumor cells and not tumor stroma. Interestingly, in contrast to Yauch *et al*., they show that recombinant SHH was able to promote relative Gli1 mRNA expression in pancreatic cancer cell lines. This might be because they used different cell lines and higher recombinant SHH concentration (10 µg/mL instead of 1 µg/mL).

Targeting Hedgehog Pathway in Pancreatic Cancer

A number of compounds have been suggested to inhibit the hedgehog pathway [28]. In an early report, cyclopamine was shown to increase the effect of paclitaxel or irradiation but not cisplatin or gemcitabine on pancreatic cancer cell lines with an activated hedgehog pathway [29]. Combination of cyclopamine and gefitinib was able to inhibit growth and enhance apoptosis in pancreatic cancer cell lines that express both epidermal growth factor receptor (EGFR) and SMO at a greater extent than any compound alone [30]. Interestingly, cyclopamine alone was able to downregulate EGFR. However, these studies do not take into account the possible role of hedgehog inhibition in tumor stroma or in pancreatic cancer stem cells.

A different approach was employed by Chun *et al*. [31] who tested the effect of the combination of SANT-1 which is a SMO antagonist with a histone deacetylase (HDAC) inhibitor, suberoylanilide hydroxamic acid (SAHA). The combination showed supra additive effects on growth inhibition and apoptosis. Besides, SAHA lead to hedgehog interacting protein (HHIP, a hedgehog antagonist) upregulation and Ptc-1 repression providing a possible mechanism for the synergistic effect of the two compounds.

Olive *et al*. [32] provided evidence that chemotherapy does not have access to the tumor cells in KRAS and p53 mutant pancreatic adenocarcinoma xenografts in a gemcitabine resistant mouse model due to poor vascularization of the tumors. When combining gemcitabine with hedgehog inhibition (IPI-926, a specific SMO inhibitor) however, tumor vasculature and subsequently gemcitabine delivery in the tumors were enhanced. Mean vessel density and CD31 positive cells increased whereas stromal myofibroblasts were reduced. Mice treated with the combination had their tumors reduced in size, undergoing apoptosis and had significantly improved survival eventually. On the other hand, IPI-926 had no effect on cell proliferation in these KRAS driven tumors.

When xenografts derived from pancreatic ductal adenocarcinoma cell lines are treated with gemcitabine, cells that express stem cell markers like aldehyde dehydrogenase (ADAC) and CD24 are enriched [33]. However, co-treatment of those xenografts with cyclopamine results in reduction of the cells which express stem cell markers, implying the possible role of hedgehog inhibition in reducing the stem cell burden of the tumor that is responsible for resistance to therapy and recurrences. Co-administration of cyclopamine with gemcitabine was tested in two studies in mouse models [13, 34]. Both studies concluded that the addition of hedgehog inhibition in chemotherapy specifically targets aldehyde dehydrogenase positive cells and reduces the number of metastases observed whereas it does not have significant effect on primary tumor volume. Interestingly, one of the two studies [34] showed that when cyclopamine is administered at the same time with implantation of the tumor rather than later, primary tumor gets significantly smaller. This is compatible with the assumption that cyclopamine targets cancer initiating cells.

There are currently five clinical studies that are recruiting patients with pancreatic adenocarcinoma and include hedgehog inhibition in their arms. The first is a phase I trial that recruits patients with metastatic pancreatic cancer or other solid tumors that cannot undergo surgery. Treatment consists of the SMO inhibitor GDC-0449 (HhAntag691) plus erlotinib or gemcitabine. The second is a placebo controlled randomized phase II study for patients with metastatic or recurrent pancreatic cancer who receive gemcitabine plus GDC-0449 or placebo. The third is a one armed

Table 1. Clinical trials that include hedgehog pathway inhibition in pancreatic cancer (source: http://clinicaltrials.gov).

Trial	Regimen	Randomization	Setting	Phase	Recruitment frame
#1	GDC-0449 plus erlotinib or gemcitabine	No	Metastatic	I	March 2009 - November 2009
#2	Gemcitabine plus GDC-0449 *versus* gemcitabine plus placebo	Yes	Metastatic or recurrent disease	II	September 2009 - September 2011
#3	GDC-0449 plus nab paclitaxel plus gemcitabine	No	First line	II	September 2010 - December 2012
#4	GDC-0449 plus gemcitabine	No	First line	0	June 2010 - June 2012
#5	IPI-926 plus gemcitabine *versus* gemcitabine plus placebo	Yes	First line	Ib/II	April 2010 - March 2012
#6	GDC-0449	No	Pre-operative	II	July 2010 - January 2012

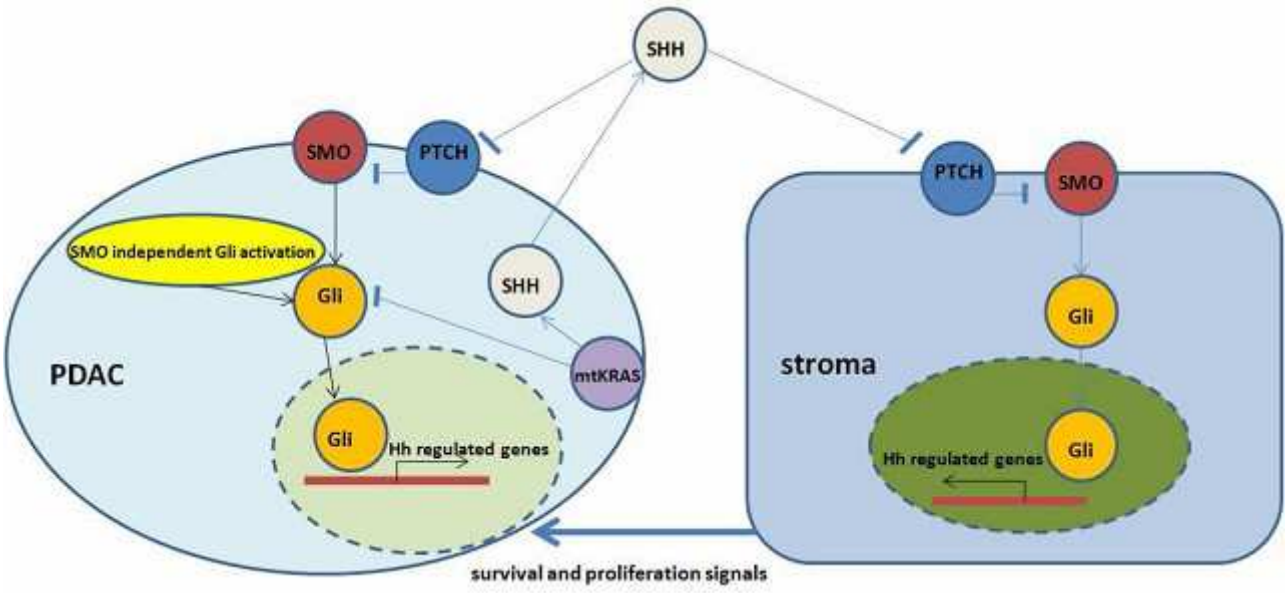

Figure 1. Sonic hedgehog (SHH) ligand is produced from pancreatic ductal adenocarcinoma cells and acts mainly in a paracrine way to release SMO from PTCH control, in the tumor microenvironment. Subsequently, smoothened homolog (SMO) causes Gli to locate to the nucleus and activate a number of genes that promote survival and proliferation of the tumor cells. The tumor cells themselves, can activate the hedgehog pathway through SMO independent signals like TGFbeta. Mutated KRAS blocks the autocrine loop of hedgehog action in favour of the paracrine loop.
mtKRAS: mutated KRAS; PDAC: pancreatic ductal adenocarcinoma; SHH: sonic hedgehog; PTCH: patched; SMO: smoothened

phase II study of the triple combination of GDC-0449, nab-paclitaxel and gemcitabine in the first line setting of pancreatic cancer. The fourth is a trial that focuses on the pancreatic cancer stem cell population after treatment with GDC-0449 plus gemcitabine in the first line as a primary objective. Another interesting randomized phase Ib/II study with a different SMO inhibitor, IPI-926 in combination with gemcitabine or placebo is under way for patients in the first line of pancreatic cancer (Table 1).
Last but not least, there is an additional clinical trial that will test GDC-0449 in pancreatic cancer patients and will start recruitment soon (Table 1).

Discussion

The unraveling of the role of the hedgehog pathway in cancer biology, and specifically in pancreatic adenocarcinoma, has been emerging during the past five years. In this review we go through the majority of studies that provide preclinical data and rationale for designing clinical trials.
The notion of a hedgehog based paracrine loop between the tumor and its microenvironment was first introduced for prostate cancer [35]. There is a wealth of data that supports the notion of paracrine action of hedgehog ligands to the adjacent stroma in pancreatic adenocarcinoma. There is also evidence that indicates the activation of the hedgehog pathway in tumor cells, by ligands like TGFbeta or the KRAS pathway rather than the hedgehog ligands themselves. However, there are a lot of questions to be answered from future research: the exact mechanism that tumor cells interact with their microenvironment through the hedgehog pathway and especially how the stroma influences tumor cell proliferation and survival is unknown to a large extent. Furthermore, the role of a possible autocrine loop where hedgehog ligands might activate Gli2 in tumor cells, especially in cancer initiating cells is still an open question. Last but not least, the cross talking of the hedgehog pathway with other pathways in pancreatic cancer remains to be further illuminated. Figure 1 illustrates the current knowledge about the hedgehog pathway in pancreatic adenocarcinoma.

Summary

The present studies provide the rationale for the first clinical trials with hedgehog inhibitors in the various settings of pancreatic adenocarcinoma. Possible synergistic role of hedgehog inhibitors with chemotherapy like gemcitabine, as well as biologics like tyrosine kinase inhibitors might introduce novel combinations as treatment options. The involvement of the hedgehog pathway in pancreatic cancer stem cell biology and the preclinical data that show prevention of metastases and inhibition of occult tumors, imply that such inhibitors can be tested in the adjuvant setting of pancreatic cancer. Poor chemotherapy delivery might be the reason for the discrepancy in the efficiency of the various regimens between clinical trials and *in vitro* or mouse models. The effect of hedgehog inhibitors in tumor vasculature and chemotherapy delivery is suggestive of a possible role of those compounds in locally advanced and metastatic disease.
Careful design of clinical trials that takes into account the preclinical data is mandatory. The translational analysis of the clinical trials can unravel exciting aspects of the role of the hedgehog pathway in the biology of pancreatic adenocarcinomas and is equally important.

Conflict of interest The authors have no potential conflict of interest

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