Deciphering the role of hedgehog signaling in pancreatic cancer

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Abstract

Pancreatic cancer, mostly pancreatic ductal adenocarcinoma (PDAC), is a leading cause of cancer-related death in the US, with a dismal median survival of 6 months. Thus, there is an urgent unmet need to identify ways to diagnose and to treat this deadly cancer. Although a number of genetic changes have been identified in pancreatic cancer, their mechanisms of action in tumor development, progression and metastasis are not completely understood. Hedgehog signaling, which plays a major role in embryonic development and stem cell regulation, is known to be activated in pancreatic cancer; however, specific inhibitors targeting the smoothened molecule failed to improve the condition of pancreatic cancer patients in clinical trials. Furthermore, results regarding the role of Hh signaling in pancreatic cancer are controversial with some reporting tumor promoting activities whereas others tumor suppressive actions. In this review, we will summarize what we know about hedgehog signaling in pancreatic cancer, and try to explain the contradicting roles of hedgehog signaling as well as the reason(s) behind the failed clinical trials. In addition to the canonical hedgehog signaling, we will also discuss several non-canonical hedgehog signaling mechanisms.

Keywords: hedgehog, pancreatic cancer, Gli1, non-canonical signaling, cancer metastasis

The hedgehog signaling pathway

In mammals, hedgehog (Hh) signaling plays a crucial role in embryonic development, adult tissue homeostasis and pathogenesis of human diseases[1-3]. In normal situation, Hh signaling is regulated by one of the three ligands: sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Hh ligands activate signaling in target cells by binding to the 12-pass transmembrane receptor patched (PTC). In the absence of these ligands, PTC prevents the 7-transmembrane protein, smoothened (SMO), from transducing signal to downstream Gli transcription factors, and the pathway is in the "off" state. Hedgehog binding to Ptc leads to SMO signaling to downstream effectors, leading to Gli-induced target gene expression, and the pathway is turned on. Numerous studies indicate a critical role of primary cilium for Hh signal transduction[4-7]. Primary cilium is a microtubule-based non-motile antenna-like structure that emanates from cell surface of virtually all mammalian cells. There are three mammalian Gli gene family members: Gli1, Gli2 and Gli3. Gli1 and Gli2 are generally regarded as transcriptional activators whereas Gli3 is often viewed as a repressor[8]. Activation of GLI proteins via the Hh-PTC-SMO route is regarded as the canonical Hh signaling pathway. In addition to the canonical pathway, the molecules can bypass the ligand-receptor signaling axis to activate Gli, and these types of regulation are regarded as non-canonical Hh signaling. RAS signaling[9-10], TGFβ[11], PI3K[12] and PKC[13] are reported to regulate Hh signaling via non-canonical pathways. Non- canonical Hh signaling is often observed in malignant diseases and have been summarized in another recent published review[14].

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Pancreatic cancer

Pancreatic cancer is a devastating malignant disease with a very high mortality. Despite its low incidence (2% of all cancer cases), pancreatic cancer is the fourth leading cause of cancer-related deaths in the US and expected to become the second cause of cancer-related deaths in a few years\(^\text{[15-16]}\). After several decades of efforts, the 5-year survival rate of pancreatic cancer remains around 5%, without dramatic improvement\(^\text{[17]}\). The high mortality rate and poor prognosis are largely due to its aggressive and metastatic nature. By the time of diagnosis, more than 80% of cases are locally advanced or distally metastasized\(^\text{[18]}\), and are not eligible for surgical resection, which is the most effective treatment option. Even in patients with resected pancreatic cancer, the outcomes are not as good as other resected solid tumors. For pancreatic cancer patients, the median survival is about 2 years after surgery and adjuvant therapy\(^\text{[19-21]}\). In contrast, patients with advanced disease can only survive a few months.

The most common histologic type of pancreatic cancer is pancreatic ductal adenocarcinoma (PDAC), accounting for >90% of pancreatic cancer cases. The exact cellular origin of PDAC is still not completely known. By histological studies and clinical observation, it is postulated that before the final formation of invasive cancer, there is a stepwise progression of precursor lesions, including pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN)\(^\text{[22]}\). PanIN, the most common precursor lesion of PDAC, is a type of microscopic precursor lesion\(^\text{[23]}\). Based on the degree of cytologic and architectural atypia, PanINs are divided in three grades: PanIN-1 (subdivided into PanIN-1A and PanIN-1B), PanIN-2 and PanIN-3/in situ carcinoma\(^\text{[23,24]}\), reflecting a progressive increase in histologic grade. Recent genetic studies indicate a possibility that PDAC arises from acinar cells instead of ductal cells\(^\text{[25,26]}\). Clinically, pancreatic cancer can be divided into four stages (I, II, III, IV) based on the tumor size, and appearance of lymph node or distal metastasis\(^\text{[27]}\). Although whether the tumor is resectable or not will require radiology data, stage-I and some stage-II tumors are generally resectable or borderline resectable whereas all stage-IV and some stage-III tumors are not resectable.

The most common genetic event in pancreatic cancer is oncogenic KRAS mutation, which is almost universally present in PDAC (>90%)\(^\text{[28]}\). Since over 90% of low-grade PanIN (PanIN-1) lesions also harbor oncogenic KRAS mutations\(^\text{[29]}\), and mice conditionally expressing mutant KRAS develop PanIN\(^\text{[30]}\), mutated-KRAS is considered an early and initiating event in PDAC development. This mutation alone, however, may not be sufficient to drive the progression of invasive cancer. Molecular profiling studies revealed that during the PanIN-to-PDAC progression, inactivating mutations of three tumor suppressor genes are commonly found: telomere shortening (PanIN-1) p16/CDKN2A (some PanIN-1B and most PanIN-2), tumor protein 53 (TP53, PanIN-3), BRCA2 (PanIN-3) and SMAD family member 4 (SMAD4, PanIN-3)\(^\text{[31]}\) (Fig. 1). Deficiency in the p16/CDKN2A axis is detectable in the early PanIN lesions (30% of PanIN-1B)\(^\text{[32]}\) and in nearly all PDAC\(^\text{[33-34]}\), whereas inactivation of TP53 and SMAD4 is mainly found in PanIN-3, and is associated with tumor progression\(^\text{[23,35-36]}\) (Fig. 1). In addition to these four frequently mutated genes (designated “mountains” in the genetic landscape of the PDAC genomes), comprehensive genetic analysis has also uncovered alterations of numerous candidate cancer genes at low frequency (designated “hills”)\(^\text{[37-40]}\), indicating the complexity and heterogeneity of PDAC.

Another important feature of pancreatic cancer is the dense stroma, which is composed of fibroblasts, stellate cells, extracellular matrix and immune cells. The direct regulation of TGFβ signaling on pancreatic cancer desmoplasia has been reviewed elsewhere, and will

**Fig. 1 Molecular alterations in pancreatic cancer development.**

Development of pancreatic cancer is a multiple-step process, involving in formation of pancreatic intraepithelial lesions (PanIN) and carcinoma. In this process, the tumor compartment starts to have activated mutation of Kras and loss of p16 in early stages whereas loss of SMAD4 and p53 are often found in later stages. Accompanying the alterations in the tumor, accumulating changes in the stroma also occur, including expansion of fibroblasts, stellate cells, and an increase in tumor stromal fibers. These cellular changes are associated with elevated expression of many growth factors, cytokines and chemokines. Shh is one of the factors secreted from the tumor compartment to affect the tumor microenvironment.
not be repeated here[43]. Moreover, rooted from genetic alterations, many cytokines, growth factors, and their receptors as well as the associated signaling pathways are involved in the development and maintenance of PDAC[33], reinforcing the heterogeneous features of this deadly disease. While genetic alterations during tumor development are well characterized, changes in PDAC metastasis are not well studied. Lack of typical symptoms at early stages, the complicated and heterogeneous genetic makeup of the tumor, the existence of extensive stroma and less well characterized metastatic tumors all increase the difficulty to make clinical advances. In the rest of the review, we will focus on the role of Hh signaling in pancreatic cancer.

**Shh signaling and pancreatic cancer**

*Hh signaling in pancreas development*

Activation of the Hh pathway is necessary for early embryonic specification of the gastrointestinal tract, but downregulation of the Hh pathway is critical for pancreatic development. Ectopic expression of Hh or aberrant activation of this pathway at the onset of pancreas organogenesis results in gain of tissues with duodenal properties and loss of pancreatic tissue[42,43], whereas the inactivation of Hh pathway promotes the development of pancreatic tissue[44,45]. In adult pancreas, the activity of the Hh pathway is very limited and restricted to beta-cells of the endocrine pancreas in regulation of insulin production[46], but is also required for regeneration of the exocrine pancreas under circumstances such as injury or disease[47].

*Hh signaling in pancreatic cancer*

The aberrant activation of the Hh pathway in human pancreatic cancer was first reported by two independent studies[48,49]. Overexpression of Shh is observed in both pre-invasive and invasive epithelium of 70% of human pancreatic cancer samples, and detectable as early as PanIN1 and throughout all disease progression, but is absent in normal pancreas[48]. Conversely, aberrant Hh ligand expression has been identified in the majority of pancreatic cancer cell lines. This observation in human PDAC was also confirmed in a genetically engineered mouse model[50]. The aberrant expression of Shh is directly associated with oncogenic Kras expression in PDAC. Ectopic expression of oncogenic KrasG12D in normal human pancreatic ductal cells leads to increase of Shh transcript[51], indicating that Shh is a downstream effector of oncogenic KrasG12D in pancreatic cancer development. It was further shown that NF-κB is constitutively active in pancreatic cancer[52], and Shh is a target gene of NF-κB[53,54]. The human SHH promoter region contains putative NF-κB binding sites and activation of NF-κB can promote the transcriptional activity of Shh in cell-based and in vivo models[54]. Moreover, oncogenic Kras is known to be an activator for NF-κB transcriptional activity[55,56]. Thus, it is possible that oncogenic Kras promotes Shh expression via NF-κB signaling (Fig. 2).

Despite the above promising data, recent studies indicate that the roles of Hh pathway for pancreatic cancer may not be that simple. Initially, it was thought that overexpressed Shh by cancer or pre-cancer cells promotes PDAC by activation of Hh signaling in the stroma or in the tumor proper, and application of Hh inhibitors will bring hope for patients with pancreatic cancer. However, gene knockout of Smo in the pancreas has no effects on Kras-mediated pancreatic cancer development[57], and removal of stromal Hh signaling actually accelerates Kras-mediated tumor development[58], a result opposite to the prediction. Below we will discuss activation and function of Hh signaling in the stroma and cancer cells of PDAC (Fig. 2).

**Paracrine hh signaling in tumor stroma**

One of the notorious features of PDAC is desmoplasia, characterized by activation and proliferation of...
fibroblasts and production of collagens, laminin, and fibronectin by stromal cells. Shh ligand secreted from cancerous epithelial cells can activate Smo-dependent signaling in adjacent stromal cells according to the canonical Hh pathway, leading to desmoplasia\cite{57,59,60}. Fibroblasts in tumor microenvironment, also named cancer-associated fibroblasts (CAFs), are widely considered to promote cancer development, and this theory is also evidenced in PDAC. Co-culture of fibroblasts, isolated from resected pancreatic adenocarcinoma samples, increased proliferation, migration, invasion, and colony formation of cancer cells. Fibroblasts also increase gemcitabine resistance in vitro and promoted tumor growth and metastasis in vivo\cite{61}. Using mouse embryonic fibroblasts (MEFs) as a substitute for CAFs, the growth of tumor after co-injection with SMO-deficient MEFs was much slower compared with those from cancer cells plus wild type MEFs\cite{60}. Furthermore, orthotopic xenograft of pancreatic tumor cell line ectopically expressing Shh induces primary tumor size and promotes metastasis\cite{62}. Taken together, all these data demonstrate that epithelium-derived Shh is a major regulator of fibrosis in PDAC, and the activated stroma promotes tumor in PDAC progression. In another word, cancer or pre-cancer cells communicate with its surroundings via Shh to create a favorable environment for PDAC development. Pharmacologic blockade of the canonical Hh pathway with Smo antagonist, such as cyclopamine, HhAntag and Shh ligand-blocking antibody 5E1 has been reported to reduce the growth and distal metastases of human pancreatic tumors in immunodeficient mice\cite{48,49,60,62-64}, and also in one genetically engineered mouse (GEM) model\cite{51}. These results reinforce the tumor promoting function of Shh.

The desmoplastic feature of PCAC not only facilitates tumor growth but also protect them from chemotherapy. It is proposed that failure to treat this disease by chemotherapy is likely due to an inability of the drugs to penetrate the dense stroma to reach cancer cells. Using a KPC mouse model (Pdx1-Cre; LSL-Kras\textsuperscript{G12D}, Trp53\textsuperscript{R172H/+} or Pdx1-Cre; LSL-Kras\textsuperscript{G12D}, Trp53\textsuperscript{R270H/+}), Olive and colleagues found that tumors contain an extensive stroma and poor vascular density and lead to the limitation of the chemotherapeutic agent delivery and reduction of the effectiveness of chemotherapy\cite{65}. Smo inhibitor treatment can decrease the fibroblastic components and transiently increases blood perfusion in the tumor by increasing vasculature density. Combination of Smo antagonist and gemcitabine leads to increased gemcitabine accumulation in the tumor, leading to enhanced mouse survival\cite{66}. These studies further support that elimination of desmoplasia by Shh inhibitors in PDAC will result in effective delivery of chemotherapeutic agents to the tumor, and thus better clinical outcomes.

However, these promising data fail to lead to better outcomes in clinical trials. Clinical trials using Smo inhibitors in PDAC patients have shown little to no efficacy when combined with gemcitabine\cite{46}. Another phase II clinical trial of SMO inhibitor IPI-926 in combination with gemcitabine on PDAC was suspended because patients receiving the combination had a worse outcome when compared to the placebo group (Infinity Corp reports, 2012). Furthermore, recent published studies from two groups revealed that either genetic ablation of Shh in KPC mice or prolonged exposure to Hh inhibitors led to more frequent ADM and PanIN lesions, less well-differentiated, more proliferative and metastatic tumors compared with the control littermates\cite{58,67,68}. These dogma-challenging studies indicate that the stromal cells may play a restraining role during PDAC development by promoting differentiation and inhibiting aggressiveness of cancer cells, a mechanism also reported in bladder cancer\cite{46}. Taken together, it is possible that the impact of stroma on pancreatic cancer is highly circumstantial, probably determined by temporal stage of cancer progression. Further studies are definitely needed to delineate the biological function of stroma in PDAC, and more importantly, SMO inhibitor should be more carefully applied in cancer patients before better understanding of stromal functions for cancer development.

**Hh signaling in tumor cells**

Earlier studies suggested that cancer cell-derived Shh signals both via paracrine fashion to communicate with stromal cells and via autocrine signaling to support self-survival. The evidence for autocrine signaling is that in vivo Hh signaling pathway components such as SMO and PTC are also expressed in PDAC and in pancreatic cancer cell lines\cite{48}. More recent studies revealed that Hh signaling is restricted to the stromal compartment during pancreatic carcinogenesis and PDAC cells do not respond to Hh ligand.

In the PDAC GEM mouse model based on oncogenic Kras expression, conditional deletion of Smo in the same cells has no effects on pancreas development or on the multistage development of PDAC, indicating that the canonical Hh signaling is indispensable for PDAC progression\cite{70}. Expression of SmoM2, an oncogenic Smoothened, using pdx1 promoter-driven cre recombinase does not result in Hh signaling activation, and has no impact on Kras\textsuperscript{G12V}-induced tumor development\cite{77}. SmoM2, however, is able to transduce
Hedgehog signaling in several pancreatic cancer cell lines and orthotopic xenograft models. Additionally, in a subcutaneous xenograft model, Yauch and colleagues showed that tumors from mouse xenografts displayed significant inhibition of tumor growth after treatment with a SMO inhibitor, followed by decreased expression of mouse Hh target genes without effects on human counterpart.

Taken all these data together, it seems that canonical (ligand-dependent) Hh signaling is not activated in the tumor compartment of PDAC. However, it is hard to rule out the possibility of non-canonical Hh signaling in human PDAC and a potential role for Hh signaling in a minor subpopulation of epithelial tumor cells, such as cancer initiating cells. In our studies, we found that Smo signaling inhibition in orthotopic xenografts of human pancreatic cancer almost completely suppresses Hh signaling in the stromal cells but only reduces 50% of the Hh signaling activity in cancer cells as indicated by Hh target gene expression (our unpublished data). These results indicate the coexistence of canonical and non-canonical Hh signaling in pancreatic cancer cells. We also found that Smo signaling inhibition in vitro reduces stem cell population, suggesting a role of ligand-dependent Hh pathway in the maintenance of cancer stem cell population in PDAC. Recently, Sharma and colleagues also demonstrated that NVP-BEZ-235, another SMO inhibitor, can also inhibit the self-renewal of pancreatic cancer stem cells (CSCs) by suppressing the ligand dependent Hh signaling pathway. Thus, Hh may play different roles in different cell types within the same tumor.

Although the involvement of the upstream part of the canonical Hh signaling pathway in pancreatic cancer cells is controversial, Gli proteins, the downstream transcription factors, do play a role in pancreatic cancer development. Nolan-Stevaux et al. demonstrated that conditional deletion of Smo doesn’t affect Gli1 expression in cancer cells, indicating that Gli transcription in cancer cells is regulated through non-canonical Hh signaling. In other studies, Rajurkar et al. showed that targeted ectopic expression of GLI1 in the pancreatic cells accelerates PDAC initiation by mutant Kras. Furthermore, inhibition of Gli transcriptional activity by dominant negative Gli3 reduced the incidence of Kras-driven PanINs and PDAC, indicating the importance of Gli transcription factors in pancreatic tumorigenesis. Recently, it has been found that GLI1 promotes the growth and migration of pancreatic cancer cells via regulation of the transcription of eukaryotic translation initiation factor 5A2 (EIF5A2).

Kras activating mutation is almost universal in sporadic PDAC, and it is reported that the Kras-MEK-ERK cascade increases Gli transcriptional activity. Ectopic expression of oncogenic Kras in normal human pancreatic cell line HPDE-c7 or BXPC3, a pancreatic cancer cell line with wild type Kras, increases transcription activity of Gli molecules. Depletion of oncogenic Kras with specific mutant Kras-targeted siRNAs inhibits Gli transcription activity, as indicated by expression of Gli1 and Ptc1 in PDAC cell lines. It is not clear how the RAS/RAF/MEK cascade affects Gli1 transcriptional activity remains to be elucidated. It is known that Gli transcriptional activity is regulated by the pattern of Gli phosphorylation. Ser130 of Gli1 protein can be phosphorylated by Erk2, but it is not clear whether this mechanism is responsible for Gli1 function in pancreatic cancer. In the mouse model of pancreatic cancer, after Smo knockout, TGFβ treatment causes marked elevation of Gli1 and Gli3. Other ligand-independent Hh signaling mechanisms in pancreatic cancer include altered expression of the co-receptor for Hh ligands and epigenetic regulation of Hh signaling molecules HIP and PTCH1.

Taken together, increasing evidence indicates that the transcription activity of Gli protein may be directly regulated by phosphorylation of the Kras-MEK-ERK cascade in the tumor compartment. On the other hand, ligand-dependent Hh signaling may be responsible for Hh signaling activation in the tumor stroma.

**Perspectives**

It becomes clear that Hh signaling is activated in both the tumor stroma and in the tumor compartment in pancreatic cancer. However, the mechanisms underlying Hh signaling activation in these two compartments are not the same. While ligand-dependent Hh signaling is mainly responsible for stromal Hh signaling, both canonical and non-canonical Hh signaling occurs in the tumor compartment. In addition to different types of Hh signaling activation, the roles of Hh signaling for pancreatic cancer development, progression and metastasis are not well studied. The poorly understood biology of Hh signaling in pancreatic cancer may account for the failed clinical trials using Smo signaling inhibitors.

With further efforts in deciphering the Hh signaling mechanisms in pancreatic cancer, we predict a new wave of novel strategies to suppress Hh signaling. To that end, we believe that efforts are needed to answer the following questions: 1) What is the role of stromal Hh signaling for tumor development of pancreatic cancer?
2) What is the best way to suppress Hh signaling in the tumor compartment? 3) In addition to tumor development, is Hh signaling responsible for pancreatic cancer metastasis? 4) Does the current GEM model (KPC) recapitulate all the features of PDAC in the humans or only a subset of them?

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