Published in final edited form as:

Pancreas. 2012 October ; 41(7): 1019–1028. doi:10.1097/MPA.0b013e31824a0eeb.

Plasma Shh levels reduced in pancreatic cancer patients

Mohamad El-Zaatari, PhD¹, Stephanie Dagnault, MS², Art Tessier, MS¹, Gail Kelsey, BSc¹, Lisa A. Travnikar, BSc¹, Esperanza F. Cantu, BSc¹, Jamie Lee, MD¹, Caitlyn M. Plonka, BSc¹, Diane M. Simeone, MD³, Michelle A. Anderson, MD, MSc¹, and Juanita L. Merchant, MD, PhD^{1,*}

¹Department of Internal Medicine-Gastroenterology, University of Michigan, Ann Arbor, MI

²University of Michigan Cancer Center, University of Michigan, Ann Arbor, MI

³Department of Surgery, University of Michigan, Ann Arbor, MI

Abstract

Objectives—Normally, sonic hedgehog (Shh) is expressed in the pancreas during fetal development and transiently after tissue injury. Although pancreatic cancers express Shh, it is not known if the protein is secreted into the blood and whether its plasma levels change with pancreatic transformation. The goal of this study was to develop an ELISA to detect human Shh in blood, and determine the levels in subjects with and without pancreatic cancer.

Methods—A human Shh ELISA assay was developed, and plasma Shh levels were measured in blood samples from normal volunteers and subjects with pancreatitis or pancreatic cancer. The biological activity of plasma Shh was tested using NIH-3T3 cells.

Results—The average levels of Shh in human blood were lower in pancreatitis and pancreatic cancer patients than in normal individuals. Hematopoietic cells did not express Shh suggesting that Shh is secreted into the bloodstream. Plasma fractions enriched for Shh did not induce Gli-1 mRNA suggesting that the protein was not biologically active.

Conclusions—Shh is secreted from tissues and organs into the circulation but its activity is blocked by plasma proteins. Reduced plasma levels were found in pancreatic cancer patients, but alone were not sufficient to predict pancreatic cancer.

Keywords

hedgehog; plasma; blood; stroma; myofibroblast; stomach

Introduction

Sonic Hedgehog (Shh) is a protein morphogen essential for embryonic development and adult tissue homeostasis¹. During embryogenesis, Shh diffuses from epithelial cells to other epithelial cells or to the mesenchyme where it binds its receptor Patched-1 (Ptch-1) expressed on responding cells. Shh triggers a signaling cascade that includes induction of glioma-associated oncogene-1 (Gli-1)². Some gastrointestinal tumors including pancreatic³, gastric⁴ and colorectal cancer⁵ overexpress the Shh ligand. In both chronic pancreatitis and pancreatic cancer, there is extensive amplification of the stroma consistent with epithelial production of the Shh ligand driving proliferation of Hh-sensitive pancreatic stromal cells.

*Correspondence: Juanita L. Merchant, M.D., Ph.D., 109 Zina Pitcher Place, BSRB, Rm. 2051, Ann Arbor, MI 48109-2200, Phone: (734) 647-2944, Fax: (734) 736-4686, Merchantj@med.umich.edu.

Disclosure: The authors have nothing to disclose and declare no conflict of interest.

Prior studies have shown that elevated *de novo* Shh expression correlates with pancreatic cancer³. Supporting this concept, Hedgehog (Hh) signaling is necessary for tumor maintenance of pancreatic cancer xenografts⁶. Indeed, overexpression of Shh occurs in early pancreatic intraepithelial neoplasia (PanIN) lesions³, suggesting that modulation of Shh protein might be an early diagnostic marker for pancreatic cancer if detectable in body fluids. Since inhibitors of Hh signaling are currently in phase 2 clinical trials^{7–9}, biomarkers are needed to non-invasively follow response to therapy.

Pancreatic cancer has been notoriously difficult to diagnose and treat. Clinically, the most widely used serologic biomarker is CA-19-9, which is fairly sensitive because of elevated levels in pancreatic cancer patients, but is not very specific since it is expressed in other cancers and inflammatory states. Therefore CA19-9 is not used as a primary screening test. Given the overproduction of the Shh ligand in pancreatic cancer^{10,11}, we queried whether Shh itself might be detected in the general circulation and if so, whether it correlated with pancreatic disease. Indeed, it has recently been reported that the Shh homolog, Indian Hedgehog (Ihh), circulates in the bloodstream by associating with very low density lipoproteins (VLDL)^{12,13}. Therefore, we developed an ELISA assay to measure Shh in human plasma, then tested whether Shh levels vary with pancreatic disease such as chronic pancreatitis and cancer. We reasoned that Shh might be elevated compared to normal subjects if excessive amounts leave the tissue and enter the circulation. However, if Shh diffusion out of the tissue is retarded or metabolized, blood levels might fall. Pancreatic cancer is known for its extensive stromal proliferation¹⁴. Since the primary target of the ligand is the stroma, we tested the hypothesis that Shh levels would fall in patients with extensive pancreatic fibrosis. An ELISA to detect circulating Shh for human plasma does not exist. Therefore, we first developed and validated a serologic assay for Shh then assessed Shh ligand levels in patients with chronic pancreatitis and pancreatic cancer.

Materials and Methods

Blood collection and plasma preparation

Blood collection from human volunteers was approved by The Institutional Review Board of the University of Michigan Medical School. All subjects provided written informed consent. Blood from 40 male and 40 female self-identified normal human subjects age 18 to 70 was collected over 1 year (2009–2010, IRB# HUM00027437) in tubes containing K3-EDTA. In addition, age and gender-matched blood was collected from 38 normal, 20 chronic pancreatitis, and 20 pancreatic cancer patients (2004–2009, IRBMed# 2005-248 and IRBMed# 2001-147). Immediately after collection, plasma was separated from blood elements by centrifugation.

Human Shh ELISA

The mouse specific ELISA assay for Shh (R&D Systems, Minneapolis, MN) was modified to detect human Shh by substituting the kit detection antibody (R&D Systems) with goat polyclonal anti-Shh (N-19, Santa Cruz Biotechnology, Santa Cruz, CA) that detects human Shh. Per the manufacturer's instructions, the R&D kit exhibits only 10% cross-reactivity with human Shh. For detection with tetramethylbenzidine (TMB), this antibody (N-19 Lot#C0509, Santa Cruz) was biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Thermo Scientific, Rockford, IL). To perform the ELISA, the capture antibody (8 µg/ml, MAB4641, R&D Systems) was incubated overnight on high-binding polystyrene microplates (DY990, R&D Systems), washed three times with 0.05% Tween-20 in PBS (PBS-T), blocked for 2 h with 1% BSA in PBS, then washed again in PBS-T. Serial dilutions (2–2000 ng/ml) of recombinant human N-terminal Shh (1314-SH/CF, R&D Systems) or Ihh peptide (1705-HH/CF, R&D Systems) were prepared in 1% BSA. A

standard curve was generated using 100 μ l of recombinant Shh peptide (in triplicates) or plasma samples (in duplicates). After binding for 2h, unbound peptide was removed with PBS-T, followed by adding the detection antibody (biotinylated N-19, 2 μ g/ml, Santa Cruz) for 2 h. After a final rinse with PBS-T, the complex was detected using streptavidin-HRP and TMB according to the manufacturer's instructions (R&D Systems).

Western Blot Analysis

Purification of bound antigen from the ELISA microplate was performed by adding 100 μ l of acidic elution buffer (pH 2.8, #21009, Pierce) for 15 min. Whole cell lysates of human stomach were obtained commercially (BioChain, Hayward, CA). Western blots were performed as described previously¹⁵ using polyclonal anti-Shh (N-19, Santa Cruz) to detect the immobilized antigen. Quantification of western blot intensity was performed using the Image J software (National Institute of Health, Bethesda, MD).

White blood cell isolation

Blood samples collected in lithium-heparin vacutainers (#366664, Becton, Dickinson, and Company, Franklin Lakes, New Jersey) were layered on top of a discontinuous Histopaque gradient consisting of 2.5 mL Histopaque 1119 (#11191, Sigma-Aldrich, St Louis, MO) topped with 2.5 mL Histopaque 1077 (#10771, Sigma-Aldrich) in a 15 mL falcon tube (#1475-1611, USA Scientific, Inc., Orlando, FL), centrifuged at $400 \times g$ for 30 min¹⁶. Briefly, lymphocytes and monocytes were collected from the interphase between plasma and Histopaque 1077, polymorphonuclear neutrophils from the interphase between Histopaque 1077 and Histopaque 1119, and eosinophils and basophils from Histopaque 1119 red blood cell-containing fraction. Red blood cells were lysed in ammonium-chloride potassium (ACK) buffer (#A1049201, Invitrogen, Carlsbad, CA). Collected cells were lysed using the RNEasy Microkit lysis buffer (#74004, Qiagen, Valencia, CA), and homogenized using Qiashredder (#79654, Invitrogen).

Fast protein liquid chromatography (FPLC)

Human plasma and normal or tumor stomach lysate (380 μ L) were injected into a Superose 12 10/300 GL column (#17-5173-01, GE Healthcare, Livonia, MI) on an ÄKTA FPLC machine (#18-1900-26, GE Healthcare). The fractions (0.5 mL) were collected and concentrated to 100 μ L using Amicon Ultra-4 Centrifugal Filters (#UFC8-010-24, Thermo Fisher Scientific, Waltham, MA) and frozen at -80°C until further use. For albumin and IgG removal, samples were incubated with anti-albumin and anti-IgG antibodies immobilized on beaded agarose (#89876, Pierce). For protein denaturation, samples were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) and β -mercaptoethanol for 7 minutes.

Cell culture and treatments

NIH 3T3 cells (American Type Culture Collection, Manassas, VA) were cultured in DMEM plus 10% FBS and 1% antibiotic/anti-mycotic solution (#15240112, Invitrogen) at 2×10^5 cells per well overnight in a 24-well plate. The cells were incubated overnight in serum-free DMEM then treated with 50 ng/mL of Shh (461-SH-025/CF, R&D Systems), 50 ng/mL or 3 μ g/ml of Ihh (1705-HH-025/CF, R&D Systems), 2 μ g/mL 5E1 antibody (Developmental Studies Hybridoma Bank, The University of Iowa, Iowa City, IA), or 40 μ l (to a total amount of 125ng Shh) of plasma FPLC fractions. Cells were lysed using RNEasy Microkit lysis buffer (Qiagen), and homogenized using Qiashredder (Invitrogen).

Quantitative PCR

RNA extraction, reverse transcription, and mouse Gli-1 qPCR (for NIH 3T3 cells) were performed as previously described¹⁵. The 18S, GAPDH, Shh, Ptch-1 and Gli-1 qPCR for

human stomach RNA (#HR-302, Zyagen, San Diego, CA), FirstChoice® Human Total RNA Survey Panel (#AM6000, Ambion, Invitrogen) and human blood cell RNA were performed using TaqMan® Gene Expression Assays: with the 18S primer #Hs99999901_s1; GAPDH primer #Hs99999905_m1; Shh primer #Hs01123832_m1; Ptch-1 primer #Hs00181117_m1; and Gli-1 primer #Hs01110766_m1 (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Immunohistochemistry

Immunohistochemistry was performed as described previously¹⁵. Briefly, pancreatic cancer tissue was sectioned in paraffin blocks (IRB# HUM00025339), and stained with polyclonal goat anti-Shh antibody (N-19, Santa Cruz) or polyclonal rabbit anti-Ptch-1 antibody (H-267, Santa Cruz). Immunofluorescent detection was visualized using Alexa Fluor-conjugated secondary antibodies (Invitrogen). For staining of primary human colorectal myofibroblasts NL-235 (kind gift from F. Rieder and C. Fiocchi, Cleveland, OH), cells were grown in chamber slides (#177399, Lab-Tek, Thermo Scientific), fixed in 4% paraformaldehyde for 10 min, washed in PBS, and blocked in 20% donkey serum (#0170-000-121, Jackson ImmunoResearch, West Grove, PA). Cells were incubated with the Shh and Ptch-1 primary antibodies overnight at 4°C. After washing with PBS, immunofluorescent detection was visualized with Alexa Fluor-conjugated secondary antibodies (Invitrogen).

Statistical Methods

The mean $\pm$ S.D., median and associated percentiles were calculated for Shh in normal, age-matched normal, pancreatitis, and pancreatic cancer subjects. A logistic regression model with pancreatic cancer as cases and normal age-matched patients as controls was used to determine the relationship between Shh, age and gender. ROC curves were constructed for Shh.

Results

Human Shh ELISA Development and Validation

To determine the levels of Shh in blood, we developed an ELISA for human Shh by modifying the mouse specific ELISA assay from R&D Systems. To determine the specificity of the ELISA for human Shh and its range of detection, recombinant human Shh was used at different concentrations to construct a standard curve. The ELISA was performed using a biotinylated Shh antibody that detects the human ligand (Fig. 1A). Indeed, the ELISA detected between 4 and 2000 ng/ml of recombinant human Shh (Fig. 1B, left panel). Since recent reports documented the presence of Indian Hedgehog (Ihh) in human blood¹², we confirmed the specificity of the ELISA for Shh versus Ihh. As expected, the ELISA did not cross-react with recombinant human Ihh (Fig. 1B, right panel). To confirm the signal specificity of the ELISA, bound recombinant protein was eluted after the capture step using a pH elution buffer. Western blot analysis of the eluted Shh peptide was analyzed using the Shh N-19 antibody. Indeed, the eluted protein was of the same size as the input indicating that the colorimetric signal detected in the ELISA was due to the captured Shh protein (Fig. 1B, left panel inset).

Plasma Shh levels in the general population

Since plasma detection of Shh in human subjects has not been reported, we first determined Shh levels in the general population (Table 1). Previous reports have shown that Shh is ubiquitously expressed and produced by a variety of different organs^{17–20}. However, it is not known which tissues secrete Shh into the peripheral circulation. We found that plasma Shh levels were highly variable among normal (non-fasting) human subjects (Fig. 2A, Table 1).

Moreover, Shh levels decreased with age (Pearson correlation (r) = -0.19) but showed no gender difference (Fig. 2A, Table 1).

Reduced plasma levels of Shh in subjects with pancreatic cancer

Prior studies have shown that Shh is highly expressed in pancreatic cancer tissue from human subjects³. Moreover, transgenic mouse models overexpressing Shh from tissue-specific promoters induce pancreatic transformation³. Therefore, we initially predicted that excessive expression of Shh in the malignant human pancreas might correlate with increased Shh secretion into the bloodstream. We therefore collected blood from pancreatitis and pancreatic cancer patients. Since pancreatitis and pancreatic cancer usually develop in human subjects after age 50, we compared the Shh levels from our patient population to age-matched controls (Table 1). Indeed, plasma Shh levels in age-matched individuals were lower than the levels from younger random volunteers (Table 1). However the levels still varied widely (Fig. 2B). The average levels in pancreatitis and pancreatic cancer patients were even lower than in age-matched self-identified normal human individuals (Fig. 2B, Table 1). ROC curves demonstrated that Shh was not a significant predictor of pancreatic cancer status (Fig. 2C), primarily due to the age-related decrease in plasma Shh levels. Nevertheless, to confirm that the reduced levels of Shh were not due to storage, we analyzed samples after prolonged freezing or repeated freeze-thaw cycles and demonstrated no significant Shh protein degradation (Fig. 2D).

Shh binds to fibrotic human pancreatic tissue

Since Shh levels were reduced in pancreatic cancer patients, we hypothesized that the expanded myofibroblast population in pancreatic cancer sequesters Shh from the circulation. Indeed, immunohistochemical analysis detected Shh in epithelial (Fig. 3B) and fibrotic (Fig. 3C) cells of pancreatic cancer lesions but not in normal pancreatic tissue (Fig. 3A). The fibrotic cells also expressed the Shh receptor, Ptch-1 (Fig. 3C). Since Shh is only produced by pancreatic epithelial cells⁶, we hypothesized that stromal cells were binding to Shh via the Ptch-1 receptor and sequestering Shh from the bloodstream. To test this hypothesis, we treated primary human myofibroblasts (NL-235) with recombinant Shh. Despite rigorous washing, recombinant Shh remained bound to these cells (Fig. 4). Therefore, the expanded stromal compartment in pancreatic cancer might sequester circulating Shh from the bloodstream, leading to the observed reduction in plasma Shh levels of pancreatic cancer patients. In fact, Shh protein was undetectable on a western blot of pancreatic cancer tissue (Suppl. Fig. 1).

Circulating Shh is not produced by hematopoietic cells

To rule out the possibility that circulating Shh might be secreted by blood cells, we isolated hematopoietic cells and normalized their Shh expression to human stomach mRNA obtained commercially. Shh was expressed in human stomach but was undetectable in monocytes, lymphocytes, polymorphonuclear neutrophils (PMNs), basophils or eosinophils (Fig. 5A). However, these cells expressed detectable amounts of Gli-1 and Ptch-1 (Fig. 5A) suggesting active hedgehog signaling. To determine the likely site of Shh production and secretion into the bloodstream, we screened a tissue array of different human organs and measured Shh expression. Shh mRNA was highly expressed in the bladder, prostate, cervix, stomach, liver, kidney and lung (Fig. 5B), suggesting that these organs were a likely source of circulating Shh.

Size fractionation of Shh from human plasma

Since plasma Shh was not detectable by western blot due to the abundance of non-specific immunoglobulin protein bands, we determined the size of plasma Shh by Fast Protein

Liquid Chromatography (FPLC). Therefore, we enriched for Shh from human plasma with FPLC (Fig. 6A, B), and determined the amount of Shh protein in each fraction by ELISA. Shh was present in fractions 7–13 and peaked in fraction 9 (Fr 9) (Fig. 6B). Since pancreatic cancer samples exhibited extensive fibrosis (Fig. 3C) and Shh levels were not readily detectable by western blot in this tissue (Suppl. Fig. 1), we obtained extracts from normal human stomach and gastric cancers to analyze by FPLC. We previously showed that the gastric mucosa produces significant amounts of processed and unprocessed Shh ligand, while gastric cancers only produce the unprocessed form²¹. Shh from human plasma (confirmed by the ELISA) eluted from the FPLC in a fraction slightly larger than that eluted from gastric tissues, suggesting a molecular weight of 60–80 kDa. The unprocessed Shh species from gastric tissues was ~45kDa and eluted in fractions 9–13. The processed form of Shh found only in normal gastric mucosa eluted in fractions 14–20 corresponding to a molecular weight of 19kDa (Fig. 6B–D). Furthermore, denaturation of plasma Shh by boiling in sample buffer did not shift the FPLC curve suggesting that Shh is likely not covalently bound to other plasma proteins, but might be posttranslationally-modified or forming undisruptable oligomers as previously reported²² (Fig. 6B, native versus denatured plasma Shh).

Plasma Shh biological potency dampened by circulating plasma proteins

To determine the biological activity of circulating Shh, we treated NIH-3T3 cells with recombinant Shh, Ihh or Fr 9 (Shh-enriched) after an overnight incubation with or without the Shh-blocking 5E1 antibody. Expectedly, recombinant Shh (50ng/ml) induced Gli-1 mRNA expression and the induction was blocked by the mouse monoclonal antibody to Shh (5E1) (Fig. 7A). However, Fr 9 inhibited Gli-1 expression in these cells irrespective of whether 5E1 was added (Fig. 7A). This suggested the presence of plasma factors remaining in Fr 9 that prevented ligand induction of hedgehog signaling. To demonstrate this inhibitory effect, we treated NIH-3T3 cells with recombinant Shh peptide diluted in Fr 9. Indeed, induction of Gli-1 was inhibited by 50% (Fig. 7A).

We considered that multiple abundant plasma proteins, e.g. albumin and IgG exerted this inhibitory effect²³. We therefore reduced albumin and IgG protein amounts using specific bead-bound antibodies (data not shown). Treatment of NIH 3T3 cells with IgG/albumin-reduced Fr 9 did not suppress Gli-1 expression below normal levels, as observed in Fr 9 without depletion (Fig. 7A). Furthermore, dilution of rShh in Fr9 (-IgG -Alb) did not inhibit its activity as observed when diluted in Fr9 without depletion (Fig. 7A). Indeed, rShh activity in Fr9 (-IgG -Alb) was also inhibited by 5E1 (Fig. 7A). In contrast to rShh, Fr9 (-IgG -Alb) did not induce Gli-1 expression and did not respond to 5E1 pre-treatment. Therefore plasma Shh lacked signaling activity and was inhibited by the presence of common plasma proteins.

To confirm the lack of signaling activity, we treated all the plasma fractions with 5E1 versus untreated and showed that no signaling was detected in any of the fractions (Fig. 7B). A similar result was obtained with mouse plasma (Fig. 7B). In contrast, recombinant Shh (50ng/ml) induced Gli-1 expression potently, while Ihh (50ng/ml) did not induce Gli-1 expression, and required a much higher concentration of the ligand (3 µg/ml), which was not inhibited by 5E1 at this concentration (Fig. 7B). This confirmed that the assay was specific for Shh and that its presence in the blood is not mediating Hh signaling, supporting the notion that the effect on target tissues is through local diffusion of the ligand.

Discussion

In the current study, we show that Shh protein can be detected in human plasma and that the levels trend downward in chronic pancreatitis or cancer. Since there are phase 2 trials

underway to block Hh signaling in pancreatic cancer, an assay to measure circulating levels of Shh would be clinically useful. Currently, the carbohydrate antigen 19-9 (CA19-9) is the most frequently used biomarker for pancreatic cancer, but this glycoprotein is not sufficiently specific to screen for pancreatic cancer²⁴. Other glycoprotein antigens that have been considered biomarkers include carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA125), but these are also inadequate due to low positive predictive values and high false-positive rates²⁴. Furthermore, these markers are not only associated with pancreatic cancer but other types of cancer and benign disease. Therefore, the identification of new pancreatic cancer markers is essential. Since Shh production is considerably increased in pancreatic cancer tissues³, we investigated whether the excess ligand might be secreted into the circulation and reflect disease in the pancreas. Unlike the glycoprotein biomarkers whose functions are not understood, a wealth of information surrounding Shh tissue expression and signaling is available^{25,26}.

We report here successful detection of the Shh ligand using a “sandwich” type ELISA assay in which the antigen is captured by antibody adherent to high-binding plastic then is detected using a chromogen-tagged secondary antibody. Since this assay method has not been used to detect human Shh previously, we first validated the specificity of the antibody by eluting bound Shh from the ELISA plate. Second, we showed that Shh was detected in the plasma of self-identified normal volunteers. Third, we confirmed that human hematopoietic cells do not produce Shh. This result revealed that the immunoreactive Shh detected in the assay was not due to contamination by circulating blood elements but likely is secreted by ligand-expressing tissues into the bloodstream. There was significant variability in the levels detected presumably reflecting variations in the secretion pattern and tissues of origin. Indeed, Shh mRNA was highly expressed in several human organs including the bladder, prostate, cervix, stomach, liver and kidney. Since we detected no differences in Shh protein levels between males and females, gender-specific organs such as the prostate and cervix are unlikely to contribute to the variability in plasma Shh levels. However, the variability might reflect differences in bladder, stomach, liver or kidney expression or function. Moreover, variables such as fasting state, smoking status, time of day and medications were not reported by the volunteers and may have contributed to the variability in Shh levels. As Shh functions as a morphogen in a concentration-dependent manner, the function of circulating Shh and the exact site of secretion remain to be determined in order to evaluate its clinical importance.

Despite the lack of Shh production, white blood cells expressed components of the hedgehog signaling pathway, e.g., Ptch-1 and Gli-1, suggesting that clinical hedgehog (Hh) inhibitors might target these cells. The role of Hh signaling components in circulating white blood cells remains poorly studied. Previous studies report that paracrine Hh signaling from the gastrointestinal epithelium targets monocytes and macrophages, by modulating immune cell responses^{27,28}. Yauch *et al.* reported that paracrine Hh signaling from the epithelium to the mesenchyme specifically targets fibroblasts⁶, which might also include antigen presenting cells in the mesenchyme subsequently affecting the inflammatory response within the tumor microenvironment. Furthermore, other studies have also implicated hedgehog signaling in T-cell differentiation and maturation²⁹. Therefore, hedgehog signaling appears to regulate immune cells, a function that is perhaps tissue specific. Indeed, Lees *et al.* showed that reduced Gli-1 and therefore canonical Hh signaling correlates with a heightened inflammatory intestinal response with an increase in IL-23, IL-12 and IL-17 (Lees *et al.* 2008). Thus in the small intestine, Hh signaling appears to inhibit inflammation.

The link between Hh signaling and immune cells might explain the decrease in circulating Shh ligand. An increase in stroma is characteristic of chronic pancreatitis and pancreatic cancer. Therefore expansion of this compartment would also include an increase in Ptch-1-

positive mesenchymal cells. Based on the evidence that Shh is detected in fibrotic cells surrounding the pancreatic cancer epithelium, and that human myofibroblasts can sequester the Shh ligand *in vitro*, we suggest that the lower levels of plasma Shh might reflect adherence to the expanded compartment of target cells present in the diseased pancreas. It might be useful to test the hypothesis that circulating Shh becomes trapped in organs with expanded mesenchyme by tagging recombinant Hh then tracking the location of the ligand *ex vivo*.

We also found that the Shh ligand in plasma displayed no signaling activity. This correlated with plasma Shh appearing in a similar FPLC fraction as the 45kDa Shh from stomach lysate and with a later fraction corresponding to the recombinant human 20kDa form. This led us to speculate that circulating Shh might be the full-length form that exhibits no signaling activity. Alternatively, since plasma Shh peaked at a slightly earlier fraction (fraction 9) than 45kDa Shh (fraction 11), circulating plasma Shh might be of a larger size (60–80kDa) than the 45kDa protein suggesting that it circulates in higher molecular weight undisruptable oligomers as previously described²². However, our attempts to conclusively determine the size of plasma Shh were unsuccessful due to an excess of plasma proteins including immunoglobulins and albumin²³ remaining in the Shh enriched fractions and the lack of sufficient amounts of plasma from the volunteers. Moreover, plasma diminished the ability of Shh to induce Gli-1, suggesting that the signaling activity of Shh in the plasma is generally suppressed until it reaches its target tissues.

In conclusion, ELISA can detect plasma Shh, but the levels were decreased in chronic pancreatitis and pancreatic cancer patients, indicating that Shh is upregulated only in the tumor microenvironment and not systemically. Nevertheless, Shh levels in the circulation are different from the mean values observed in age-matched controls. Since Shh levels also decrease with age, changes in the levels do not necessarily predict pancreatic cancer. We speculate that the decrease with age might reflect inflammatory or fibrotic processes that increase with age. Thus while a decrease in Shh can be detected in human subjects with pancreatic cancer, it alone is not sufficiently predictive for disease in this organ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant support: This study was supported by Public Health Service Grants P01-DK62041 (J.L.M.), K23-DK082097 (M.A.A.), UL1RR024986 (MICHR), and P30 CA46592 (Cancer Center). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

The study was supported by Public Health Service (PHS) Grant P01 DK-62041 (JLM). The 5E1 antibody developed by Thomas M. Jessell and Susan Brenner-Morton was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. We acknowledge the assistance of the Michigan Institute for Clinical and Health Research (MICHR) (PHS UL1-RR024986). We wish to express gratitude to Ms. Melissa Tuck and Dr. Mack Ruffin for their assistance with control specimens, and to Dr. Peter Higgins, Dr. Florian Rieder, and Dr. Claudio Fiocchi for providing us with the primary human myofibroblast (NL-235) cells.

Abbreviations

Shh	Sonic hedgehog
Ihh	Indian hedgehog
Ptch-1	Patched-1

Gli-1	glioma-associated oncogene homolog 1
ELISA	Enzyme-linked immunosorbent assay
FPLC	fast protein liquid chromatography

References

1. Ingham PW, McMahon AP. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* 2001; 15(23):3059–3087. [PubMed: 11731473]
2. Nybakken K, Perrimon N. Hedgehog signal transduction: recent findings. *Curr Opin Genet Dev.* 2002; 12(5):503–511. [PubMed: 12200154]
3. Thayer SP, di Magliano MP, Heiser PW, et al. Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature.* 2003; 425(6960):851–856. [PubMed: 14520413]
4. Fukaya M, Isohata N, Ohta H, et al. Hedgehog signal activation in gastric pit cell and in diffuse-type gastric cancer. *Gastroenterology.* 2006; 131(1):14–29. [PubMed: 16831586]
5. Monzo M, Moreno I, Artells R, et al. Sonic hedgehog mRNA expression by real-time quantitative PCR in normal and tumor tissues from colorectal cancer patients. *Cancer Lett.* 2006; 233(1):117–123. [PubMed: 16473672]
6. Yauch RL, Gould SE, Scales SJ, et al. A paracrine requirement for hedgehog signalling in cancer. *Nature.* 2008; 455(7211):406–410. [PubMed: 18754008]
7. Garber K. Stromal depletion goes on trial in pancreatic cancer. *J Natl Cancer Inst.* 2010; 102(7):448–450. [PubMed: 20339135]
8. Stanton BZ, Peng LF. Small-molecule modulators of the Sonic Hedgehog signaling pathway. *Mol Biosyst.* 2010; 6(1):44–54. [PubMed: 20024066]
9. Furukawa T. Molecular targeting therapy for pancreatic cancer: current knowledge and perspectives from bench to bedside. *J Gastroenterol.* 2008; 43(12):905–911. [PubMed: 19107333]
10. Li C, Heidt DG, Dalerba P, et al. Identification of pancreatic cancer stem cells. *Cancer Res.* 2007; 67(3):1030–1037. [PubMed: 17283135]
11. Morton JP, Mongeau ME, Klimstra DS, et al. Sonic hedgehog acts at multiple stages during pancreatic tumorigenesis. *Proc Natl Acad Sci U S A.* 2007; 104(12):5103–5108.
12. Queiroz KC, Tio RA, Zeebregts CJ, et al. Human plasma very low density lipoprotein carries Indian hedgehog. *J Proteome Res.* 2010; 9(11):6052–6059. [PubMed: 20839884]
13. Panakova D, Sprong H, Marois E, et al. Lipoprotein particles are required for Hedgehog and Wingless signalling. *Nature.* 2005; 435(7038):58–65. [PubMed: 15875013]
14. Korc M. Pancreatic cancer-associated stroma production. *Am J Surg.* 2007; 194(4 Suppl):S84–86. [PubMed: 17903452]
15. El-Zaatari M, Zavros Y, Tessier A, et al. Intracellular calcium release and protein kinase C activation stimulate sonic hedgehog gene expression during gastric acid secretion. *Gastroenterology.* 2010; 139(6):2061–2071. e2062. [PubMed: 20816837]
16. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl.* 1968; 97:77–89. [PubMed: 4179068]
17. Odent S, Atti-Bitach T, Blayau M, et al. Expression of the Sonic hedgehog (SHH) gene during early human development and phenotypic expression of new mutations causing holoprosencephaly. *Hum Mol Genet.* 1999; 8(9):1683–1689. [PubMed: 10441331]
18. Wang BE, Shou J, Ross S, et al. Inhibition of epithelial ductal branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells. *J Biol Chem.* 2003; 278(20):18506–18513. [PubMed: 12626524]
19. Unger S, Copland I, Tibboel D, et al. Down-regulation of sonic hedgehog expression in pulmonary hypoplasia is associated with congenital diaphragmatic hernia. *Am J Pathol.* 2003; 162(2):547–555. [PubMed: 12547712]

20. Bitgood MJ, McMahon AP. Hedgehog and Bmp genes are coexpressed at many diverse sites of cell-cell interaction in the mouse embryo. *Dev Biol.* 1995; 172(1):126–138. [PubMed: 7589793]
21. Zavros Y, Waghray M, Tessier A, et al. Reduced pepsin A processing of sonic hedgehog in parietal cells precedes gastric atrophy and transformation. *J Biol Chem.* 2007; 282(46):33265–33274. [PubMed: 17872943]
22. Dierker T, Dreier R, Migone M, et al. Heparan sulfate and transglutaminase activity are required for the formation of covalently cross-linked hedgehog oligomers. *J Biol Chem.* 2009; 284(47):32562–32571.
23. Gerszten RE, Accurso F, Bernard GR, et al. Challenges in translating plasma proteomics from bench to bedside: update from the NHLBI Clinical Proteomics Programs. *Am J Physiol Lung Cell Mol Physiol.* 2008; 295(1):L16–22. [PubMed: 18456800]
24. Steinberg WM, Gelfand R, Anderson KK, et al. Comparison of the sensitivity and specificity of the CA19-9 and carcinoembryonic antigen assays in detecting cancer of the pancreas. *Gastroenterology.* 1986; 90(2):343–349. [PubMed: 2416628]
25. Ruiz i Altaba A, Sanchez P, Dahmane N. Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat Rev Cancer.* 2002; 2(5):361–372. [PubMed: 12044012]
26. Rubin LL, de Sauvage FJ. Targeting the Hedgehog pathway in cancer. *Nat Rev Drug Discov.* 2006; 5(12):1026–1033. [PubMed: 17139287]
27. Zacharias WJ, Li X, Madison BB, et al. Hedgehog is an anti-inflammatory epithelial signal for the intestinal lamina propria. *Gastroenterology.* 2010; 138(7):2368–2377. 2377 e2361–2364. [PubMed: 20206176]
28. van Dop WA, Heijmans J, Buller NV, et al. Loss of Indian Hedgehog activates multiple aspects of a wound healing response in the mouse intestine. *Gastroenterology.* 2010; 139(5):1665–1676. 1676 e1661–1610. [PubMed: 20682322]
29. Rowbotham NJ, Hager-Theodorides AL, Cebecauer M, et al. Activation of the Hedgehog signaling pathway in T-lineage cells inhibits TCR repertoire selection in the thymus and peripheral T-cell activation. *Blood.* 2007; 109(9):3757–3766. [PubMed: 17227833]

Figure 1.

Validation of human Shh ELISA. *(A)* Schematic diagram of the sandwich ELISA using high-affinity binding polystyrene plates, rat monoclonal and goat polyclonal antibodies against human Shh for capture and detection respectively. *(B)* The ELISA standard curve was constructed using non-linear regression analysis of the optical density against the logarithm of the concentration of recombinant human Shh (rhShh) vs recombinant human lhh (rhIhh). Each concentration was performed in triplicate wells (shown is the mean of three replicates per concentration $\pm$ standard error of the mean). *(B, inset)* Western blot analysis of the eluate from the ELISA plate after capture of rhShh protein compared to input rhShh protein that was not subjected to the ELISA.

Figure 2.

Plasma Shh levels in normal, pancreatitis and pancreatic cancer patients. (A) Shh levels (ng/ml) in self-identified normal male and female volunteers (shown is the mean of 40 individuals per group $\pm$ standard error of the mean; median values and 25th–75th percentiles are listed in Table 1). (B) Shh levels (ng/ml) in pancreatitis and pancreatic cancer patients versus age-matched controls (shown is the mean per group $\pm$ standard error of the mean; median values and 25th–75th percentiles are listed in Table 1). (C) ROC curve of plasma Shh levels as a predictor of pancreatic cancer. (D) The levels of Shh protein as a function of time. Blood plasma was collected and tested for Shh over different times after freeze-thaw cycles. The dates (x-axis) illustrate the time of testing and the lines illustrate the variability in values between assays run at different time points.

Figure 3. Binding of Shh to fibrotic cells in human pancreatic cancer. (A) H&E and Shh (green) immunofluorescent analysis in serial sections of normal human pancreas. (B) H&E and Shh (green) immunofluorescent analysis in serial sections of pancreatic cancer epithelial cells. (C) Serial section showing H&E and Shh (green)/Ptc-1 (red) co-immunofluorescent localization in a fibrotic area of human pancreatic cancer. Black/white and yellow arrows annotate enlarged nuclei and fibrotic cells respectively.

Figure 4. Immunofluorescent analysis of Shh (green) and Ptch-1 (red) after incubating primary human myofibroblasts with or without recombinant Shh.

Figure 5.

Expression of Shh by various organs in adult humans. (A) RT-qPCR expression of Shh, Ptch-1 and Gli-1 in human stomach versus human lymphocytes and monocytes, polymorphonuclear neutrophils, and basophils and eosinophils. (B) RT-qPCR expression of Shh in a panel of human organ tissue. Shown is the mean of samples from 3 individuals $\pm$ standard error of the mean.

Figure 6.

Size determination of plasma Shh by FPLC. (A) FPLC chromatogram of blood plasma showing the amount of protein collected in each fraction. mAU, milli-absorbance units. (B) The concentration of native Shh and denatured Shh (by boiling in SDS for 7 min) in each FPLC fraction as determined by the human Shh ELISA compared to the concentrations of 45kDa and 19kDa proteins (from normal human stomach and gastric cancer lysate) in each fraction quantified from western blots. (C) Western blot analysis of FPLC fractions of the 45kDa and 19kDa Shh obtained from normal human gastric tissue. (D) Western blot analysis of FPLC fractions of 45kDa Shh obtained from human gastric tumor lysate.

Figure 7.

Biological activity of plasma Shh. (A) Gli-1 mRNA expression in NIH 3T3 cells treated with recombinant Shh (rShh) peptide, Shh-specific 5E1 antibody, plasma Fr 9, or albumin- and IgG-depleted plasma Fr9 (Fr9 (-IgG -Alb)). (B) Gli-1 mRNA expression in NIH-3T3 cells treated with each human plasma fraction (1–15) or mouse plasma $\pm$ 5E1 chelating antibody. Recombinant Shh or Ihh were used as positive controls. Shown is the mean of 3 experiments $\pm$ standard error of the mean for all experiments. **P < 0.01.

Table 1

Age, gender, and plasma Shh levels in normal, pancreatitis, and pancreatic cancer patients.

Group tested	Gender		Age (years)		Shh (ng/ml)		Median	Q1-Q3*	Maximum
	Male	Female	Mean	S.D.	Mean	S.D.			
General population (n = 80)	50%	50%	40.2	14.7	200	495	8.1	0.0 – 51.0	2000
Control group (n = 39)	41%	59%	69.6	13.8	138	412	3.2	0.5 – 22.9	1987.8
Chronic pancreatitis patients (n = 20)	80%	20%	56.9	11.8	45	98	0.5	0.5 – 29.1	390.1
Pancreatic cancer patients (n = 20)	45%	55%	64.6	13.0	17	44	1.2	0.5 – 13.8	196.9

* Q1–Q3 values represent the 25th–75th percentiles.