HHS Public Access

Author manuscript

Clin Exp Metastasis. Author manuscript; available in PMC 2018 April 01.

Published in final edited form as:

Clin Exp Metastasis. 2017 April ; 34(3-4): 229–239. doi:10.1007/s10585-017-9840-3.

Genome-wide *in vivo* RNAi screen identifies ITIH5 as a metastasis suppressor in pancreatic cancer

Ken Sasaki[1], **Hiroshi Kurahara**[1,2], **Eric D. Young**[1], **Shoji Natsugoe**[2], **Asami Ijichi**[2], **Tomoo Iwakuma**[1], and **Danny R Welch**[1]

[1]Department of Cancer Biology, The University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA

[2]The University of Kansas Cancer Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA

[3]Department of Digestive Surgery, Breast and Thyroid Surgery, Graduate School of Medical Sciences, Kagoshima University, 8-35-1, Sakuragaoka, Kagoshima 890-8520, Japan

Abstract

The overwhelming majority of pancreatic ductal adenocarcinoma (PDAC) is not diagnosed until the cancer has metastasized, leading to an abysmal average life expectancy (3-6 months post-diagnosis). Earlier detection and more effective treatments have been hampered by inadequate understanding of the underlying molecular mechanisms controlling metastasis. We hypothesized that metastasis suppressors are involved in controlling metastasis in pancreatic cancer. Using an unbiased genome-wide shRNA screen, an shRNA library was transduced into the non-metastatic PDAC line S2-028 followed by intrasplenic injection. Resulting liver metastases were individually isolated from these mice. One liver metastatic nodule contained shRNA for ITIH5 (Inter-alpha-trypsin inhibitor heavy chain 5), suggesting that ITIH5 may act as a metastasis suppressor. Consistent with this notion, metastatic PDAC cell lines had significantly lower protein expression of ITIH5 compared to immortalized pancreatic ductal epithelial cells and non-/poorly-metastatic PDAC cell lines. By manipulating expression of ITIH5 in different PDAC cell lines (over-expression in metastatic, knockdown in non-metastatic) functional and selective regulation of metastasis was observed for ITIH5. Orthotopic tumor growth of PDAC cells was not blocked following orthotopic injection. In vitro ITIH5 over-expression inhibited motility and invasion. Immunohistochemical analysis of a human PDAC tissue microarray revealed that ITIH5 expression inversely correlated with both survival and invasion/metastasis. ITIH5 is, therefore, functionally validated as a PDAC metastasis suppressor and shows promise as a prognostic biomarker.

Corresponding Authors: Danny R. Welch and Tomoo Iwakuma, Department of Cancer Biology, The University of Kansas Medical Center, 3901 Rainbow Blvd., Mailstop 1071, Kansas City, KS 66160. Phone: 913-945-7739; Fax: 913-588-4701; Dwelch@KUMC.edu or TIwakuma@KUMC.edu.

Conflict of interest: The authors declare no conflict of interest.

Author Contributions: Conception and design: TI and DRW; Acquisition of data: KS, HK, EDY, SN, AI, TI, DRW; Writing, review and/or revision of manuscript: KS, EDY, TI, DRW; Study supervision: DRW and TI

Keywords

ITIH5; Pancreatic cancer; Metastasis suppressor; RNAi screen

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a particularly difficult malignancy to cure because the majority of tumors have aggressive local invasion and/or metastatic disease by the time of diagnosis; most tumors are asymptomatic until late in disease progression; and, most PDAC are resistant to therapies. It is the fourth most common cause of cancer-related death in the United States, and the overall 5-year survival rate is only 6-7% [1]. Furthermore, the incidence of PDAC has been increasing, and it is projected to become the second leading causes of cancer-related death by 2030 [2]. In addition to early detection and development of more effective treatments, prevention of metastatic disease after curative surgery is crucial for improving prognosis of patients with PDAC. Specifically, control of liver metastasis is important, because liver is the most common site of metastasis followed by the peritoneum and lung [3].

Metastasis suppressors are defined by their ability to block metastasis at any step of the metastatic cascade with little to no effect on primary tumor growth [4-6]. Discovery and characterization of metastasis suppressors, and understanding of their mechanisms could lead to the identification of novel metastasis markers and potential therapeutic targets for PDAC treatment. Two metastasis suppressors, KISS1 and KAI1, have been reported in PDAC [5]. Both KISS1 and KAI1 were originally discovered by subtractive hybridization and differential display comparing metastatic cells and metastasis suppressed hybrids (i.e., complemented with human chromosome 6 or 11 via microcell-mediated chromosome transfer) in human melanoma cells and rat prostatic cancer cells, respectively [7, 8]. Subsequently, they were tested in PDAC by orthotopic xenograft or subcutaneous mouse models [9, 10]. The therapeutic potential of these proteins has not yet been analyzed.

We recently utilized an unbiased systematic forward *in vivo* RNA interference (RNAi) screen to identify metastasis suppressors in PDAC [11-13]. To identify candidate metastasis suppressor for PDAC liver metastasis, we infected a non-metastatic variant of the SUIT2 human PDAC cell line S2-028 with a whole genome short hairpin RNA (shRNA) lentiviral library and injected them into spleens of nude mice. We identified 2 liver metastatic nodules which contained shRNA for ITIH5 (Inter-alpha-trypsin inhibitor heavy chain 5) and HMP19 (Hypothalamus Golgi apparatus expressed 19 kDa protein). In this study, we focused on ITIH5, and characterized for its function on liver metastasis and tumor growth using multiple PDAC cell lines. We also investigated relationship between ITIH5 expression and clinicopathological factors in human PDAC.

Materials and Methods

Cell lines and cell culture

Five human PDAC cell lines (S2-007, S2-028, MIAPaCa-2, BxPC-3 and Panc-1) were obtained from Dr. M. Anthony Hollingsworth (Eppley Cancer Center). S2-007, S2-028, Panc-1 and MIAPaCa-2 cells, which contain KRAS mutation, and HPNE were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), BxPC-3 cells, which does not have KRAS mutation, were cultured in RPMI1640 medium (Invitrogen), supplemented with 10% FBS at 37°C in a 5% CO_2-humidified atmosphere, respectively. Immortalized human pancreatic duct normal epithelium cells (HPNE) were kindly provided by Animesh Dhar at the University of Kansas Medical Center. All cells were most recently validated as human and having expected STR polymorphisms and K-Ras mutations. Following transduction, experiments were completed using cells within 5–10 passages. All cells were screened for *Mycoplasma spp*. and used for experiments in this study when approximately 70-80% confluent. Cells were harvested using a solution containing 0.05% Trypsin and 2 mM EDTA.

shRNA and *in vivo* screen

Non-metastatic S2-028 cells were infected with pGIPZ lentiviral microRNA-adapted shRNA (shRNAmir) library consisting of 74,468 shRNA directed against 21,416 human genes (S2-028 shRNA library) or pGIPZ lentiviral vector containing non-silencing control shRNAmir (S2-028 control) (ThermoScientific Open Biosystems, Huntsville, AL) at multiplicity of infection of 0.2. Transduced cells were cultured in medium with puromycin for 1 wk for selection. We used 7-wk old female athymic nude mice (Harlan Sprague Dawley Inc., Indianapolis, IN) for screening to identify genes that suppress liver metastasis. Cells (5×10^5 cells) in 100 μL of Hank's balanced salt solution of S2-028 cell and S2-028 control were injected into the spleens of 5 mice, and cells of S2-028 shRNA library was injected into the spleens of 10 mice, respectively. The spleen injected with the cell suspension was removed 2 min after the injection by surgical ligation. Splenic injection was performed as previously described with some minor modifications [14, 15]. Mice were euthanized 4 wk after the injection and liver metastases were cultured individually. The cultures derived from each liver metastasis were re-injected into the spleen to reduce the possibility of a false positive result. After 2 rounds of intrasplenic injection, genomic DNA was isolated from each liver metastasis. The individual shRNAmir integrated into cells was identified by sequencing after recovery by PCR amplification using primers designed to flank the shRNAmir sequence.

Plasmids and viral transductions

For knockdown of ITIH5, two different shRNA sequences, shRNA 1 (5'-CCCATCTACTGTCATTAACCAA-3') (ThermoScientific Open Biosystems, Pittsburgh PA, USA) and shRNA 2 (5'-AGGACCTTTGCTCAAGAAG-3') (GeneCopoeia, Inc. Rockville, MD, USA) were used. The sequence of shRNA1 was same as that used in shRNAmir library screening. The lentiviral vector non-target shRNA was purchased from GeneCopoeia. For overexpression, full-length human ITIH5 cDNA and empty vector were purchased from GeneCopoeia. Transduction of shRNA and cDNA were performed using Lenti-Pac™ HIV

expression packaging kit (GeneCopoeia, Inc.) according to the manufacturer's instructions. For lentivirus production, 2.5 μg of lentiviral open reading frame/ shRNA expression plasmid together with 2.5 μg of Lenti-Pac HIV mix were transfected into 293FT cells with EndoFectin Lenti. Viral culture medium was collected 48 hr after transfections and cleared through a 0.45 μm polyethersulfone low protein-binding filters. Cells were infected with viral culture medium containing 4 μg/mL hexadimethrine bromide (Sigma, St. Louis, MO) and selected with puromycin for 1 wk. Transduced cells were characterized by immunoblot for ITIH5 expression.

Western blotting

Cells were lysed with RIPA buffer supplemented with protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, Rockford, IL, USA). Protein concentration was determined using a BCA assay. Whole-cell extracts (40 μg) were denatured with Laemmli buffer at 95°C for 5 min before separation using 4–20% gradient SDS-PAGE (Bio-Rad Laboratories, Hercules, CA, USA) and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% non-fat milk in 1 × Tris-buffered saline with 0.1% Tween-20, PVDF membranes were incubated with anti-ITIH5 (ThermoFisher Scientific, Waltham, MA), anti-GAPDH (Santa Cruz, Dallas, TX, USA), overnight at 4°C followed by secondary antibodies conjugated with horseradish peroxidase (GE Healthcare Biosciences, Piscataway, NJ, USA). To visualize signals, Super Signal West Dura or Femto (ThermoFisher) was used according to the manufacturer's instructions. The signals were detected using a FLuorChem M MultiFluor System (Protensimple, Santa Clara, CA).

Orthotopic injection | Spontaneous Metastasis Assay

To assess tumor growth of generated PDAC cell lines, 6- to 8-wk-old athymic nude mice (10 each) were injected orthotopically with PDAC cell lines (1×10^6 cells/50 μL) suspended in a 1:1 mixture of HBSS and growth factor-reduced Matrigel (BD Biosciences, Franklin Lakes, NJ) as previously described with some modifications [16]. Tumor size was measured using calipers following euthanasia. Tumor volume was calculated using the following formula (1/2 × length × breadth × width)].

Intrasplenic injection | Experimental Metastasis Assay

To assess an ability of experimental liver metastasis, 6- to 8-week-old athymic nude mice (10 ea) were injected intrasplenically with PDAC cell lines. Mice injected with S2-007 cells, and mice injected with S2-028 and MIAPaCa-2 cell were euthanized 4 and 8 wk after the injection, respectively. And then the incidence of liver metastasis and the numbe of liver metastasis were evaluated.

Ethics statement

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by University of Kansas Medical Center Institutional Animal Care and Use Committee (Protocol #2014-2208).

Motility | Wound healing assay

An *in vitro* wound healing (a.k.a., scratch assay) was used to analyze effects of ITIH5 on cell migration. Cells were plated onto 6-well plates in normal culture medium and allowed to reach ~90% confluence. Sterile 200 μL pipet tips were use to create an acellular line in the monolayer; plates were washed for several times with PBS to remove cell debris; grow medium was replaced and cells were incubated. The average distances of wound closure were measured 12-24 hours after scratching.

Trans-well cell invasion assay

Invasion assays were performed in 24-well BioCoat™ Matrigel invasion assay kit containing 8-μm pore polyethylene terephthalate membrane with a thin layer of Matrigel basement membrane matrix (BD Biosciences, San Jose, CA, USA) according to product instructions. Cells were suspended at 5×10^4 cells/ml in medium with 0.5% FBS. Five hundred μL of cell suspension was placed in the upper chamber and 750 μL of medium with 10% FBS were added in the lower chamber, and allowed to invade for 24-48 hours at 37°C in 5% CO_2 incubator. After incubation, upper surfaces of the filters were scraped twice with cotton swabs to remove non-invading cells, and invading cells were fixed and stained, and the number of non-invading cells in five high-power fields per filter was counted microscopically at 400 × magnifications.

Proliferation assay

To measure proliferation rate, cells were inoculated onto 6-well culture dishes at 1×10^5 cells per well in normal culture medium at 37°C in a 5% CO_2 humidified atmosphere. At the indicated time points, the cells were labeled with trypan blue and counted.

Patients and tumor samples

Surgical specimens were obtained from 81 patients with pancreatic cancer, who received surgical treatment at Kagoshima University Hospital between January 1990 and December 2007. The study group comprised 52 men and 29 women ranging in age from 42 to 81 years (median, 66.43 years). All patients underwent macroscopically curative resection. None of these patients had received any preoperative chemotherapy or radiotherapy. All resected specimen were examined histologically by hematoxylin and eosin (H&E) staining according to the TNM classification system [17, 18]. Pathologically, all tumors were invasive PDAC. Of these 81 patients, 33 (40.7%) experienced liver metastasis after surgery. This study was approved by the Institutional Review Board of Kagoshima University.

Immunohistochemistry

Paraffin sections (3 μm) of formalin-fixed tissue were dewaxed and rehydrated. After heat-induced antigen retrieval, endogenous peroxidase activity and nonspecific reactions were blocked by 3% hydrogen peroxide in methanol and 1.5% normal goat serum, respectively. Sections were then incubated with anti-ITIH5 antibody (Sigma-Aldrich, St. Louis, MO) *a* dilution of 1:100 overnight at 4°C. The antigen and antibody complexes were visualized with an avidin biotin complex detection kit (Vector Laboratories, Inc. Burlingame, CA) and a diaminobenzidine substrate (Life Technologies, Grand Island, NY). The sections were

counterstained with hematoxylin, and mounted. Normal placenta tissue, known to abundantly express ITIH5[16], was used as positive control for ITIH5, and negative control was created by replacing the primary antibody with PBS. ITIH5 staining was considered as positive if at least 5% cancer cells were stained and was scored as follows: 0 (negative), 1 (weak), 2 (moderate), or 3 (strong).

Statistical analysis

Statistical analyses were performed using Sigmaplot 12.0 (Systat Software, Inc. San Jose, CA) and JMP 10.0.2 (SAS Institute, Inc., Cary, NC) was used. Associations between different categorical variables for the tissue microarray and clinical variable studies were assessed using the χ^2 test. Continuous variables were compared between 2 groups using the Mann-Whitney U test. Survival rates were calculated using the Kaplan-Meier method, and significant differences in survival were estimated by the log-rank test. Multivariate analysis was calculated using a Cox regression model including all parameters that were significantly prognostic in univariate analysis. In vitro experiments were performed at least twice independently using triplicate samples. In vivo studies were performed at least twice independently using 8-10 mice per experimental group. Statistical significance for all studies was defined as $P < 0.05$.

Results

Identification of ITIH5

To better understand the mechanisms underlying pancreatic cancer metastasis, we performed a whole genome shRNA library screen to identify candidate genes that, when knocked down, enabled a non-metastatic PDAC cell line S2-028 to metastasize to the liver using an *in vivo* intrasplenic injection mouse model (Fig. 1A). A total of 6 liver metastases were observed in 3 mice. In contrast, no liver metastasis was observed in 10 athymic nude mice injected with parental S2-028 cells or S2-028 infected with a control shRNA lentiviral vector. We validated these 6 metastases-derived cells by second intrasplenic injections and found that only 2 out of the 6 clones consistently formed liver metastases. Sequencing of shRNA in the genomic DNA of these 2 clones revealed ITIH5 and HMP19 as candidate metastasis suppressors. Cells with shRNA for ITIH5 and HMP19 formed 4 and 20 liver metastases, respectively, in this second metastasis assays (Figs. 1B and 3A). We recently reported functionality and preliminary mechanistic characterization of HMP19 [19]. In this study, we focused on ITIH5.

ITIH5 suppresses metastasis in PDAC cell lines

To compare endogenous expression of ITIH5 among a pancreatic duct normal epithelium HPNE and 5 human PDAC cell lines (S2-007, S2-028, MIAPaCa-2, BXPC-3, Panc-1), immunoblotting was performed (Fig. 2A). Non-tumorigenic HPNE showed highest ITIH5 protein expression, while all of the cancer cell lines had lower ITIH5 expression. Consistent with its putative role as a metastasis suppressor, ITIH5 protein was lower in metastatic (S2-007, MIAPaCa-2 and Panc-1) compared to low/non-metastatic PDAC cell lines (S2-028 and BxPC3).

To further validate involvement of ITIH5 in regulating metastasis independent shRNA were used. The first (designated shRNA 1) was used in the screen identifying ITIH5 and the second (designated shRNA2) was commercially available. Cells infected with both shRNA1 and shRNA2 resulted in more mice with liver metastasis (7/10 and 8/10 mice, respectively) and more liver metastatic nodules per mouse (3-5 metastases per mouse); whereas, none of mice injected with control S2-028 cells developed metastasis (Figs. 2B and 3B).

We next examined the effects of ITIH5 overexpression on the metastatic potential of highly metastatic S2-007 cells by intrasplenic injection. S2-007 cells were transduced with empty vector (control) or with ITIH5 cDNA-encoding lentiviral vectors. Although 9/10 mice developed metastases whether ITIH5 was overexpressing or not, the number of liver metastasis in ITIH5-overexpressing cells were significantly fewer compared to control transduced cells (˜6 *vs* ˜20 foci, $P<0.05$, Fig.2B). Additional experiments over-expressing ITIH5 in MIAPaCa-2 cells also completely blocked experimental metastasis to the liver (0/10 mice), whereas 3/10 control MIAPaCa-2 cells formed multiple liver metastases ($P<0.001$, Fig.2C).

ITIH5 marginally slows orthotopic tumor growth

Down-regulation of ITIH5 in S2-028 cells resulted in increased primary tumor growth (Fig. 4 top row). In S2-007 cells, over-expression of ITIH5 slightly reduced sizes of tumors (Fig. 4 middle row), while over-expression of ITIH5 in MIAPaCa-2 cells did not significantly change the tumor size (Fig. 4 bottom row). Thus, effects of ITIH5 on tumor cell proliferation and primary tumor growth by intrapancreatic, orthotopic injection assays were not as robust as those observed in metastasis assays and dependent on cell lines used.

ITIH5 suppresses *in vitro* PDAC cell migration and invasion

To begin understanding the potential mechanism(s) by which ITIH5 suppresses PDAC metastasis, we employed a *in vitro* assays modeling steps of metastasis. To examine the effects of ITIH5 on cell growth following down-regulation or over- expression of ITIH5 in multiple PDAC cell lines was measured *in vitro*. Down-regulation of ITIH5 using two different shRNA in S2-028 cells did not alter cell proliferation. Similarly, over-expression ITIH5 in S2-007 and MIAPaCa-2 cell lines did not lead to any changes in tumor cell proliferation (Fig. 5A-C).

Wound-healing motility assays following manipulation of ITIH5 expression in PDAC cells revealed that knockdown of ITIH5 in S2-028 cells significantly accelerated wound closure compared to control cells (Fig. 5D), while over-expression of ITIH5 in S2-007 and MIAPaCa-2 cells significantly inhibited it (Figs. 5E and 5E). Similarly, knockdown of ITIH5 in S2-028 cells significantly increased cells invading through Matrigel compared with the control (Fig. 4G), while over-expression of ITIH5 in S2-007 and MIAPaCa-2 cells decreased invasion (Fig. 5H and 5I).

Attempts to rescue the shRNA knockdowns were unsuccessful for several reasons (EDY and DRW, unpublished observations). The primary reason was that we could not use cDNA with mutations in the coding region since we do not know precisely how ITIH5 works at a biochemical level. Introduction of mutations in the cDNA could alter function, making

interpretation of negative results impossible. Additionally, there are other splice variants of ITIH5 reported (http://uswest.ensembl.org/Homo_sapiens/Transcript/Summary?db=core;g=ENSG00000123243;r=10:7559270-7666998;t=ENST00000397146). We are currently characterizing different variants.

ITIH5 loss is associated with extrapancreatic plexus invasion, postoperative liver metastasis and poor prognosis in human PDAC

To examine the clinical significance of ITIH5, we performed immunohistochemistry for ITIH5 using a tissue microarray comprised of 81 human PDAC tissues. Placenta served as a positive control, since it has high ITIH5 expression (Fig. 6A). ITIH5 was intensely expressed in the cytoplasm of normal pancreatic duct epithelial cells and islet cells (Fig. 6B). Staining patterns for ITIH5 in tumor tissues were heterogeneous and hence were categorized as negative (0, Fig. 6C), weak positive (1, Fig. 6D), moderately positive (2, Fig. 6E), and strongly positive (3, Fig. 6F). A summary of relationship between ITIH5 expression and clinicopathological parameters is shown in Table 1. ITIH5 was observed in 55.6% (45/81) of cases. Negative expression of ITIH5 was correlated with high incidence of extrapancreatic plexus invasion and postoperative liver metastasis. Tumors without ITIH5 expression (44.4%; 36/81) had significantly poorer prognosis after surgery (P = 0.0299, Fig. 6G). The median survival time of patients with tumors positive or negative for ITIH5 were 12.6 months and 9.4 months, respectively. Using a stratified univariate analysis, the prognostic value of ITIH5 was more remarkable in the early stage clinical subgroups of node-negative (P=0.0198, Fig. 6H) and plexus invasion-negative patients (P=0.0174, Fig. 6I). We also compared the prognostic impact of ITIH5 expression with other clinicopathological parameters using univariate and multivariate analyses. Lymph node metastasis, extrapancreatic plexus invasion and ITIH5 expression were significantly associated with overall survival by univariate analyses. However, multivariate regression analysis indicated that lymph node metastasis and extrapancreatic plexus invasion, but not ITIH5 expression, were independent prognostic factors.

Discussion

Because of the complexity of the metastatic process, identification of and, more importantly, validation of metastasis-associated molecules has proven to be quite challenging. But an increasing number of *bona fide* metastasis genes have been discovered, but most have been discovered in breast and prostate cancers. Using a forward genetic screen using a genome-wide RNAi library and a non-metastatic S2-028 PDAC cell line, we identified two novel metastasis suppressor candidates, ITIH5 and HMP19. The latter was recently validated as a metastasis suppressor [19] and ITIH5 was functionally characterized in this study.

Inter-α-trypsin inhibitors (ITI) are a family of serine protease inhibitors that comprise a common light chain of chondroitin sulfate proteoglycan, bikunin, and a variable set of two homologous heavy chains (ITI heavy chains, ITIH). Bikunin plays an important role, not only in the protection of organ injury during severe inflammation, but also in the inhibition of tumor invasion [20] and metastasis [21]. ITIH were originally referred to as serum-derived hyaluronic acid-associated proteins (SHAP) owing to the covalent linkage with

hyaluronic acid (HA), and ITIH interaction with HA were thought to stabilize the extracellular matrix [22]. At least 6 ITIH have been identified in the human genome, and ITIH appear to have biological function independently of bikunin [23, 24].

Various ITIH family members are down-regulated in a variety of human malignant tumors [25-27]. Paris *et al.* demonstrated that over-expression of ITIH in a human lung cancer cell line reduced the number of lung metastasis in a mouse model [28]. ITIH5 has only recently characterized via bioinformatics approaches and is the only gene within the ITIH family having a CpG-rich promoter region [29]. ITIH5 down-regulation by aberrant promoter hypermethylation is associated with regional lymph node and distant metastasis as well as unfavorable clinical outcomes in invasive node-negative breast cancer [30]. Recent examination of the ITIH5 promoter in circulating free DNA in serum suggests that ITIH5 may be a biomarker for early breast cancer detection [31]. Down-regulation of ITIH5 due to the promoter hypermethylation is associated with progression of acute myeloid leukemia, squamous cell carcinoma of the tongue, bladder carcinoma, and colon cancers [32-35]. Decreased ITIH5 expression is also associated with development of thyroid and gastric cancers [36, 37]. Thus, accumulating anecdotal evidence suggests roles of ITIH5 expression on the progression of various types of cancer.

The present study reports, for the first time, that ITIH5 plays an important role in the PDAC progression using reciprocal up- and down-regulation experimental models using multiple PDAC models. ITIH5 appears to affect PDAC metastasis by altering cell migration and invasion with affecting cell proliferation. These observations are consistent with the results by Rose *et al.* in a human bladder cancer cell line [34]. However, *in vitro* growth of colon and breast cancer cell lines in which ITIH5 was over-expressed has been observed [30, 35]. The suppressive effects of ITIH5 may be cell/tissue-type specific. Additionally, we observed more lamellipodia when ITIH5 is expressed in BxPC3 cells (EDY and DRW, unpublished observations), a pattern opposite of what we would expect with a metastasis suppressor. Thus, ITIH5 effects on PDAC behavior may be combinatorically associated with oncogenic drivers as well. Experiments to explore such associations will be required.

Further supporting the role of ITIH5 as a metastasis suppressor in PDAC is the association of reduced ITIH5 expression with a high incidence of liver metastasis and plexus invasion in post-surgical specimens. IHC analyses reveals that ITIH5 can be a useful prognostic biomarker, especially early stage PDAC. Importantly, ITIH5 appears not to serve a prognostic factor for advanced PDAC. These findings are consistent with the observations of Veeck *et al.* [30], who observed some predictive value of ITIH5 in breast cancer metastasis to liver.

In summary, our studies have identified a novel prognostic biomarker for PDAC. Both experimental and clinical analyses support the conclusion that ITIH5 is a *bona fide* metastasis suppressor, although its precise mechanism of action is not yet clear. Moreover, since ITIH5 expression correlated with 20-30% multi-year survival of PDAC patients, it is interesting to speculate that ITIH5 may also help stratify patients considering pancreaticoduodenectomy. Since the Whipple procedure is associated with significant morbidity, a challenging recovery, but only 25–30% 5-year survival, it is interesting to

speculate that ITIH5 could help identify the patients who are mostly likely to benefit from the procedure, while sparing other patients surgical complications that do not improve likelihood for long-term survival. This possibility is among the many future investigations required to dissect the molecular mechanisms underlying ITIH5-mediated PDAC metastasis suppression.

Acknowledgments

The authors are extremely grateful to Dr. Tony Hollingsworth (Eppley Cancer Center) for the gift of the SUIT-2 cell lines and appreciate helpful advice from Drs. Animesh Dhar, Shrikant Anant and Dan Von Hoff. They thank Dr. Christopher Bohl and Carolyn Vivian for technical assistance and other members of our labs for thoughtful suggestions and advice from the University of Kansas Cancer Center Biostatistics Shared Resource. DRW is the Hall Family Professor of Molecular Medicine, a Kansas Bioscience Authority Eminent Scholar and a Komen Scholar. The authors also thank the funding agencies who supported this research.

Grant support. These studies were funded primarily by a grant from the National Foundation for Cancer Research (DRW), with additional support from U.S. Public Health Service grants CA134981 (DRW), CA168524 (DRW) and CA174735 (TI), the American Cancer Society (RSG-09-169-01-CS to TI), Susan G. Komen for the Cure SAC11037 (DRW) and The Kansas Biosciences Authority (DRW).

Reference List

1. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011; 378(9791):607–20. [PubMed: 21620466]
2. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. Cancer Res. 2014; 74(11):2913–21. [PubMed: 24840647]
3. Cameron JL, Riall TS, Coleman J, Belcher KA. One thousand consecutive pancreaticoduodenectomies. Annals of surgery. 2006; 244(10-5)
4. Liu W, Vivian CJ, Brinker AE, Hampton KR, Lianidou E, Welch DR. Microenvironmental influences on metastasis suppressor expression and function during a metastatic cell's journey. Cancer Microenvironment. 2014; 7(3):117–31. [PubMed: 24938990]
5. Bohl CR, Harihar S, Denning WL, Sharma R, Welch DR. Metastasis suppressors in breast cancers: mechanistic insights and clinical potential. J Mol Med (Berlin). 2013; 92(1):13–30. [PubMed: 24311119]
6. Hurst DR, Welch DR. Metastasis suppressor genes: At the interface between the environment and tumor cell growth. Intl Rev Cell Molec Biol. 2011; 286(107-80)
7. Lee J-H, Miele ME, Hicks DJ, et al. *KiSS-1*, a novel human malignant melanoma metastasis-suppressor gene. J Natl Cancer Inst. 1996; 88(23):1731–7. [PubMed: 8944003]
8. Dong JT, Lamb PW, Rinker-Schaeffer CW, et al. KAI1, a metastasis suppressor gene for prostate cancer on human chromosome 11p11.2. Science. 1995; 268(5212):884–6. [PubMed: 7754374]
9. Guo XZ, Friess H, Shao XD, et al. KAI1 gene is differently expressed in papillary and pancreatic cancer: influence on metastasis. World J Gastroenterol. 2000; 6(6):866–71. [PubMed: 11819710]
10. McNally LR, Welch DR, Beck BH, et al. KISS1 over-expression suppresses metastasis of pancreatic adenocarcinoma in a xenograft mouse model. Clin Exptl Metastasis. 2010; 27(8):591–600. [PubMed: 20844932]
11. Gumireddy K, Li AP, Gimotty PA, et al. KLF17 is a negative regulator of epithelial-mesenchymal transition and metastasis in breast cancer. Nature Cell Biol. 2009; 11(11):1297–U69. [PubMed: 19801974]
12. Murugaesu N, Iravani M, van WA, et al. An in vivo functional screen identifies ST6GalNAc2 sialyltransferase as a breast cancer metastasis suppressor. Cancer Discov. 2014; 4(3):304–17. [PubMed: 24520024]

13. Duquet A, Melotti A, Mishra S, et al. A novel genome-wide in vivo screen for metastatic suppressors in human colon cancer identifies the positive WNT-TCF pathway modulators TMED3 and SOX12. EMBO Mol Med. 2014; 6(7):882–901. [PubMed: 24920608]
14. Giavazzi R, Jessup JM, Campbell DE, Walker SM, Fidler IJ. Experimental nude mouse model of human colorectal cancer liver metastases. J Natl Cancer Inst. 1986; 77(6):1303–8. [PubMed: 3467119]
15. Giavazzi R, Campbell DE, Jessup JM, Cleary KR, Fidler IJ. Metastatic behavior of tumor cells isolated from primary and metastatic human colorectal carcinomas implanted into different sites of nude mice. Cancer Res. 1986; 46(4):1928–33. [PubMed: 3948174]
16. Vonlaufen A, Joshi S, Qu CF, et al. Pancreatic stellate cells: Partners in crime with pancreatic cancer cells. Cancer Res. 2008; 68(7):2085–93. [PubMed: 18381413]
17. Gospodarowicz MK, Miller D, Groome PA, Greene FL, Logan PA, Sobin LH. The process for continuous improvement of the TNM classification. Cancer. 2004; 100(1):1–5. [PubMed: 14692017]
18. Sobin LH. TNM, sixth edition: new developments in general concepts and rules. Semin Surg Oncol. 2003; 21(1):19–22. [PubMed: 12923912]
19. Kurahara H, Bohl C, Natsugoe S, et al. Suppression of pancreatic cancer growth and metastasis HMP19 identified through genome-wide shRNA screen. Int J Cancer. 2016; 139(3):628–38. [PubMed: 27012470]
20. Kobayashi H, Fujie M, Shinohara H, Ohi H, Sugimura M, Terao T. Effects of urinary trypsin inhibitor on the invasion of reconstituted basement membranes by ovarian cancer cells. Int J Cancer. 1994; 57(3):378–84. [PubMed: 8168999]
21. Kobayashi H, Shinohara H, Fujie M, et al. Inhibition of metastasis of Lewis lung carcinoma by urinary trypsin inhibitor in experimental and spontaneous metastasis models. Int J Cancer. 1995; 63(3):455–6. [PubMed: 7591248]
22. Huang L, Yoneda M, Kimata K. A serum-derived hyaluronan-associated protein (SHAP) is the heavy chain of the inter alpha-trypsin inhibitor. J Biol Chem. 1993; 268(35):26725–30. [PubMed: 7504674]
23. Chen L, Mao SJ, McLean LR, Powers RW, Larsen WJ. Proteins of the inter-alpha-trypsin inhibitor family stabilize the cumulus extracellular matrix through their direct binding with hyaluronic acid. J Biol Chem. 1994; 269(45):28282–7. [PubMed: 7525572]
24. Zhuo L, Kimata K. Structure and function of inter-alpha-trypsin inhibitor heavy chains. Connec Tissue Res. 2008; 49(5):311–20.
25. Subrungruanga I, Thawornkunob C, Chawalitchewinkoon-Petmitrc P, Pairojkul C, Wongkham S, Petmitrb S. Gene expression profiling of intrahepatic cholangiocarcinoma. Asian Pac J Cancer Prev. 2013; 14(1):557–63. [PubMed: 23534794]
26. Hamm A, Veeck J, Bektas N, et al. Frequent expression loss of Inter-alpha-trypsin inhibitor hea chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. BMC Cancer. 2008; 8(25)
27. Abdullah-Soheimi SS, Lim BK, Hashim OH, Shuib AS. Patients with ovarian carcinoma excret different altered levels of urine CD59, kininogen-1 and fragments of inter-alpha-trypsin inhibitor heav chain H4 and albumin. Proteome Sci. 2010; 8(58)
28. Paris S, Sesboue R, Delpech B, et al. Inhibition of tumor growth and metastatic spreading by overexpression of inter-alpha-trypsin inhibitor family chains. Int J Cancer. 2002; 97(5):615–20. [PubMed: 11807786]
29. Himmelfarb M, Klopocki E, Grube S, et al. ITIH5, a novel member of the inter-alpha-trypsin inhibitor heavy chain family is downregulated in breast cancer. Cancer Lett. 2004; 204(1):69–77. [PubMed: 14744536]
30. Veeck J, Chorovicer M, Naami A, et al. The extracellular matrix protein ITIH5 is a novel prognostic marker in invasive node-negative breast cancer and its aberrant expression is caused by promoter hypermethylation. Oncogene. 2008; 27(6):865–76. [PubMed: 17653090]
31. Kloten V, Becker B, Winner K, et al. Promoter hypermethylation of the tumor-suppressor genes ITIH5, DKK3, and RASSF1A as novel biomarkers for blood-based breast cancer screening. Breast Cancer Res. 2013; 15(1):R4. [PubMed: 23320751]

32. Oing C, Jost E, Dahl E, Wilop S, Brummendorf TH, Galm O. Aberrant DNA hypermethylation of the ITIH5 tumor suppressor gene in acute myeloid leukemia. Clin Epigenetics. 2011; 2(2):419–23. [PubMed: 22704354]
33. Zhang S, Feng XL, Shi L, et al. Genome-wide analysis of DNA methylation in tongue squamous cell carcinoma. Oncol Rep. 2013; 29(5):1819–26. [PubMed: 23446731]
34. Rose M, Gaisa NT, Antony P, et al. Epigenetic inactivation of ITIH5 promotes bladder cancer progression and predicts early relapse of pT1 high-grade urothelial tumours. Carcinogenesis. 2014; 35(3):727–36. [PubMed: 24265292]
35. Kloten V, Rose M, Kaspar S, von SS, Knuchel R, Dahl E. Epigenetic inactivation of the novel candidate tumor suppressor gene ITIH5 in colon cancer predicts unfavorable overall survival in the CpG island methylator phenotype. Epigenetics. 2014; 9(9):1290–301. [PubMed: 25093535]
36. Mai C, Zhao JJ, Tang XF, et al. Decreased ITIH5 expression is associated with poor prognosis in primary gastric cancer. Med Oncol. 2014; 31(7):53. [PubMed: 24913813]
37. Pita JM, Banito A, Cavaco BM, Leite V. Gene expression profiling associated with the progression to poorly differentiated thyroid carcinomas. Br J Cancer. 2009; 101(10):1782–91. [PubMed: 19809427]

Abbreviations

RNAi	RNA interference
PDAC	pancreatic ductal adenocarcinoma
shRNA	short hairpin RNA
ITIH5	Inter-alpha-trypsin inhibitor heavy chain 5
HMP19	Hypothalamus Golgi apparatus expressed 19 kDa protein
HPNE	human pancreatic duct normal epithelium cells
FBS	fetal bovine serum
shRNAmir	microRNA-adapted shRNA
ITIs	Inter-α-trypsin inhibitors
ITIHs	ITI heavy chains
HA	hyaluronic acid
SHAP	serum-derived hyaluronic acid-associated protein

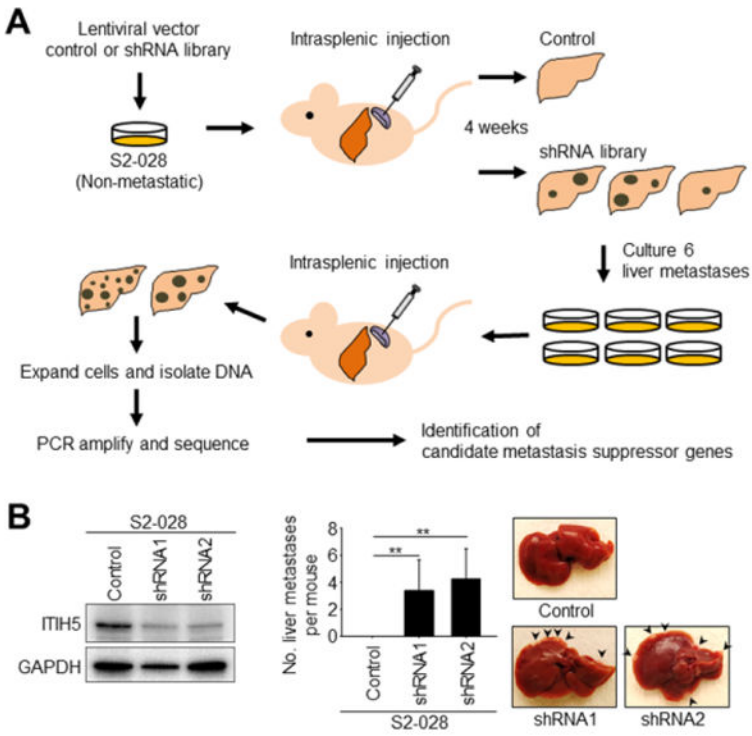

Fig. 1. Whole-genome *in vivo* shRNA screen identifies ITIH5 as a metastasis suppressor gene in PDAC

A, Schematic diagram of the *in vivo* shRNA forward screen to identify genes regulating liver metastasis in PDAC. Briefly, following transduction, S2-028 PDAC cells were injection intrasplenically. After 4 wk, liver metastases were isolated, cultured and re-injected into the spleens of athymic mice. Hepatic metastases were isolated and established in cell culture before isolating DNA and identification of shRNA integrated. After the second round of intrasplenic injections, two target genes were identified from 2 cell clones, HMP19 and ITIH5. B, Representative images of livers from mice injected with control S2-028 or shRNA library-infected S2-028. Scale bar, 10 mm.

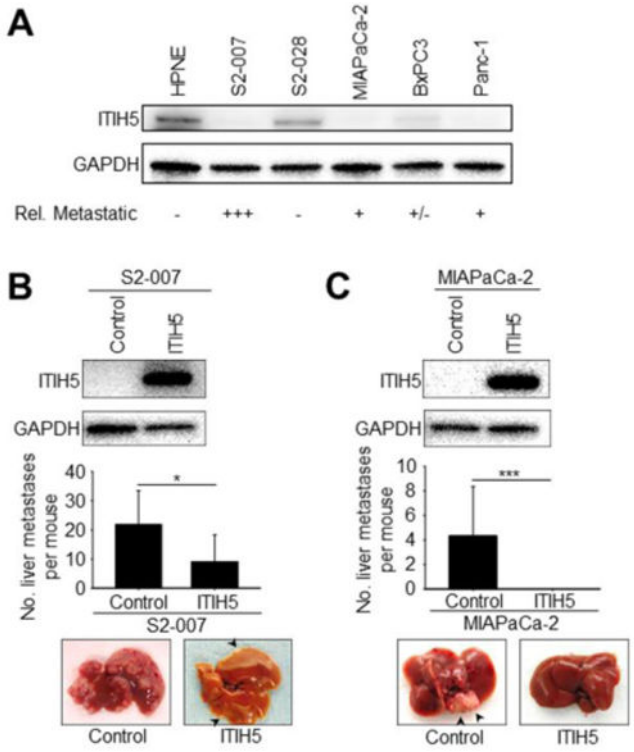

Fig. 2. ITIH5 suppresses experimental PDAC liver metastasis

A, The relative expression ITIH5 levels of in a panel of PDAC cell lines and HPNE, as determined by immunoblotting. Relative metastatic potential is provided for comparison. B, C Mixed pools of S2-007 and MIAPaCa-2 cells transduced with ITIH5 cDNA over-express ITIH5 and the number of liver metastasis are compared following intrasplenic injections. Representative livers are shown. Arrows highlight liver foci. Results are means + SE of liver nodules from positive mice. * $P < 0.05$ and ** $P < 0.001$; Mann-Whitney U test.

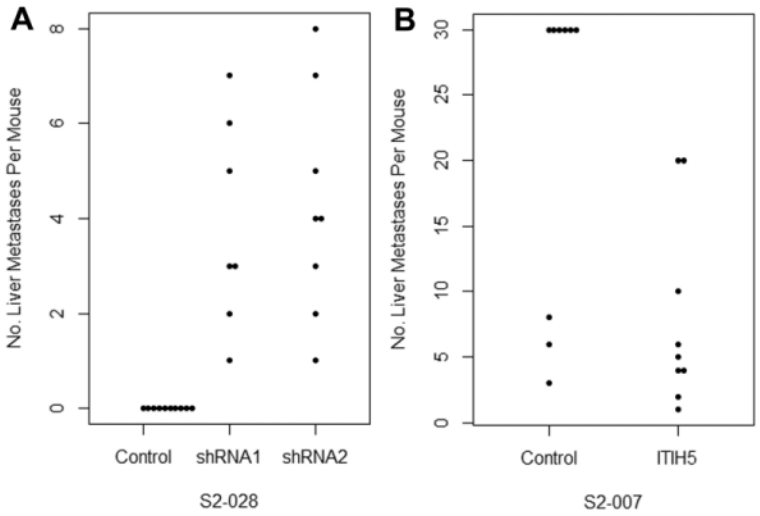

Fig. 3. Liver metastases formed in S2-028 and S2-007

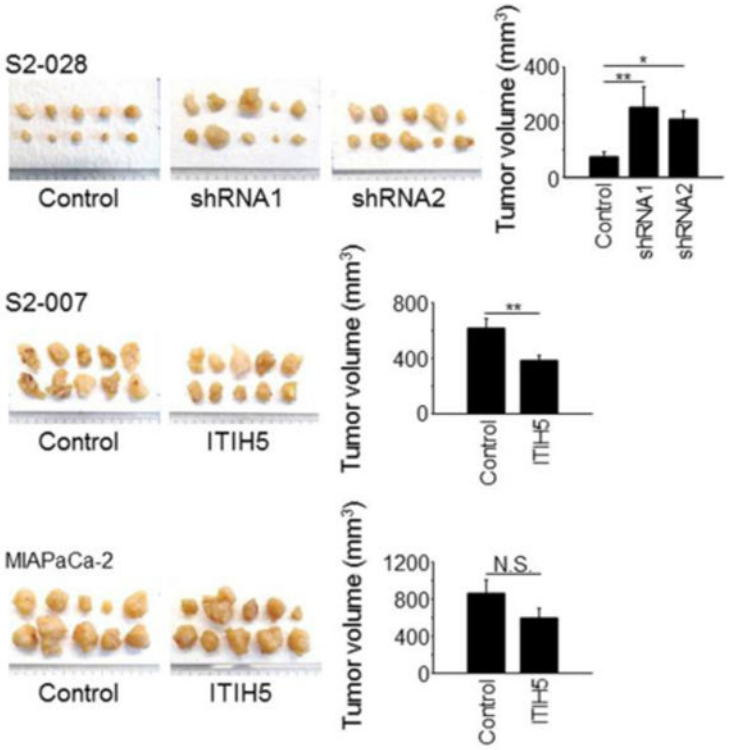

Fig. 4. ITIH5 suppresses orthotopic PDAC tumor growth

A-C, Images of pancreatic tumors and average tumor volume following orthotopic injections of PDAC cells in which ITIH5 expression levels have been altered by transduction of shRNA (in S2-028 cells) or cDNA (in S2-007 or MIAPaCa-2 cells). Graphical representation of orthotopic tumor size at time of euthanasia. Results are means + SE of the orthotopic tumor volume. * $P < 0.05$ and ** $P < 0.01$; Mann-Whitney U test.

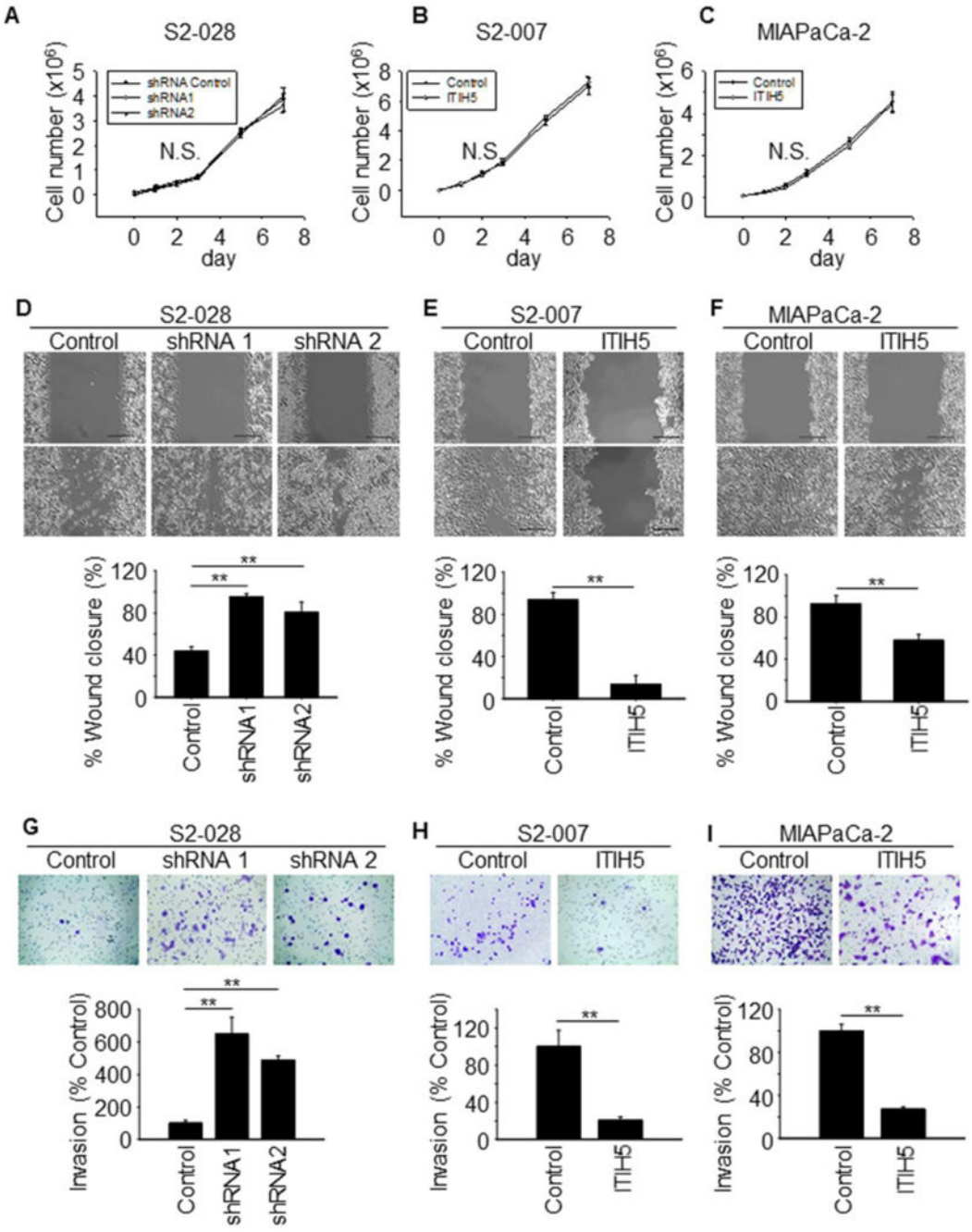

Figure 5. ITIH5 suppresses PDAC cell migration and invasion

Representative photographs showing migration (A-C) and invasion (D-F) are inhibited when ITIH5 levels are high. Results are means + SE of the % wound closure and number of invaded cells, respectively. G-I, Growth curves of PDAC cell lines altered ITIH5 expression levels. * $P < 0.01$ and ** $P < 0.001$; Mann-Whitney U test. Scale bar, 200 μm.

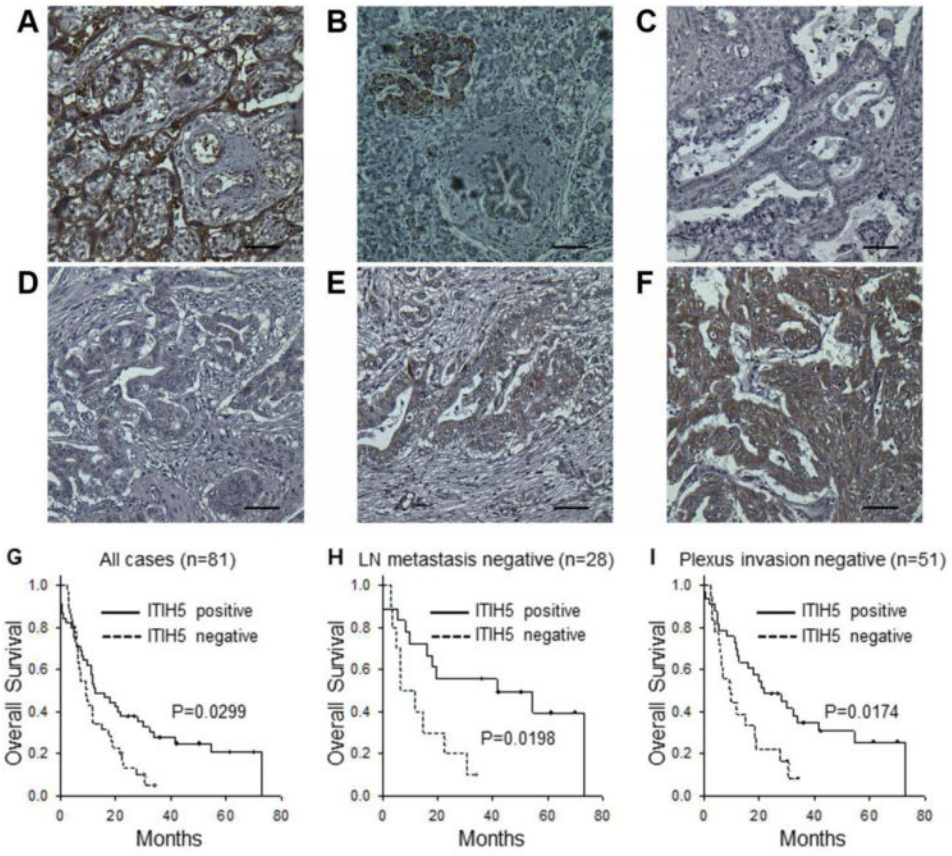

Figure 6. ITIH5 protein expression predicts overall survival in PDAC

A-F, Representative images of ITIH5 immunohistochemical staining in human pancreatic cancer and normal pancreatic tissues adjacent to cancerous lesion. A, Normal placenta, known to highly express ITIH5, was used as positive control for ITIH5 staining. B, Strong ITIH5 expression in normal pancreatic duct epithelial cells and islet cells. C, negative expression of ITIH5, D, positive expression of CXCR4 with weak intensity, E, positive expression of CXCR4 with moderate intensity, F, positive expression of CXCR4 with strong intensity. Counterstaining, hematoxylin; magnification, ×200: scale Bar, 200 μm. G, Kaplan-Meier survival curves of PDAC patients. H, I, Survival curves of node-negative or node-positive PDAC patients J, K, Survival curves of plexus invasion negative or plexus invasion positive PDAC patients.

Table 1

Relationship between the ITIH5 protein expression clinicopathological factors in human PDAC

	Total (*n* =81)	ITIH5 expression		*P value*
		Positive	Negative	
		n = 45 (55.6%)	n = 36 (44.4%)	
Age (y)		69	65.5	0.605
Sex				
Female	29	16	13	0.958
Male	52	29	23	
Histological grade				
Grade 1	30	17	13	0.877
Grade 2/3	51	28	23	
Primary tumor				
Tl/T2	6	4	2	0.565
T3/T4	75	41	34	
Lymph node metastasis				
Negative	28	18	10	0.248
Positive	53	27	26	
Plexus invasion				
Negative	51	33	18	0.030
Positive	30	12	18	
Liver metastasis				
Negative	48	31	17	0.048
Positive	33	14	19	