HAb18G/CD147 Promotes pSTAT3-Mediated Pancreatic Cancer Development via CD44s

Ling Li, Wenhua Tang, Xiaoqing Wu, David Karnak, Xiaojie Meng, Rachel Thompson, Xinbao Hao, Yongmin Li, Xiaotan T. Qiao, Jiayuh Lin, James Fuchs, Diane M. Simeone, Zhi-Nan Chen, Theodore S. Lawrence, and Liang Xu

Laboratory of Cancer Biology, Fourth Military Medical University, Xi’an; Engineering Research Centre and Department of Cell Biology, State Key Laboratory of Cancer Biology, University of Michigan, Ann Arbor, Michigan; Departments of Molecular Biosciences and Radiation Oncology, University of Kansas, Lawrence, Kansas; Department of Pediatrics, College of Medicine; and Division of Medical Chemistry and Pharmacognosy, College of Pharmacy, Ohio State University, Columbus, Ohio

Abstract

**Purpose:** Signal transducer and activator of transcription 3 (STAT3) plays a critical role in initiation and progression of pancreatic cancer. However, therapeutically targeting STAT3 has failed clinically. We previously identified HAb18G/CD147 as an effective target for cancer treatment. In this study, we aimed to investigate the potential role of HAb18G/CD147 in STAT3-involved pancreatic tumorigenesis in vitro and in vivo.

**Experimental Design:** The expression of HAb18G/CD147, pSTAT3, and CD44s was determined in tissue microarrays. The tumorogenic function and molecular signaling mechanism of HAb18G/CD147 were assessed by in vitro cellular and clonogenic growth, reporter assay, immunoblot assay, immunofluorescence staining, immunoprecipitation, and in vivo tumor formation using loss or gain-of-function strategies.

**Results:** Highly expressed HAb18G/CD147 promoted cellular and clonogenic growth in vitro and tumorogenicity in vivo. Cyclophilin A (CyPA), a ligand of CD147, stimulated STAT3 phosphorylation and its downstream genes cyclin D1/survivin through HAb18G/CD147-dependent mechanisms. HAb18G/CD147 was associated and colocalized with cancer stem cell marker CD44s in lipid rafts. The inhibitors of STAT3 and survivin, as well as CD44s neutralizing antibodies suppressed the HAb18G/CD147-induced cell growth. High HAb18G/CD147 expression in pancreatic cancer was significantly correlated with the poor tumor differentiation, and the high coexpression of HAb18G/CD147-CD44s-STAT3 associated with poor survival of patients with pancreatic cancer.

**Conclusions:** We identified HAb18G/CD147 as a novel upstream activator of STAT3, which interacts with CD44s and plays a critical role in the development of pancreatic cancer. The data suggest that HAb18G/CD147 could be a promising therapeutic target for highly aggressive pancreatic cancer and a surrogate marker in the STAT3-targeted molecular therapies.

**Clin Cancer Res; 19(24); 6703–15. © 2013 AACR.**

**Introduction**

Pancreatic ductal adenocarcinoma (PDCA) remains a devastating and almost uniformly lethal disease with the 5-year survival offless than 5% (1, 2). Most patients with advanced unresectable PDCA either do not respond, or respond transiently and modestly to systemic chemo/radio-therapy (3, 4). This dire clinical situation cries for our deep understanding of the genetics and biology of PDCA and our efforts on identifying novel therapeutic approaches.

It has been widely accepted that PDCA arises from K-ras mutations, followed by acquisition of additional epigenetic and genetic somatic alterations, including inactivation or point mutation of p16/CDKN2A, TP53, and DPC4/SMAD4 (2, 5). However, the above genetic information has not yet led to the development of effective targeted therapeutic strategies.

Signal transducer and activator of transcription 3 (STAT3) integrates signals from cytokines and growth factors into transcriptional responses in target cells. It is an important regulator of stem cell self-renewal, cancer cell survival, and...
inflammation (2, 6, 7). It was recently reported that STAT3 has critical roles in the development of PDCA, especially the initiation and progression of PDCA by controlling expression of target genes survivin, cyclin D1, and matrix metalloproteinase 7 (MMP7; refs. 5, 8, 9). In the context of acute pancreatitis and K-ras–induced pancreatic intraepithelial neoplasias (PanIN) lesions, STAT3-mediated tumor initiation are related to its ability to promote cell survival and proliferation, and to induce reprogramming of normal pancreatic epithelial cells into progenitor-like phenotype, a process assuming a proneoplastic fate (5, 8).

In addition, constitutive activation of STAT3 is frequently detected in pancreatic cancer and has been associated with a poor prognosis, and served as a therapeutic target (10). Because of lack of enzyme activity, targeting STAT3 is not easy. Inhibition of STAT3 phosphorylation/activation using monoclonal antibody or small molecules that antagonize growth factor and cytokine receptor shows modest efficacy of treatment of pancreatic cancer and develops resistance finally (10). Because multiple factors can activate STAT3, blockade of a single molecule related to STAT3 activation may not sufficiently abrogate STAT3. Therefore, a novel upstream signaling molecule responsible for STAT3 activation would sufficiently abrogate STAT3. Therefore, a novel upstream activator in STAT3-mediated pancreatic cancer initiation and progression induced by STAT3. Our results also suggest HAb18G/CD147 as a novel therapeutic target for highly aggressive pancreatic cancer and as a surrogate marker in clinical trials of molecular therapy targeting STAT3.

HAb18G/CD147, which belongs to the CD147 (also called EMMPRIN or basigin) family, is a transmembrane protein identified by screening a human hepatocellular carcinoma cDNA library using a monoclonal antibody HAb18 in our laboratory (11). HAb18G/CD147 is capable of promoting tumor invasion and metastasis via inducing MMP production (12) and cell motility (13), and affecting tumor cell angiogenesis (14), chemoresistance (15), and glycolysis (16). Because of its high expression in many carcinomas, HAb18G/CD147 acts as a cancer-associated biomarker for detection (17) and an effective target for treatment (18). Licartin, a 131I-labeled antibody HAb18 F(ab')2 against HAb18G/CD147 has been used to treat primary hepatocellular carcinoma and prevent tumor recurrence of post liver transplantation in advanced hepatocellular carcinoma patients in China (19, 20). These results suggest that HAb18G/CD147 plays an important role in cancer metastasis and progression. Recently, we showed that HAb18G/CD147 promotes epithelial–mesenchymal transition (21), anoikis resistance and anchorage-independent growth in vitro (22, 23), and tumorigenic potential of liver cancer in vivo (21), indicating a possible role of HAb18G/CD147 in tumor initiation.

Nevertheless, the function of HAb18G/CD147 has not yet been fully understood in pancreatic cancer. Highly expressed CD147 has been reported in human PDCA tissues and cell lines (24–26), these studies either, however, have a relatively small sample size of patients (e.g. 39–55 cases), or are lack of a clinicopathologic data. We also showed that HAb18G/CD147 was highly expressed in breast carcinomas and sarcomas (17), but its expression in pancreatic cancers was not included in that analysis. Although targeting CD147 by siRNA (27, 28) or monoclonal antibody (29, 30) can reduce cell growth and invasion in vitro and inhibits tumor growth and metastasis in a xenograft model, the role of HAb18G/CD147 in the early promotion of PDCA, especially in STAT3-involved PDCA initiation, remains largely unknown.

To explore the potential molecular targets of HAb18G/CD147, we searched the oncomine database for genes coexpressed with CD147 in pancreas (31). We observed that CD147 highly expressed in primary pancreatic carcinoma patients (32–34), and STAT3 is among the top listed genes that highly correlated with CD147 (Supplementary Fig, S1). It has been reported that cyclophilin A (CyPA), as a CD147 ligand, promotes pancreatic cancer cell growth (35, 36), and contributes to STAT3-mediated cell survival (37). These evidences promote us to investigate the role of HAb18G/CD147 in STAT3-mediated cell growth signaling using CyPA as a stimulus of HAb18G/CD147 activation. Typically, CD147 transmits extracellular signal by forming complexes with another membrane protein upon CyPA stimulation (38); whereas activated STAT3 promotes the transcription of target genes by phosphorylating and translocation from cytoplasm into nucleus. CD44 (CD44 v3-10) has been reported to activate STAT3 signaling (39), and colocalizes with CD147 in cancer cells (40, 41), suggesting a potential role of CD44 in the CyPA-HAb18G/CD147-STAT3–mediated cell growth signaling in pancreatic tumorigenesis.

Here, we perform a systematic study to investigate the relationship among HAb18G/CD147, CD44, and STAT3 in the development of pancreatic cancer. We identified HAb18G/CD147 interacting with CD44s as a novel upstream
activator of STAT3 signaling, which plays a critical role in the development of pancreatic cancer. Our data suggest that HAb18G/CD147 could be a promising therapeutic target for highly aggressive pancreatic cancer and a surrogate marker for the STAT3-targeted molecular therapies.

Materials and Methods

Antibodies and reagents

Antibodies to phospho-STAT3 (Tyr705) and total STAT3 were purchased from Cell Signaling Technology. CD44s clone MEM-263 from Abnova, survivin from Novus, cyclin D1 and a-tubulin from Santa Cruz, goat anti-rabbit Texas Red and goat anti-mouse fluorescent isothiocyanate (FITC) from Jackson ImmunoResearch, green fluorescent protein (GFP), β-actin, goat anti-rabbit horseradish peroxidase (HRP), goat anti-mouse HRP, mouse immunoglobulin G (IgG), cholera toxin subunit B conjugates CTB-594, and genixin (G418) from Invitrogen. Antimouse CD44s antibody H4C4 was purchased from the University of Iowa Developmental Studies Hybridoma Bank (Iowa, IA), and anti-mouse HAb18G/CD147 antibody HAb18 was prepared as reported (20). Recombinant human CyPA, cycloheximide, and doxycycline were purchased from Sigma. Puromycin, WP1066, and YM-155 were purchased from InvivoGen, Calbiochem, and Active Biochem, respectively. FLLL32 were prepared as previously described (42).

Cell lines and constructs

Human pancreatic cancer cell lines and normal human embryonic lung fibroblast cell line WI-38 and embryonic kidney cell line HEK293 were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle Medium (DMEM; HyClone), supplemented with 10% FBS (HyClone). All cell lines from ATCC have been tested and authenticated with genotyping by ATCC with 10% FBS (HyClone). All cell lines from ATCC have been tested and authenticated with genotyping by ATCC. Established cell lines were maintained in 10% FBS (HyClone) supplemented with 10% FBS (HyClone).

Patient samples

Fresh and paraffin-blocked pancreatic tumor and adjacent nontumor tissues from patients with pancreatic cancer were obtained from University of Michigan Comprehensive Cancer Center (UMCCC; Ann Arbor, MI) Histology Core according to an Institutional Review Board-approved human protocol (H7094) and from National Engineering Center for Biochip (NECB) according to an approved human protocol. Tissue microarrays (TMA) from 193 patients were obtained from UMCCC Histology Core for analyzing HAb18G/CD147 expression in the progress of pancreatic tumorigenesis. They were constructed by two different recipient paraffin blocks: “control tissue array” and “tumor tissue array.” The “control tissue array” included 53 cases of normal pancreatic tissues and seven cases of pancreatitis; the “tumor tissue array” included two cases of PanINs, six cases of cystadenoma, 17 cases of IPMN, and 108 cases of PDCA.

Establishment of stable cell lines

The CD147 lentiviral shRNA or control shRNA was introduced into cells using FuGene6 (Roche), whereas CD147/EGFP cDNA and pEGFP control vector was transfected with Hek293 cells using Lipofectamine 2000 (Invitrogen). Knockdown or knock-in cells were selected by adding 4 to 6 μg/mL of puromycin or 1 mg/mL of G418 to the culture medium, and expression of inducible shRNA was induced in the presence of 1 μg/mL doxycycline. Silencing or increase of HAb18G/CD147 expression was verified by quantitative real-time PCR (qRT-PCR) and immunoblot analysis.

Cell growth assay

Cells were plated in 24-well plates and were counted every 24 hours for 3 to 5 days using a hemocytometer, or measuring WST-8 dye absorbance at 570 nm. A cell growth curve was drawn according to the cell numbers with the specified incubation time. For growth inhibition of STAT3/survivin inhibitor or CD44s antibody, cells were serum-starved for 24 hours before adding 5 μmol/L FLLL32 or 0.05 to 0.1 μmol/L YM155 or 10 μg/mL H4C4 or nIG for one hour followed by 72 to 96 hour 100 nmol/L CyPA treatment. The results are shown as relative cell growth inhibition, which normalized to their individual control.

Colony formation assay

Cells were cultured in DMEM containing 10% FBS with 250 cells in each 6-well plate. Plates were maintained at 37°C in a humidified incubator for 7 to 10 days. The colonies were stained by 0.1% crystal violet and calculated under microscopy.

In vitro Matril gel invasion assay

Cells were seeded into upper chambers of 8 μm pore Transwells coated with Matrigel and then allowed to invade the Matrigel for 36 hours. Invaded cells were stained with Diff-Quik stain (Allegiance), and the invasive potential of the cells was determined by counting the number of cells that had invaded to the lower surface of the filter in 10 different areas under an inverted light microscope (Olympus BX41).
STAT3 reporter assay

Cells were seeded in 48-well plates and then transfected with 0.5 μg STAT3-luc reporter constructs or 0.5 μg of a GL3 vector control using Lipofectamine 2000. Sixteen hours after transfection, cells were treated with CyPA for 18 hours. Luciferase activity was then measured in cell lysates by a POLARstar OPTIMA Microplate Reader using the Bright-Glo Luciferase Assay System (Promega) according to the manufacturer’s protocol. For comparison, luciferase activity was normalized to β-galactosidase expression using the Beta-Glo Assay System (Promega).

Animal studies

Animal studies of tumor formation and tumor growth were performed as described previously (44). Briefly, 1 × 10⁶ cells in 0.2 mL DMEM inoculated into five- to six-week-old female athymic NCr-nu/nu nude mice subcutaneously on both flanks. Five mice with two injection sites (up and down) on each mouse were used for each type of construct (n = 10 per group). The tumor sizes and animal body weights were measured twice weekly and plotted. Tumor volume was calculated using the formula: (length × width²)/2. All animal experiments were performed according to the protocol approved by the University of Michigan Animal Care and Use Committee and in accordance with NIH guidelines.

Quantitative real-time PCR

Total RNA extraction and cDNA synthesis were carried out as previously described (45). qRT-PCR was carried out using an ABI 7700 real-time PCR system (Applied Biosystems) with gene-specific primers for HAb18G/CD147, CD44, STAT3, CyclinD1, survivin, MMP1, MMP2, MMP9, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), or 18sRNA (Supplementary Table S1). Individual genes of interest (GOI) were normalized to housekeeping genes (HKG): β-actin, 18sRNA, or GAPDH. Relative mRNA levels are presented as unit values of 2[ΔΔCt] = 2[−(Ct (HKG) − Ct (GOI)).

Immunohistochemistry

TMA staining was performed by standard immunohistochemistry procedures. To confirm the specificity of the primary antibodies, tissue sections were incubated in the absence of the primary antibodies and with control mouse IgG. The number of positively stained cells and the intensity of positive staining on epithelium and stromal cells were independently scored by two pathologists in a blinded manner. The percentage of positive stained cells was scored as: 0, 1% to 25%, 26% to 75%, and >75%. The intensity of positive immunostaining on the cells was graded by an experienced pancreatic pathologist in a blinded manner and classified into four categories: 0, 1, 2, and 3 representing no visible staining; light brown, mid-brown, and dark brown staining, respectively, with the same intensity covering more than 75% of the staining area. For statistical analysis, the stained tumor tissues were divided into two groups: the low-expression group and the high-expression group. For membranous HAb18G/CD147, only 3+ specimens were defined as high expression; for membranous CD44s and phosphorylated STAT3-positive nuclei, the samples with 1+ staining in >50% of cells or 2+ staining in >20% or all 3+ staining of cells were defined as high expression.

Immunofluorescence staining

Cells grown on chambered cover slips were either left untreated or treated with 100 nmol/L CyPA for 30 minutes. After fixation, cells were blocked and probed with anti-HAb18G/CD147 antibody HAb18 and anti-CD44s antibody H4C4, and were then detected with fluorochrome-conjugated FITC, Texas-Red, or CTB594. Cover slips were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen) for visualization of nuclei. Cell images were observed under a fluorescent microscope (Olympus).

Immunoblot and immunoprecipitation assays

Immunoblot assays were performed using standard methods (45). Cells were treated with CyPA and/or H4C4 and/or mouse IgG as required in each assay. Membranes were probed with total and phosphorylated antibodies as detailed above in Materials and Methods. Immunoprecipitation assays were performed using the Pierce Co-Immunoprecipitation (Co-IP) Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions with minor modifications (45). We used 25 μg of each monoclonal antibody (complete IgG molecule) specific to either HAb18G/CD147 (HAb18) or CD44s (H4C4) for immobilization. A mouse IgG1 isotype antibody (Sigma-Aldrich) served as a negative control. Adsorbed immune complexes were washed, eluted, and then subjected to immunoblot analysis using HAb18 and H4C4.

Statistical analysis

All data shown are mean ± SEM of triplicate values from three separate experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 as compared with the control group. Independent Student t tests or one-way ANOVA were used to compare the continuous variables between the two groups or more than two groups, and categorical variables were compared using the χ² test. Spearman rank correlation was conducted to analyze expression correlations among HAb18G/CD147 and CD44s. The Kaplan–Meier method and the log-rank test were used to compare overall survival, defined as the time of patients from surgery until death (patients alive were censored at the time of their last follow-up). Statistical analyses were carried out with statistical analysis software program SPSS 13.0 software (IBM) and Prism 5.0 software (GraphPad).

Results

HAb18G/CD147 is highly expressed in pancreatic cancer cell lines

To determine how HAb18G/CD147 contributes to pancreatic tumor development, we first determined the HAb18G/CD147 expression in a panel of pancreatic cancer...
cell lines, a human pancreatic primary tumor earlypassage xenograft (12) and immortalized human pancreatic epithelial cell HPDE, as well as normal human embryonic lung fibroblast cell line WI-38 and embryonic kidney cell line HEK293. HAb18G/CD147 mRNA and protein expression were significantly higher in pancreatic cancer cell lines and HPDE than that in WI-38 and HEK293 cells (Supplementary Fig. S2). On the basis of the cells’ endogenous HAb18G/CD147 expression and capacity for tumor formation (44), we selected PANC-1 and MIA PaCa-2 cells for further knockdown studies and HEK293 with low endogenous HAb18G/CD147 expression for knock-in studies.

**HAb18G/CD147 promotes cellular and clonogenic growth in vitro and tumor formation in vivo**

To determine the potential biologic function of HAb18G/CD147 in pancreatic cancer, we adopted a loss-of-function strategy using three different lentiviral vectors: untagged plKO CD147-shRNA targeting the coding region, a GFP-tagged pGIPZ CD147-shRNA targeting the coding regions and the 3’ untranslated region (UTR), and an RFP-tagged inducible pTRIPZ CD147-shRNA targeting the coding regions and the 3’UTR. We also adopted a gain-of-function strategy to knock-in HAb18G/CD147 cDNA into HEK293 cells.

When HAb18G/CD147 expression was effectively silenced (typically 60%–80% reduction of the total; Supplementary Fig. S3D and Supplementary Table S5), which functionally validated by decrease of cell invasion (Supplementary Fig. S3), cellular and clonogenic growth was significantly decreased (Fig. 1A and B). In contrast, HAb18G/CD147 knock-in HEK293 cells with significantly increased HAb18G/CD147 expression (6.32-fold increases in mRNA levels and 3-fold increases in protein levels, comparable with the average expression in pancreatic cancer cells), have a significantly increased cell invasion (Supplementary Fig. S3) and cellular and clonogenic growth (Fig. 1A and B). These results were confirmed with pGIPZ-CD147 shRNA and pTRIPZ-inducible CD147 shRNA (Supplementary Fig. S4A and S4B).

Next, we examined the effects of HAb18G/CD147 on tumor formation in a xenograft mouse model. After knockdown of HAb18G/CD147, both tumor formation and tumor growth were significantly decreased, with a 71.8% to 87.9% reduction in tumor sizes (n = 10; Fig. 1C and D and Supplementary Fig. S4C). These results were confirmed in MIA PaCa-2 xenograft mouse model using pGIPZ CD147 shRNA (Supplementary Fig. S4D). Conversely, HAb18G/CD147 knock-in promoted tumorogenicity indicated by a significantly increased tumor incidence (10/10 vs. 2/10, P < 0.001, n = 10; Fig. 1C) and tumor volume (793.9 ± 549.3 vs. 110.1 ± 58.8; Fig. 1D and Supplementary Fig. S4C) in HEK293 xenograft model. These data suggest that highly expressed HAb18G/CD147 is associated with high tumorogenicity of pancreatic cancer cells, and thus support the potential role of HAb18G/CD147 in promoting pancreatic tumor development.

**HAb18G/CD147 is involved in the STAT3 signaling pathway**

We first determined the effects of CyPA, a natural ligand of HAb18G/CD147, on the pancreatic cancer cell growth as well as its effect on the modulation of HAb18G/CD147, STAT3, and the downstream genes cyclin D1 and survivin. We observed that CyPA induced HAb18G/CD147 protein expression in a dose- and time-dependent manner (Supplementary Fig. S5). The protein levels of pSTAT3 were significantly increased in cells treated with CyPA, whereas the levels of HAb18G/CD147 were not changed. To further validate these results, we used the CyPA inhibitor WP1066 and YM155. We observed that FLLL32/ WP1066 and YM155 significantly inhibited CyPA-induced cell growth (Fig. 2D and Supplementary Fig. S5F), and CyPA rescued the inhibition of cell growth by WP1066 and YM155.
survivin shRNA (Fig. 3A). Furthermore, the cell growth inhibition ratio of WP1066 increased up to 2-folds by knockdown of HAb18G/CD147 (Fig. 3B). These data suggest that the STAT3 and its downstream gene survivin play an important role in CyPA-HAb18G/CD147-mediated cell growth.
CD44s is involved in the activation of STAT3 by HAb18G/CD147 in lipid rafts

As shown in Fig. 3C and D, CD44s siRNA and CD44s antibody H4C4 abolished the CyPA-induced STAT3 phosphorylation and downstream signaling, indicating that CD44s is required for CyPA-HAb18G/CD147-mediated STAT3 transcription activation. CD44s protein, but not mRNA, levels were significantly affected upon CyPA stimulation (Supplementary Fig. S5D) or HAb18G/CD147 knock-in (Supplementary Fig. S6A and S6B), indicating that transcriptional regulation may not be the clue that connects HAb18G/CD147 with CD44s. Our immunoprecipitation assay showed that HAb18G/CD147 and CD44s were colocalized and significantly associated in the untreated cells (Fig. 4A and B). This association was much stronger in the HAb18G/CD147 knock-in HEK293/CD147 cells than that in HAb18G/CD147 knockdown MIA PaCa-2/A6 cells, and both were enhanced further upon CyPA stimulation. By immunofluorescence staining, we observed that both HAb18G/CD147 and CD44s were evenly located on the cell membrane before treatment, but were translocated to the GM1-enriched lipid rafts (identified by CTB-594 staining) after CyPA stimulation (Fig. 4C and Supplementary Fig. S6C). However, this translocation of CD44s in the lipid rafts was blocked after knocking down HAb18G/CD147. These results suggest that HAb18G/CD147 may colocalize and form a signaling complex with CD44s in the lipid rafts, and CyPA stimulation, though not a prerequisite, may promote the association of the two proteins.

Furthermore, we observed that the CD44s blocking antibody H4C4 attenuated the CyPA-induced cell growth.
to the level of the IgG control and decreased the level of EGF receptor (EGFR) but not HAb18G/CD147 (Figs. 3D and 4D). These data indicate that HAb18G/CD147 promotes pSTAT3-mediated cell growth through CD44s.

**HAb18G/CD147, CD44s, and STAT3 are highly expressed in human pancreatic cancer and correlate with pancreatic cancer patients’ survival**

We found that HAb18G/CD147 mRNA levels in human pancreatic tumor tissues are significantly higher (average 4.27-fold) than that in the adjacent normal tissues (Fig. 5A). In a TMA with 193 pancreatic tissues, HAb18G/CD147 is highly expressed in only 22.6% and 14.3% of normal pancreatic tissue and chronic pancreatitis, respectively, but highly expressed in 56% and 55.6% of pancreatic preneoplasia and PDCA, respectively (Fig. 5B; Table 1).

We next investigated the correlation between HAb18G/CD147 expression and clinicopathological parameters in 157 cases of pancreatic cancer (Supplementary Table S2). No significant correlation exists in age, gender, tumor type, size and location, American Joint Committee on Cancer (AJCC) stage, and patient survival. But high HAb18G/CD147 expression seems to have more lymph node invasion (52.94% vs. 42.59%) and advanced tumor–node–metastasis (TNM) stage (51.35% vs. 37.5%). Moreover, high HAb18G/CD147 expression was found to be significantly associated with a poor tumor differentiation (51.43% vs. 15.65%, P < 0.0001). Taken together, being upregulated in both pancreatic preneoplasma and PDCA, and being correlated to poor tumor differentiation and advanced TNM stage, HAb18G/CD147 may have a role in pancreatic tumor development.

To investigate whether HAb18G/CD147-mediated tumor growth and CD44s-pSTAT3 expression are associated in patient samples, we analyzed the coexpression of CD44s-pSTAT3 with high HAb18G/CD147 in 157 pancreatic cancer tissues. As indicated, high HAb18G/CD147 expression was significantly correlated with high CD44s expression (Spearman r = 0.4961, P < 0.001; Supplementary Fig. S6D and S6E), but not with high pSTAT3 nuclear positivity (Table 2). With regard to the clinical pathologic factors, patients with high expression of HAb18G/CD147 and CD44s had poor tumor differentiation (P < 0.0001; Supplementary Table S3) and a lower median survival, as compared with patients with low expression of HAb18G/CD147 and CD44s, although the latter lacks statistical difference (P = 0.311; Fig. 5C, top).

Moreover, patients with high expression of all three genes had a higher incidence of poor tumor differentiation (60.87% vs. 7.69%, P = 0.002) and mortality (60% vs. 28.57%, P = 0.218), as well as a 75% survival of 4 months, as compared with that of 10 months for patients with low expression of the three genes (HR of death = 3.024; 95% confidence interval, 0.642–15.983, P = 0.12; Fig. 5C and Supplementary Table S4). These results suggest that HAb18G/CD147-CD44s-pSTAT3 association might be used as a prognosis marker for pancreatic cancer.

**Figure 3.** HAb18G/CD147 activates pSTAT3 signaling via CD44s. A, cell growth assay in survivin shRNA or empty vector (EV)-transfected MIA PaCa-2 cells with or without 100 nmol/L CyPA treatment. Efficiency of the knockdown was confirmed by immunoblot analysis (right). B, cell growth assay in HAb18G/CD147 knockdown cells after the exposure to STAT3 inhibitor WP1066 (5 μmol/L). C, HAb18G/CD147, CD44s, pSTAT3, and STAT3 protein expression (left) and STAT3 transcription activity (right) in CD44s siRNA knockdown PANC-1 cells with or without CyPA treatment. Negative siRNA was included as control. D, HAb18G/CD147, EGFR, pSTAT3, and STAT3 protein expression in PANC-1 cells with or without H4C4 or and CyPA treatment. DMSO, dimethyl sulfoxide.

---

Li et al.
Discussion

In this study, we found that HAb18G/CD147 was highly expressed in pancreatic cancer cell lines, chronic pancreatitis, preneoplasma, and PDCA. Knockdown of HAb18G/CD147 significantly inhibited the tumor cell invasion, cellular and clonogenic growth in vitro and reduced tumor formation and tumor growth in xenograft mouse model. Moreover, we showed that HAb18G/CD147 and CD44s are involved in the activation of STAT3 signaling pathway. Finally, we demonstrated that patients with high expression of HAb18G/CD147, CD44s, and pSTAT3 had higher mortality and poorer tumor differentiation, and poorer survival, as compared with the patients with low expression of these three genes. These data support that HAb18G/CD147 plays a novel oncogenic role in pancreatic tumor development, besides its well-known role in regulating MMP-related metastasis.

We previously showed that HAb18G/CD147 plays an important role in liver tumor metastasis under different experimental and clinical conditions (13, 20). In this study, we showed that HAb18G/CD147 plays a role in early promotion of pancreatic cancer. HAb18G/CD147 antibody has been used in clinical practice for liver cancer therapy, and other CD147 antibodies are in preclinical development for pancreatic cancer (29, 30, 46), suggesting the potential clinical application of HAb18G/CD147 antibody for patient with PDCA.

Both HAb18G/CD147 activator CyPA and STAT3 were related to inflammation and cancer, suggesting a potential link between inflammation and pancreatic tumor initiation via CyPA-HAb18G/CD147-STAT3 signaling. STAT3, as a mediator of inflammation-associated processes, plays a critical role in PDCA initiation and progression and has been under active investigation as a potential target for PDCA therapy (5, 8, 9). However, STAT3-targeted anticancer drug, either anti-EGFR antibodies or small-molecule tyrosine kinase inhibitors, only showed a limited efficacy (10). As STAT3 occupies a point of convergence for many signaling pathways, blockade of existed upstream signaling to STAT3 activation may not sufficiently abrogate STAT3.

Figure 4. HAb18G/CD147 and CD44s are coexpressed and associated in pancreatic cancer cells. A, immunoprecipitation analysis of the HAb18G/CD147 and CD44s in HAb18G/CD147 knock-in HEK293 cells with or without CyPA treatment. Normal mouse nlgG was used as a control antibody. B, immunoprecipitation analysis of the HAb18G/CD147 and CD44s in HAb18G/CD147 knockdown MIA PaCa-2 cells with or without 100 nmol/L CyPA treatment. C, communolabeling of HAb18G/CD147 (green) or CD44s (green) with lipid raft marker CTB (red) in HAb18G/CD147 knockdown PNAC-1 cells. Magnification: ×400. Arrows indicate colocalization of HAb18G/CD147 or CD44s with CTB in lipid rafts. D, cell growth assay in PNAC-1 and MIA PaCa-2 cells with CD44s blocking antibody H4C4 (10 μg/mL) and/or CypA (100 nmol/L). *, P < 0.05; **, P < 0.01.
this study, we showed that CyPA induced STAT3 phosphorylation through HAb18G/CD147-dependent mechanism, suggesting that HAb18G/CD147 is a real upstream regulator of STAT3 activation. Pancreatic cancer cells with high HAb18G/CD147 levels and/or activity had higher active STAT3 signaling, on which the cells depend for survival and

![Image](257x363 to 430x586)

![Image](217x598 to 430x722)

**Figure 5.** HAb18G/CD147-CD44s-pSTAT3 coexpression in pancreatic cancer is clinically correlated. A, HAb18G/CD147 mRNA levels in seven pairs of pancreatic adenocarcinoma and the adjacent normal tissues. Levels were normalized against 18sRNA levels. B, HAb18G/CD147 protein levels in normal pancreas, chronic pancreatitis, PanIN, cystadenoma (CYSTADEMA), IPMN, and PDCA. Representative morphology of HAb18G/CD147 and CD44s immunostaining in pancreatic cancer tissues. C, Kaplan–Meier analysis of overall survival for 34 patients based on HAb18G/CD147 and CD44s scores by immunohistochemistry staining (top); and Kaplan–Meier analysis of overall survival for 17 patients based on immunohistochemistry staining scores of HAb18G/CD147, CD44s, and pSTAT3 (bottom). D, a proposed working model for HAb18G/CD147 interaction with CD44s signaling during pSTAT3-activated pancreatic tumor development.

<table>
<thead>
<tr>
<th>Table 1. HAb18G/CD147 expression in pancreatic tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HAb18G/CD147 expression levels</strong></td>
</tr>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Pancreatitis</td>
</tr>
<tr>
<td>Preneoplasms</td>
</tr>
<tr>
<td>PanIN</td>
</tr>
<tr>
<td>CYSTADEMA</td>
</tr>
<tr>
<td>IPMN</td>
</tr>
<tr>
<td>PDCA</td>
</tr>
</tbody>
</table>

Boldface: compared with normal, the significance of PanIN was 0.963 and that of CYSTADEMA was 0.335.

\(^a\)HAb18G/CD147 expression levels are scored as: 0 (no staining), 1 (light staining), 2 (intermediate staining), and 3 (intense staining).

\(^b\)Estimated by \(\chi^2\) test as compared with normal tissues by dividing into low- and high-expression group. Low-expression group are cases with staining intensity scores of 0 to 2; high-expression group are cases with staining intensity scores of 3.
HAb18G/CD147, CD44s, STAT3, and Pancreatic Cancer

Table 2. Spearman correlation coefficients for the correlation among expression of HAb18G/CD147, STAT3, and CD44s

<table>
<thead>
<tr>
<th>Gene expression</th>
<th>HAb18G/CD147</th>
<th>Nuclear pSTAT3</th>
<th>Cytoplasm pSTAT3</th>
<th>CD44s</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAb18G/CD147</td>
<td>-0.035</td>
<td>-0.098</td>
<td>-0.98</td>
<td>0.496a</td>
</tr>
<tr>
<td>Nuclear pSTAT3</td>
<td>-0.035</td>
<td>-0.098</td>
<td>0.415a</td>
<td>0.165b</td>
</tr>
<tr>
<td>Cytoplasm pSTAT3</td>
<td>-0.098</td>
<td>0.415a</td>
<td>-0.035</td>
<td>0.019</td>
</tr>
<tr>
<td>CD44s</td>
<td>0.496a</td>
<td>0.165b</td>
<td>-0.035</td>
<td>---</td>
</tr>
</tbody>
</table>

*P < 0.001.
bP < 0.05.

Proliferation, much like the so-called "oncogene addiction." Actually, CyPA has previously been reported to regulate STAT3 tyrosine phosphorylation and nuclear translocation (37). We also showed that HAb18G/CD147 promotes tumor growth by regulating the expression of survivin and cyclin D1. This result is consistent with previous reports that STAT3 promotes tumor early promotion by controlling the transcription of antiapoptotic gene survivin and proliferative gene cyclin D1 (8). These data suggest that HAb18G/CD147 may exert its tumor-promoting function by activating the STAT3 phosphorylation, indicating a novel role for HAb18G/CD147 in the STAT3-mediated tumor early promotion in PDCA. It has been reported that the expression of EMMPRIN (CD147) may be modulated by STAT3 ODN (47), indicating a positive feedback loop between HAb18G/CD147 and STAT3. In other word, CyPA-HAb18G/CD147 activates STAT3 phosphorylation, and then pSTAT3 promotes HAb18G/CD147 transcription. Thus, the CyPA-HAb18G/CD147-STAT3-mediated growth-promoting signaling could be amplified, in which case STAT3 inhibitors may have better effects for cancer cells with high expression of HAb18G/CD147. In a rationally designed clinical trial of STAT3-targeted cancer therapy, the HAb18G/CD147 level in patients with cancer should also be considered. This will help us to select a patient subgroup that is more likely to respond to the molecularly targeted therapy. HAb18G/CD147 may thus become an important surrogate marker in clinical trials of molecular therapies targeting STAT3.

HAb18G/CD147 expression is highly correlated with CD44s expression in our analysis. Patients with HAb18G/CD147-high/CD44s-high/pSTAT3-high coexpression show high mortality, implying a potential link of HAb18G/CD147 to tumor initiation by associating with the cancer stem cell (CSC) marker CD44s (48, 49), and by regulating the CSC signaling molecule STAT3 (50). CD147 has been shown to possess the potential in forming complexes with membrane proteins like MCT, CD98, caveolin, integrins, basigin-2 (CD147), and basigin-3, thereby shielding the charge in an energetically stable state (18, 38, 43). We show that HAb18G/CD147 forms a signaling complex with CD44s, and CD44s may mediate part of the oncogenic activities of HAb18G/CD147 by activating the STAT3 signaling. The colocalization and coexpression of CD147 and CD44 have been reported in breast and prostate cancer (40, 41, 51), our data provide new evidence of functional link and clinical relevance between them in pancreatic cancer. The HAb18G/CD147 interaction with CD44s, but not CD44v, plays a role in pancreatic tumor development. Furthermore, HAb18G/CD147 and CD44s distribute together in the specific signaling platform, lipid rafts, which facilitate efficient downstream signal transduction. The distribution and activity of CD44s depend on CyPA-HAb18G/CD147, which is consistent with a previous report indicating that HAb18G/CD147 membrane localization is not affected by hyaluronan oligomers that antagonize the hyaluronan–CD44 interaction (51). Therefore, we propose that upon CyPA stimulation, HAb18G/CD147 first clusters and recruits CD44s in the lipid rafts to form a signaling complex, and then promotes STAT3 phosphorylation and cyclin D1/survivin transcription, finally leading to cell survival and cell-cycle progression (Fig. 5D).

In conclusion, our study suggests that HAb18G/CD147 is a novel upstream activator in STAT3-mediated pancreatic tumor development by forming signaling complex with the CSC marker CD44s, and that coexpression of HAb18G/CD147-CD44s-STAT3 indicates poor prognosis in patients with pancreatic cancer. This information will be valuable for a better understanding of the relationship between inflammation and pancreatic cancer initiation and progression induced by STAT3; in addition, our results establish HAb18G/CD147 as a novel therapeutic target for highly aggressive pancreatic cancer and as a surrogate marker in clinical trials of molecular therapies targeting STAT3.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Li, Z.-N. Chen, T.S. Lawrence, L. Xu
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Li, W. Tang, D. Karnak, X. Wu, X. Meng, R. Thompson, X. Hao, X.T. Qiao, J. Fuchs, L. Xu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Li, W. Tang, X. Meng, X.T. Qiao, D.M. Simeone, T.S. Lawrence, L. Xu

www.aacjrournals.org


Published OnlineFirst October 16, 2013; DOI: 10.1158/1078-0432.CCR-13-0621

Downloaded from clincancerres.aacrjournals.org on January 6, 2018. © 2013 American Association for Cancer Research.
Writing, review, and/or revision of the manuscript: L. Li, R. Thompson, X.T. Qiao, D.M. Simone, Z.-N. Chen, T.S. Lawrence, L. Xu
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Li, X. Meng, X. Hao, Y. Li, X.T. Qiao, J. Lin, L. Xu
Study supervision: L. Li, Z.-N. Chen, L. Xu

Acknowledgments

The authors thank Dr. Susan Harris for help with the editing of the manuscript. Drs. Thomas Godano and DaYfdyl Thomas and Michelle Vinco in the UMCCC Tissue Core for providing tissues and TMAs and help on immunohistochemistry staining. The authors also thank the UMLCC Unit of Laboratory Animal Medicine (ULAM) for help with the animal experiments.

References


Grant Support

This work was supported in part by U.S. NIH grants R01 CA121830 S1 (to L. Xu), R01 CA134655 (to L. Xu), and 5P30 CA46592 (University of Michigan Cancer Center Support Grant). Kansas Bioscience Authority Rising Star Award (to L. Xu), China National Science and Technology Major Project 2012ZX09301301 (to Z.-N. Chen), and National Basic Research Program 2009CB521704 (to Z.-N. Chen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 11, 2013; revised September 11, 2013; accepted October 3, 2013; published OnlineFirst October 16, 2013.

Published OnlineFirst October 16, 2013; DOI: 10.1158/1078-0432.CCR-13-0621

Downloaded from clinicanerres.aacrjournals.org on January 6, 2018. © 2013 American Association for Cancer Research.


HAb18G/CD147 Promotes pSTAT3-Mediated Pancreatic Cancer Development via CD44s

Ling Li, Wenhua Tang, Xiaoqing Wu, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-13-0621

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2013/10/16/1078-0432.CCR-13-0621.DC1

Cited articles
This article cites 50 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/19/24/6703.full#ref-list-1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://clincancerres.aacrjournals.org/content/19/24/6703. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.