The NOTCH Ligand JAGGED2 Promotes Pancreatic Cancer Metastasis Independent of NOTCH Signaling Activation

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive and lethal disease with a high rate of metastasis. Numerous signaling events have been implicated in the molecular pathogenesis of this neoplasm. Ablantly high expression of JAGGED2, one of the NOTCH ligands, often occurs in human PDAC. However, what role JAGGED2 plays in the disease development and whether JAGGED2 executes its function through activating NOTCH signaling remain to be determined. We report here that JAGGED2 plays a critical role in promoting PDAC metastasis in vitro and in vivo. Depletion of JAGGED2, but not its homolog JAGGED1, profoundly inhibited both migration and invasion without influencing cell proliferation. Furthermore, reconstitution of JAGGED2 expression rescued the migratory defect. Surprisingly, neither pharmacologic nor genetic inhibition of NOTCH downstream signaling resulted in obvious defect in metastasis. Instead, depletion of NOTCH1 expression per se gave rise to migratory defects similar to JAGGED2 ablation. Moreover, blockade of ligand–receptor interaction by a specific JAGGED2-Fc fusion protein dramatically inhibited PDAC cell migration, suggesting that tumor metastasis relies on physical interactions of JAGGED2–NOTCH1 but not Notch downstream signaling activation. Taken together, our data reveal a novel role of NOTCH in regulation of PDAC metastasis, and identify JAGGED2 as a critical mediator in this event. These findings also provide rationale for developing small molecules or biologic agents targeting JAGGED2 for therapeutic intervention.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal types of malignancies with the annual diagnosis rate almost the same as the mortality rate. The overall 5-year survival rate is about 4% and the median survival after diagnosis is approximately 6 months (1). Early-stage pancreatic cancer is usually clinically undetectable, so most patients who present with symptoms attributable to the neoplasm have advanced disease, often associated with metastasis with no opportunities of pancreatic resection (2). Moreover, gemcitabine-based combination treatments, the current mainstay of chemotherapy, yield limited responses in metastatic PDAC patients. It is, therefore, important to develop a better understanding of the molecular mechanism that leads to the development and metastasis of the disease to identify novel potential therapeutic targets to interfere rapid spread of the neoplasm.

JAGGED2, highly expressed in PDAC cells (3, 4), has been linked to metastasis of epithelial-derived tumors such as lung cancer (5), breast cancer (6), colon cancer (7), and urothelial carcinoma of bladder (8). Although the precise mechanism is not fully determined, it is generally believed through NOTCH signaling activation. NOTCH receptor signaling is a conserved pathway controlling cell fate during development of many tissues through interaction with ligands of the Delta/Serrate family, including the Delta-like (DLL1, DLL3 and DLL4) and JAGGED (JAG1 and JAG2) ligands. Interaction of these ligands with one of the NOTCH receptors (NOTCH1-4) results in proteolytic cleavage (JAG1 and JAG2) ligands. Interaction of these ligands with one of the NOTCH receptors (NOTCH1-4) results in proteolytic cleavage that releases intracellular domain (ICN; ref. 9). ICN then translocates into nucleus and activates specific target genes through forming transcriptional complexes with CBF1–RBP–kappa-suppressor of hairless–LAG-1 (CSL) and MAML (10, 11). The signaling cascade plays critical roles during pancreatic development and becomes dispensable in adult pancreas. However, it appears to be reactivated in human pancreatic cancer, given that the expressions of receptors, ligands, and downstream targets are induced compared with normal epithelial tissues (12).

The role of NOTCH signaling in PDAC development has been controversial (13). Although initial studies reveal oncogenic roles of NOTCH signaling, blockade of the cascade using γ-secretase inhibitors results in only limited effect in proliferation and invasion (14, 15). Also, recent studies using mouse models have revealed both oncogenic and tumor-suppressive roles for
NOTCH signaling in PDAC development (16, 17). These conflicting results necessitate further investigation into how NOTCH participates in the development of PDAC.

Using human PDAC cell lines, primary specimen, and xenograft mouse models, we show that tumor cell migration, invasion, and metastasis are dependent on JAGGED2. Unexpectedly, the JAGGED2-mediated effect is independent of NOTCH activation but relies on the physical interaction of JAGGED2 and NOTCH1. Specific blockade of JAGGED2 impedes metastasis in vitro and in vivo, so we highlight JAGGED2 as a potential therapeutic target in interfering tumor metastasis.

Materials and Methods

Cell lines and reagents

Immortalized human normal pancreas cell HPDE6C7 was obtained from Beijing North Carolina Chuanglian Biotechnology research institute (Beijing, China) and pancreatic cancer cell lines BxPC3, Mia PaCa-2, PANC-1, and SW1990 from ATCC. Obtained in 2013 and used for less than 6 months after resuscitation, cells have been authenticated by the providers via examining their karyotypes, images, and specific gene expression, and cultured in RPMI-1640 or DMEM medium (Hyclone; supplemented with 10% FBS (Hyclone) and antibiotics of penicillin/streptomycin (Hyclone). Human full-length NOTCH1 Val1744 (Cell Signaling Technology), E-Cadherin (GeneCopoeia). Primary antibody specific to JAGGED2 (Abcam), NOTCH1 Val1744 (Cell Signaling Technology), E-Cadherin (Santa Cruz Biotechnology), and Vimentin (Cell Signaling Technology) were used according to the manufactures' recommendations. Purified human IgG, recombinant human JAGGED1-Fc and JAGGED2-Fc were purchased from R&D System. γ-secretase inhibitors Compound E and DAPT were obtained from Merck and Sigma.

Lentivirus production and infection

The shRNAs were constructed in the pLKO.1 vector, and DNMAML was cloned in the pCDH-CMV-MCS-EF1 vector. Purified plasmids (1 μg) were transfected into HEK-293T cells by using X-tremeGENE HP DNA transfection Reagent (Roche), along with helper plasmids psPAX2 and pMD2.G. Appropriate amount of virus supernatant was added in cell culture in the presence of 8 μg/mL polybrene (18, 19). Positive clones were obtained using puromycin selection. The sequences of shRNA were listed in the Supplementary Table S1.

Wound-healing assays

Cells were seeded in a 24-well or 96-well plate up to form a 100% confluent monolayer, followed by 24 hours serum starvation. A wound was generated by running a 10-μL pipette tip homogeneously across the monolayer, followed by washing with PBS to remove cell debris. The wounded areas were photographed and recorded at each time points. The migrating index was calculated according to the following formula: Migration index = (width of the wound at 0 hour—width of the wound at 24 or 48 hours) × 100/width of the wound at 0 hour (15).

Cell migration and invasion assays

Migration and invasion assays were performed using 24-well Transwell plates (Corning; refs. 5, 20). Briefly, 50,000 to 100,000 cells were plated in serum-free medium in the upper chambers, then the chambers were placed in the bottom wells containing medium supplemented with 10% FBS for 24 hours at 37°C. The migrated or invaded cells were fixed with 4% paraformaldehyde followed by staining with 1% crystal violet. Three random microscopic fields (×100) of the chamber were photographed and counted.

Cell proliferation assays

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Geneview). Five thousand wells were plated in each well of a 96-well plate and incubated at 37°C. MTT dye was added at each time point and incubated for 4 hours at 37°C. Absorbance at 490 nm was then measured using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek).

RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol (Ambion). Random primed total RNAs (1 μg) were reverse-transcribed with ReverTraFirst strand cDNA synthesis kit (Thermo Scientific) according to the manufacturer's instructions. qPCR was conducted using FAST SYBR Green Master Mix (Bio-Rad) on CFX Connect Real-Time PCR System (Bio-Rad). Relative expression of the mRNA was estimated using the 2−ΔΔCt method (21). Primer sequences for real-time PCR were listed in Supplementary Table S2.

Western blot analysis

Cell lysates were electrophoresed in SDS-PAGE and transferred onto PVDF membranes (Bio-Rad). The membranes were blocked with 5% skim milk before primary antibodies incubation overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour. Protein bands were then revealed using Femto ECL substrates (Thermo Scientific) and visualized in SynGENE Bio Imaging System (Synoptic Ltd).

IHC

Sections of human patient PDAC tumors were obtained from Tongji Hospital (Wuhan, P.R. China) with informed consent and approval from the Clinical Research Ethics Committee of Tongji Medical School. The procedures involving human subjects were in accordance with the Helsinki Declaration. Sections of primary samples were stained with the primary antibodies against human JAGGED2 overnight at 4°C. The remaining steps were performed using the Histostain-Plus Kit (Invitrogen; refs. 22, 23).

PDAC metastatic mouse model

Metastatic mouse models were established as described (24). Stable BxPC-3 cell lines expressing scramble or JAGGED2 shRNA were infected with pCDH-CMV-MCS-EF1 lentivirus and sorted for GFP+ cells. A total of 1 × 10⁶ GFP+ cells were injected into male mice via tail veins. All mice were sacrificed in the sixth week after injection, livers dissected to assess metastasis based on hematoxylin and eosin (H&E) staining and fluorescence detection. Animals were maintained under specific pathogen-free conditions and experimentation was approved by the Animal Ethics Committee of Huazhong University of Science and Technology (Hubel, P. R. China).
Statistical analysis

Data were analyzed by Student t test or $\chi^2$ statistical analysis using SPSS version 20. P values that are less than 0.05 are considered statistically significant.

Results

JAGGED2 expression was elevated in pancreatic ductal carcinoma cells and primary tissues

We first confirmed that JAG2 expression was highly expressed in PDAC cell lines. Comparing the JAG2 expression in four human pancreatic cancer cells with that in an immortalized epithelial cell (HPDE6-C7), we found that, consistent with previous reports (3), three of four (75%) pancreatic cancer cells had greater JAG2 transcript levels (Fig. 1A). We next assessed the JAGGED2 expression in 15 pairs of tumor and adjacent normal tissues from patients with PDAC. Consistently, we found that 11 out of 15 tumor samples exhibited stronger JAGGED2 staining in histologic analysis compared with corresponding adjacent tissues (Fig. 1B and C). As shown in Fig. 1D, $\chi^2$ statistical analysis manifested greater JAGGED2 expression in PDAC tissues. Our data provide strong evidence that JAGGED2 expression is considerably elevated in human PDAC.

Abrogation of JAGGED2 inhibited PDAC metastasis

To determine the role of JAGGED2 in PDAC cells, BxPC-3 and MIA PaCa-2 cells were infected with lentivirus-expressing human JAGGED2 shRNA or control scramble shRNA. As expected, downregulation of JAGGED2 reduced NOTCH activation evidenced by less ICN1 production (Fig. 2A and Supplementary Fig. S1A and S2A). When assessing cell survival rates, we failed to observe the inhibition of monolayer cell growth in the absence of JAGGED2 in either BxPC-3 (Fig. 2B and Supplementary Fig. S1B) or MIA PaCa-2 cells (Supplementary Fig. S2B). Similar results were also observed in other pancreatic cancer cell lines (data not shown). We next conducted wound-healing assays to examine the effect of JAGGED2 on cell migration, and revealed profound migratory defect in JAGGED2-depleted cells (Fig. 2C and Supplementary Fig. S1C and S2C). By Transwell assays, we further assessed both migratory and invasive capacities of BxPC-3 and MIA PaCa-2 cells upon JAGGED2 ablation, and observed a consistent reduction (Fig. 2D and Supplementary Fig. S2D). However, knockdown of JAGGED1 (Supplementary Fig. S3A), which is the homolog of JAGGED2, did not affect tumor cell proliferation and migration (Supplementary Fig. S3B and S3C), suggesting that JAGGED2 was specifically required for pancreatic carcinoma metastasis. Our data are also consistent with previous findings showing that JAGGED2 but not JAGGED1 promotes adenocarcinoma metastasis (5).

To explore the molecular basis for metastatic inhibition in JAGGED2-depleted pancreatic cancer cells, we examined whether JAGGED2 knockdown prevented cells from undergoing epithelial-to-mesenchymal transition (EMT), the initial step of cancer metastasis. Indeed, JAGGED2 depletion changed the EMT phenotype by upregulation of epithelial cell marker (E-CADHERIN) and downregulation of mesenchymal cell markers (N-CADHERIN and VIMENTIN) in BxPC-3 (Fig. 2E), MIA-PaCa-2 (Supplementary Fig. S2E), PANC-1, and AsPC-1 cells (data not shown).
shown). We further examined key transcription factors that govern the expression of EMT markers and found that ZEB1, ZEB2, SNAIL1, and SNAIL2 were all decreased in JAGGED2-depleted cells (Fig. 2F and Supplementary Fig. S2F). Moreover, the downregulation of JAGGED2 also reduced the expression of key factors participating in tumor metastasis in vivo, such as matrix metalloproteinase-9 (MMP-9) and VEGF (Fig. 2F and Supplementary Fig. S2F). Taken together, these molecular analyses suggest that JAGGED2 promotes metastasis through regulating EMT.

To assess the function of JAGGED2 in promoting PDAC metastasis in vivo, we intravenously injected one million GFP + BxPC-3 cells expressing JAGGED2 shRNA or scramble control shRNA into nude mice. At the 35th day postinjection, nude mice having control shRNA-expressing cells exhibited much more dramatic weight loss than mice with JAGGED2-shRNA–expressing cells. We sacrificed the mice to analyze PDAC metastasis in livers, revealing much more control tumor cells than JAGGED2-depleted cells migrated to livers (Fig. 2G). H&E staining was used to examine tumor metastasis. In addition, fluorescence was quantitated using ImageJ and shown at the bottom.
staining confirmed more tumor cells as well as greater GFP fluorescence in the livers of control mice (Fig. 2G). These results manifest that JAGGED2 ablation in PDAC cells impairs their migratory and metastatic capability.

**Enforced JAGGED2 expression promoted PDAC cell migration**

We next address the functional role of JAGGED2 using a complementary gain-of-function approach. Expression vector encoding the open reading frame (ORF) of JAG2 was transfected into BxPC-3 cells infected with scramble or JAGGED2 3'UTR shRNAs. JAGGED2 depletion resulted in downregulation of mRNA levels (Fig. 3A) and protein activity reflected by γ-secretase-mediated cleavage of NOTCH1 (data not shown), thus hampering tumor cell migration (Fig. 3B and C). Enforced expression of JAG2 ORF, which is resistant to shRNA targeting the 3'UTR of JAG2 mRNA, retained strong JAG2 expression in the presence or absence of 3'UTR shRNA (Fig. 3A). Constitutive JAGGED2 expression noticeably accelerated cell movement and substantially rescued migratory defect in JAGGED2-depleted cells (Fig. 3B and C), confirming the critical role of JAGGED2 in promoting tumor cell migration.

**Figure 3.**

Constitutive JAGGED2 expression promotes PDAC cell migration. A, analysis of JAG2 mRNA levels in JAGGED2-depleted and/or overexpressed BxPC-3 cells. Cells were infected with viruses expressing scramble or JAGGED2 shRNA and transfected by pReciever-M13 JAG2 ORF or empty vector, total RNA extracted to analyze JAG2 mRNA abundance. B, wound healing in the presence or absence of JAGGED2 ORF or 3'UTR shRNA was determined at 24 and 48 hours by measuring the width of gaps, representative images of migration are shown. Quantification of cell migration is plotted in C. Data are expressed as the mean values (±SD) of triplicate wells.
NOTCH inhibition had no effect on PDAC cell migration and invasion.

The involvement of a NOTCH ligand in PDAC prompted us to examine whether the JAGGED2-induced activation of NOTCH signal pathway is responsible for cell migration and metastasis. To this end, BxPC-3 cells were treated with γ-secretase inhibitors (GSIs), Compound E or DAPT. Both compounds effectively blocked NOTCH activity, evidenced by decreased HES1 expression (Fig. 4A and Supplementary Fig. S4A) and reduced intracellular NOTCH1 (ICN1) production (data not shown). Yet neither Compound E (1 μmol/L) nor DAPT (1 μmol/L) treatment significantly affected cell proliferation (Fig. 4B and Supplementary Fig. S4B), migration, and invasion (Fig. 4C and D and Supplementary Fig. S4C). To exclude the possibilities of nonspecific effects resulting from GSIs, we infected BxPC-3 with lentiviruses expressing dominant negative MAML (DNMAML) to block NOTCH activity more precisely. Although downregulating NOTCH target genes c-MYC and HES1 (Fig. 4E), DNMAML expression had minimal effect on cell migration (Fig. 4F). We thus conclude that NOTCH signaling activation is not required for PDAC cell migration and invasion at least in vitro.

NOTCH1 depletion led to reduced PDAC cell migration

To determine whether NOTCH receptors were relevant in promoting PDAC cell migration, MIA PaCa-2 cells were infected with lentivirus-expressing human NOTCH1 shRNA or NOTCH2 shRNA, which specifically decreased NOTCH1 or NOTCH2 transcripts in comparison with control scramble shRNA (Fig. 5A). Interestingly, we observed noticeable morphologic changes in cells expressing NOTCH1 shRNA but not in those expressing NOTCH2 shRNA or empty control. NOTCH1-ablated cells became difficult to adhere onto culture...
were repeated at least three times in triplicates and data are presented as mean ± SD. 

**Figure 5.**

**A**  NOTCH1 ablation inhibited PDAC cell migration. A, NOTCH1 and NOTCH2 mRNAs were knocked down in MIA PaCa-2 cells. NOTCH1 or NOTCH2 mRNAs were analyzed by real-time PCR. B, cell adhesion morphology changes in NOTCH1 and NOTCH2-depleted cells. C, inhibition of MIA PaCa-2 cell migration by abrogation of NOTCH1 but not NOTCH2. Represented photographs (above) and quantification of cell migration index (bottom) are shown. Experiments were repeated at least three times in triplicates and data are presented as mean ± SD.

JAGGED2-Fc fusion proteins blocked pancreatic cancer cell migration and invasion

To determine the function of JAGGED2 as an adhesion molecule for PDAC metastasis, we antagonized JAGGED2–receptor interaction using soluble JAGGED2-Fc in BxPC-3 cell culture, human IgG, and JAGGED1-Fc as negative controls. Although both JAGGED1 and JAGGED2-Fc were equally active inducing HES1 expression (Supplementary Fig. S5), only JAGGED2-Fc inhibited cell migration in a dose-dependent manner in the Transwell assay (Fig. 6A) and the wound-healing experiment (Fig. 6B). Consistent with the prior data showing that JAGGED2 ablation specifically impaired cell migration, we herein provide strong evidence that the JAGGED2-NOTCH adhesion promotes PDAC cell migration.

**Discussion**

We present here that the NOTCH ligand JAGGED2 promotes PDAC cell migration and invasion, thereby supporting a positive role of NOTCH pathways in regulation of pancreatic cancer progression. We describe a new mechanism whereby NOTCH family members mediate cell–cell communication independent of downstream signaling activation but serve as adhesion molecules facilitating cell migration. Our findings not only provide an important insight into molecular basis of tumor metastasis, but also highlight JAGGED2 as a potential therapeutic target to inhibit PDAC development.

JAGGED2 is associated with poor prognosis and overall survival in a variety of cancer types (25–27), and suggested to participate in metastasis of adenocarcinomas (5–7). Generally, the JAGGED2-mediated effect is believed through activation of NOTCH signaling and induction of downstream gene expression (5–7). In lung adenocarcinoma, for instance, JAGGED2 induces GATA3 expression via NOTCH activation, and GATA3 transcriptionally inhibits miR-200 and consequently EMT (5). Interestingly, our studies reveal a distinct mode that JAGGED2-promoted metastasis was independent of NOTCH activation in PDAC. GSI treatments or DNMAML expression were able to repress GATA3 (data not shown), but failed to affect tumor cell migration and invasion (Fig. 4 and Supplementary Fig. S4), implying that NOTCH target genes do not account for JAGGED2-mediated effects. We cannot exclude that NOTCH transcriptional activation may be involved in scenarios such as hypoxia (5, 6, 23). It could be possible that JAGGED2 applies distinct mechanisms in promoting metastasis in different tumor microenvironments, and we here highlight a previously unappreciated signaling-independent mechanism of action involved in tumor metastasis.

Our data strongly suggest that JAGGED2-NOTCH interaction plays a vital role in tumor cell migration. Most likely, diminishing either JAGGED2 or NOTCH1 presentation on the cell surface hampers the formation of focal adhesion complexes required for cell migration. Soluble JAGGED2-Fc that blocks NOTCH–ligand interactions specifically inhibited cell migration, probably attributable to disruption of adhesion complexes (Fig. 6). It has been previously reported that NOTCH–ligand interactions affected cell–cell contacts and adhesion in cardiovascular abnormalities (28) and chronic inflammation (29). Similarly, NOTCH signaling activation was dispensable in their findings. Our work provides first evidence suggesting that NOTCH–ligand interactions per se regulate tumor progression. The involvement of cell–cell contact and adhesion provides a new perspective in understanding potential roles of NOTCH pathways in tumor development.

JAGGED2 regulation of tumor metastasis could involve other mechanisms. For instance, JAGGED2-NOTCH interaction also triggers a "retrograde" signaling in ligand-presenting cells through ligand processing and turns intracellular ligands into transcriptional activators (30, 31). However, it seems unlikely that JAGGED2-mediated transcription contributes to metastasis as ligand processing also requires γ-secretase–mediated cleavage (30, 31), inhibition of which by GSI did not affect tumor cell movement (Fig. 4 and Supplementary Fig. S4). Alternatively, the
JAGGED2-mediated effects may involve a noncanonical and nuclear NOTCH-independent mechanism (32) that was previously reported through mTORC2-regulated AKT activation in nutrient deprivation-induced cell death (33) or sequestering β-CATENIN (34). Regardless of mechanisms of action, our findings suggest a tempting strategy that JAGGED2 could be used as a diagnostic marker predicting PDAC metastasis. More promisingly, peptides mimicking extracellular JAGGED2 or antibodies against JAGGED2 may prevent metastasis in patients with PDAC. Such an approach may prolong recurrence-free survival, particularly for those after surgical resection. Hence, our findings provide a rationale for developing small molecules or biologic agents targeting JAGGED2 for therapeutic benefits of patients with PDAC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: G. Qing, H. Liu
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Hu, H. Su, X. Li, G. Guo
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Hu, G. Guo, R. Qin, H. Liu
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Su, X. Li, L. Cheng, R. Qin, H. Liu
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Acknowledgments
The authors thank Ming Xiang’s laboratory in Tongji Medical College for sharing reagents.

Grant Support
H. Liu was supported by National Natural Science Foundation of China (NSFC; 81272211 and 81200381), Scientific Research Foundation for the Returned Oversea Chinese Scholars, State Education Ministry of China (2013-792-439), Program for Youth Chutian Scholar of Hubei Province of China, New Investigator Foundation, State Education Ministry of China (20120142120097), and Innovative Research Award from Huazhong University of Science and Technology (2014ZH015). G. Qing was supported by NSFC (81171928) and R. Qin by NSFC (81272659).
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Received June 12, 2014; revised September 26, 2014; accepted October 14, 2014; published OnlineFirst October 28, 2014.

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Molecular Cancer Therapeutics

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doi:10.1158/1535-7163.MCT-14-0501

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