Anti-DLL4 Has Broad Spectrum Activity in Pancreatic Cancer Dependent on Targeting DLL4-Notch Signaling in Both Tumor and Vasculature Cells

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Abstract

Purpose: We previously showed that targeting Delta-like ligand 4 (DLL4) in colon and breast tumors inhibited tumor growth and reduced tumor initiating cell frequency. In this report, we have extended these studies to pancreatic cancer and probed the mechanism of action in tumor and stromal cells involved in antitumor efficacy.

Experimental Design: Patient-derived pancreatic xenograft tumor models were used to evaluate the antitumor effect of anti-DLL4. To investigate the mechanism of action, we compared the activity of targeting DLL4 in tumor cells with an anti-human DLL4 antibody (anti-hDLL4) and in the host stroma/vasculature with an anti-mouse DLL4 antibody (anti-mDLL4). The effect of these antibodies on cancer stem cell frequency was examined by in vivo limiting dilution assays.

Results: The combination of anti-hDLL4 and anti-mDLL4 was efficacious in a broad spectrum of pancreatic tumor xenografts and showed additive antitumor activity together with gemcitabine. Treatment with either anti-hDLL4 or anti-mDLL4 delayed pancreatic tumor recurrence following termination of gemcitabine treatment, and the two together produced an additive effect. Anti-hDLL4 had a pronounced effect in reducing the tumorigenicity of pancreatic cancer cells based on serial transplantation and tumor-sphere assays. In contrast, disruption of tumor angiogenesis with anti-mDLL4 alone or with anti-VEGF had minimal effects on tumorigenicity. Gene expression analyses indicated that anti-DLL4 treatment regulated genes that participate in Notch signaling, pancreatic differentiation, and epithelial-to-mesenchymal transition.

Conclusions: Our findings suggest a novel therapeutic approach for pancreatic cancer treatment through antagonism of DLL4/Notch signaling. Clin Cancer Res; 18(19); 5374–86. ©2012 AACR.

Introduction

Pancreatic cancer is a disease with an extremely poor prognosis. The American Cancer Society currently estimates about 43,000 new cases per year of pancreatic cancer in the United States, and 36,800 deaths per year resulting from this disease (1). Depending on the extent of tumor growth at the time of diagnosis, disease progression is typically rapid and, overall, less than 5% of patients are alive 5 years after diagnosis and complete remissions are extremely rare (1). The mortality rate of pancreatic cancer remains largely unchanged, in part because of the late presentation and lack of effective therapies for surgically unresectable disease (2). Conventional chemotherapeutic agents, including gemcitabine, commonly used in the treatment of pancreatic cancer, have low response rates and limited effects on improving patient survival. The combination of multiple chemotherapeutic agents using the FOLFIRINOX regimen has been shown to result in higher objective response and survival rates than gemcitabine alone; however, the utility of this regimen has a corresponding increase in toxicity (3). Thus, there is a significant need for new strategies and therapeutic agents for the treatment of pancreatic cancer.

Evidence has accumulated that tumors are composed of heterogeneous cell types and that tumor growth is driven by a subset of cells termed cancer stem cells (CSCs) or tumor initiating cells (4). CSCs share certain similarities with normal stem cells including upregulation of self-renewal...
Pancreatic cancer is the fourth leading cause of cancer death in the United States with 5-year survival rate of only 3% and a median survival of less than 6 months. Findings from the present study suggest that agents that reduce cancer stem cell frequency may improve cancer treatment by delaying or preventing tumor recurrence and reducing the metastatic spread of the disease. In particular, blocking DLL4-mediated tumor angiogenesis, proliferation of bulk tumor cells, and cancer stem cell self-renewal provides an attractive strategy for a new cancer treatment.

Translational Relevance
Pancreatic cancer is the fourth leading cause of cancer death in the United States with 5-year survival rate of only 3% and a median survival of less than 6 months. Findings from the present study suggest that agents that reduce cancer stem cell frequency may improve cancer treatment by delaying or preventing tumor recurrence and reducing the metastatic spread of the disease. In particular, blocking DLL4-mediated tumor angiogenesis, proliferation of bulk tumor cells, and cancer stem cell self-renewal provides an attractive strategy for a new cancer treatment.

pathways and are defined by 2 key attributes: (i) the ability to propagate a continuously growing tumor and (ii) the ability to differentiate into multiple cell types including nontumorigenic cancer cells (4). The existence of a subpopulation of cells with increased tumorigenicity was shown in acute myeloid leukemia (5) and was then extended to many types of epithelial tumors, including pancreatic cancer (6–14). Furthermore, several research reports have indicated that CSCs are relatively resistant to many existing therapies including conventional chemotherapy and radiation treatments (15–20). Therefore, therapeutic strategies that are able to reduce CSC frequency could have a major impact on pancreatic cancer response rates and patient survival.

Notch signaling controls various cellular processes including stem cell self-renewal, cell fate specification, differentiation, proliferation, and survival (21). In the pancreas, Notch signaling plays a crucial role in embryonic development and cell differentiation (22). Notch receptors, ligands, and their downstream targets have been found to be upregulated in preneoplastic lesions and invasive pancreatic cancers in humans and mice, suggesting that inappropriate activation of Notch signaling could be an early event leading to accumulation of undifferentiated precursor cells in pancreatic cancers (23). Although the role of Notch signaling in promoting pancreatic cancer and CSCs is not completely well understood, several lines of evidence suggest that the Notch pathway is involved in this disease. For example, Mullendorf and colleagues reported that endogenous overexpression of Notch ligands DLL4 and Jagged2 are associated with an increase in Notch signaling that has been observed in the pancreatic cancer (24). Higher Notch1 expression was found in pancreatic CSC compared with the non-CSC population (25). Similarly, Li and colleagues reported that CD44+/CD133+ expressing pancreatic CSCs contain high levels of Notch1 and Notch2 and that restoration of mirR-34 downregulates both receptors (26). Collectively, these data suggest that activation of Notch signaling may be involved in promoting development of pancreatic cancer, and in particular Notch signaling may be involved with CSC self-renewal in this disease setting.

DLL4 is an important component of Notch-mediated stem cell self-renewal and vascular development. DLL4 overexpression is found in tumor vasculature and in tumor cells and has been shown to activate Notch signaling in CSCs (27, 28). Yan and colleagues showed that transplantation of DLL4-expressing bone marrow resulted in the induction of T cell acute lymphoblastic leukemia/lymphoma (T-ALL) in mice, suggesting that aberrant DLL4-mediated activation of Notch signaling may promote T-ALL leukemogenesis (28). Inhibition of DLL4 in epithelial tumors resulted in an antitumor effect through an increase of vasculature sprouting resulting in dysfunctional vasculature (29, 30).

We have developed neutralizing antibodies against human and mouse DLL4 to investigate the contributions of selectively targeting DLL4 in tumor cells or in the host vasculature and stroma in a panel of xenograft models derived from pancreatic cancer patients. We evaluated the effects and mechanisms of action of anti-DLL4 as a single agent and in combination with gemcitabine to assess the potential clinical utility of this drug in pancreatic cancer.

Materials and Methods
Chemicals and reagents
Gemcitabine (Lilly) was obtained from Pharmaceutical Buyers International, Inc. Hank’s balanced salt solution (HBSS), Dulbecco’s Modified Eagle Medium (DMEM) and Medium 199 were from Gibco-Invitrogen. FBS was obtained from Hyclone. Fluorescence-activated cell sorting buffer, FACS buffer, consisted of HBSS medium supplemented with 2% heat-inactivated FBS and 20 mmol/L Hepes.

Antibodies
Production of anti-hDLL4 antibody (21M18) and anti-mDLL4 (21R30) has been previously described (31).

Pancreatic tumorsphere assays
Tumorsphere assays were conducted using human pancreatic tumor cells from a patient-derived xenograft as has been previously described (13). Briefly, a thousand single cells were cultured with or without the presence of control antibody or hDLL4 antibody in tumorsphere medium containing 1% N2 supplement (Invitrogen), 2% B27 supplement (Invitrogen), 1% Antibiotic-Antimycotic (Invitrogen), 20 ng/ml bFGF-2 (Invitrogen), and 20 ng/ml EGF (Invitrogen) in 6-well ultralow attachment plates (Corning) for 4 days. All cultures were analyzed by tumorsphere formation using an inverted microscope. The experiments were repeated 3 times.

In vivo animal studies
Nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice were purchased from Harlan Laboratories and maintained under specific pathogen-free conditions and provided with sterile food and water ad libitum. The animals used in this study were housed in a U.S. Department of Agriculture-registered facility in accordance
with NIH guidelines for the care and use of laboratory animals. The mice were allowed to aclimate for several days before the studies. The establishment and characterization of in vivo primary pancreatic cancer xenograft models were described previously (13). OMP-PN4 and PN21 were initially established at OncoMed Pharmaceuticals, Inc. The PN4 tumor was obtained from the Cooperative Human Tissue Network that is funded by the National Cancer Institute and PN21 was obtained from the National Disease Research Interchange. OMP-PN8, PN9, PN13, PN16, and PN17 were established at the University of Michigan. Single cell suspensions from either frozen cell stocks or freshly dissociated tumors were used for antibody testing studies. Briefly, xenograft tumors at passage 2 or 3 were removed, cut into small pieces with a scalpel and minced with a sterile razor blade after removing necrotic portions and connective tissues. Single cell suspensions were prepared as described previously (31).

For subcutaneous xenograft models, fifty thousand cells/100 μL were implanted into the left flank region of 6- to 8-week-old NOD/SCID mice with a 25-gauge needle. To determine the effect of antibody on established subcutaneous pancreas xenograft tumor growth, tumor-bearing animals were randomized and treatment started when the mean tumor volumes reached 100 to 150 mm³. Gemcitabine was given at various doses ranging from 5 to 50 mg/kg in various experiments as specified in the figure legends. In certain experiment, that is, Fig. 1B, gemcitabine was given at 40 mg/kg with or without antibodies for 2 weeks followed by anti-DLL4 alone to determine the effect of antibody on tumor regrowth. Antibodies were dosed once per week throughout the course of study. Both antibodies and chemotherapeutic agents were administered intraperitoneally. To evaluate the effect of the antibodies on tumor recurrence, animals were treated with gemcitabine 50 mg/kg twice a week in the OMP-PN4 experiment and 100 mg/kg once a week in the OMP-PN8 experiment (these doses were determined to result in efficient tumor regression in these models) until the tumors regressed (about 3–4 weeks). Thereafter, animals were given either the control antibody or the anti-DLL4 antibodies once a week for an additional 6 to 8 weeks. Subcutaneous tumor growth was measured with an electronic caliper (Coast Tools Company). Tumor volumes were calculated using the formula $V = \frac{L \times W^2}{2}$, where $L$ was the longest and $W$ was the shortest axis of the tumor. The control antibody for the PN4 and PN8 experiments was 1B7.11, a murine monoclonal antibody directed against dinitrophenol. For the other xenograft experiments, the control antibody was LZ-1, a human monoclonal antibody raised against bacterial lysozyme.

To generate the gemcitabine-resistant OMP-PN8 tumor xenograft, tumor bearing mice were treated with 100 mg/kg gemcitabine once per week and tumors were passaged in vivo in the presence of gemcitabine until their growth rate was the same as vehicle treated tumors indicating that they no longer responded to the chemotherapeutic agent (7 cycles of in vivo selection). As a control, the parental cell suspensions were used to establish xenografts in the presence of gemcitabine until their growth rate was the same as vehicle treated tumors indicating that they no longer responded to the chemotherapeutic agent (7 cycles of in vivo selection).

For tumorigenicity studies, single cell suspensions from control and treated tumors were incubated with biotinylated mouse antibodies (α-mouse CD45-biotin 1:100 dilution and rat α-mouse H2Kd-biotin 1:50 dilution, BioLegend) on ice for 30 minutes followed by addition of streptavidin-labeled magnetic beads (Invitrogen) to remove infiltrating mouse cells. The remaining human cells in the suspension were collected, counted, and diluted to appropriate cell doses, then mixed with a mixture of 1:1 (v/v) FACS buffer and Matrigel and injected subcutaneously in NOD/SCID mice. Tumor growth was monitored for up to 3 months. CSC frequency was determined using L-Calc Version 1.1 software program (StemCell Technologies, Inc.). Differences in frequency between groups were analyzed by Fisher’s exact test. A difference of $P < 0.05$ was considered significantly different.

**Immunohistochemistry**

Formalin-fixed, paraform-embedded sections were cut 4 to 5 μm thick. Immunohistochemistry (IHC) was conducted by...
All RNA samples had 260/280 ratios more than 1.8, by the presence of intact 28S and 18S ribosomal peaks. RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) with DNAse treatment. RNA was visualized on an Olympus BX51 microscope using IPLabs v4.0 (BD Biosciences). RNA Preparation and Quantitative PCR

Biosciences). The results were analyzed using the SDS2.3 software (Applied Biosystems). All primer and probe sets were obtained from Applied Biosystems. The level of expression of target genes were normalized to the expression level of the housekeeping gene GAPDH and expressed as relative quantity.

Data analysis

Data are expressed as mean ± SEM. Differences in mean values between groups were analyzed by nonparametric t tests. Multiple comparisons used 2-way ANOVA test followed by Bonferroni posttest comparisons. Differences of P < 0.05 were considered significantly different. Software for statistical analysis was by GraphPad Prism5 (GraphPad Software Inc.).

Results

Anti-DLL4 treatment inhibits the growth of human pancreatic xenograft tumors

We evaluated the effects of anti-DLL4 in our collection of human pancreatic cancer xenograft models. This set of tumors contains both moderately differentiated (OMP-PN4, PN7, and PN21) and poorly differentiated adenocarcinomas (OPM-PN8, PN9, PN13, PN16, and PN17) that were obtained from surgical samples and established as xenografts in immunocompromised mice. Quantitative PCR (Q-PCR) analysis indicated each tumor expressed DLL4 mRNA at a level higher than that of normal human pancreatic tissue (Supplementary Fig. S1). For most of our experiments, we combined anti-human DLL4 (OMP-21M18) and anti-mouse DLL4 (OMP-21R30) in a 1:1 ratio as one treatment regimen. This combination of antibodies inhibits DLL4-Notch signaling in both the tumor cells and the murine stromal/vascular cells in the xenografts (31, 32) and is referred to as “anti-DLL4” in this manuscript. In certain experiments, we tested the effects of the antibodies separately as noted.

Our data indicated that anti-DLL4 treatment was efficacious against each of the pancreatic xenograft tumors tested, inhibiting tumor growth by 30% to 50% in these tumors (Table 1). We also examined whether there was a therapeutic benefit of combining anti-DLL4 with gemcitabine, a chemotherapeutic agent commonly used in the treatment of pancreatic cancer. We observed that the combination of anti-DLL4 and gemcitabine produced a statistically significant greater antitumor effect than either agent alone in all tumors examined (Table 1). Representative growth curves for OMP-PN4 and OMP-PN8 are shown in Fig. 1. In OMP-PN4 tumors, anti-DLL4 and gemcitabine reduced tumor growth by 53% and 71%, respectively, compared with the control groups (Fig. 1A). The combination of both agents decreased tumor volumes by 94% compared with the control and an additional 20% tumor volume decrease compared with gemcitabine treatment alone (P < 0.001 vs. control mAb and P < 0.05 vs. gemcitabine, Fig. 1A). Similar observations were also found in the poorly differentiated pancreatic xenograft tumor OMP-PN8, where combination therapy showed a greater antitumor effect relative to gemcitabine alone (Fig. 1B). Notably, in 3 of 7 xenograft tumors (OMP-PN4, PN8, and PN17), the combination of anti-DLL4 and gemcitabine resulted in tumor regression (Fig. 1 and Table 1). In addition, using an orthotopic model, we showed that anti-DLL4 inhibited OMP-PN8 primary tumor growth and decreased the incidence of distant metastases in the lung and the liver (Supplementary Fig. S2).

Blockade of DLL4 in the tumor cells induces pancreatic cell differentiation and cell death

To investigate the molecular mechanisms of action of anti-DLL4 in pancreatic tumors, we examined the effect of...
Table 1. Effect of anti-DLL4 in combination with gemcitabine on growth of pancreatic xenograft tumors

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Anti-DLL4</th>
<th>Gemcitabine</th>
<th>Combination</th>
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<td>OMP-PN4</td>
<td>53 ± 7%</td>
<td>71 ± 6%</td>
<td>94 ± 1%†</td>
</tr>
<tr>
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<td>45 ± 7%</td>
<td>38 ± 5%</td>
<td>69 ± 2%†</td>
</tr>
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<td>80 ± 1%</td>
<td>96 ± 0.4%†</td>
</tr>
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<td>15 ± 8%</td>
<td>69 ± 4%†</td>
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<td>73 ± 6%</td>
<td>87 ± 3%†</td>
</tr>
<tr>
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<td>37 ± 5%</td>
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<td>25 ± 5%</td>
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Data expressed as mean ± SEM, n = 7–10 mice per group. †, P < 0.01 versus control mAb; ††, P < 0.05 versus single agents by 2-way ANOVA followed by Bonferroni posttest comparison; †, tumor regression.

anti-DLL4 on expression of genes related to proliferation, apoptosis, pancreas differentiation, and stem/progenitor cell maintenance. Q-PCR analysis indicated that anti-DLL4 inhibited the expression of HES1 and increased expression of ATOH1 in OMP-PN4 (Fig. 2A), characteristic of Notch pathway antagonism (33). In another experiment, we tested the effects of anti-hDLL4 and anti-mDLL4 separately for their roles in regulating gene expression in human tumor and murine stromal cells in xenografts. We found that anti-DLL4 regulated Notch target genes HES1 and ATOH1 in the tumor cells but not the stroma (Fig. 2B). Conversely, anti-mDLL4 regulated Notch target genes Hes1, Atoh1, and the endothelial cell marker gene Cd31 in the mouse stromal tissues but had no effect on the expression of these genes in tumor cells (Fig 2B).

We found that anti-DLL4 as a single treatment or in combination with gemcitabine increased the expression of programmed cell death protein 4 (PDCD4; Fig. 2A), a proapoptotic gene that has properties of a tumor suppressor (34). We also observed an increase in expression of several genes indicative of promoting pancreatic cell differentiation, including chromogranin A (CHGA), mucin2 (MUC2), neurogenin-3 (NGN3), and paired-box homeoprotein 4 (PAX4; Fig. 2A). NGN3 and PAX4 play critical roles specifying differentiation of the endocrine cell lineage in pancreatic development (22). CHGA is important in islet cell physiology and is a marker for endocrine cells in the pancreas (35). MUC2 is a molecular marker for cell differentiation and tumor formation in the exocrine pancreas (36).

Gene expression changes in PDCD4, MUC2, and CHGA, were also observed by IHC (Fig. 2C). Immunohistochemical analysis in OMP-PN4 tumors also revealed that anti-DLL4 treated tumors exhibited a decrease in the frequency of proliferating cells as measured by Ki67 staining (Fig. 2C). The effect on Ki67 was more pronounced in the combination treated group, consistent with the antitumor effect where the combination produced greater growth inhibitory effect than either agent used alone. Thus, anti-DLL4-mediated inhibition of tumor growth is associated with antagonism of Notch signaling and induction of genes indicative of pancreatic differentiation and cell death.

**Anti-DLL4 delays tumor recurrence following gemcitabine termination**

Frequent tumor relapse after treatment is a major obstacle in the clinical management of pancreatic cancer patients. To determine whether anti-DLL4 treatment had an impact on tumor recurrence after chemotherapeutic treatment, we developed tumor recurrence models by treating OMP-PN4 and OMP-PN8 tumors with a high dose of gemcitabine. As seen in Fig. 3A, gemcitabine induced OMP-PN4 tumor regression; however, tumors reappeared about 4 weeks after gemcitabine treatment was terminated and grew progressively. Addition of anti-DLL4 delayed tumor recurrence following termination of chemotherapy compared with control antibody treated tumors (Fig. 3A). Similar results were obtained with OMP-PN8 tumors where anti-DLL4 significantly delayed tumor regrowth after gemcitabine treatment (Fig. 3B).

To determine the contribution of targeting DLL4-Notch signaling in tumor cells and in stromal/vascular cells in mediating the delay of tumor recurrence, we examined treatment with anti-hDLL4 and anti-mDLL4 separately in the OMP-PN8 xenograft tumor model. Blockade of DLL4 signaling in tumor vascular cells by targeting murine DLL4 in xenograft models is known to disrupt angiogenesis through an increase in endothelial proliferation that leads to nonfunctional vessels (29, 30). Our data indicate that both anti-hDLL4 and anti-mDLL4 delayed OMP-PN8 tumor regrowth after gemcitabine treatment and simultaneous treatment with both antibodies had an additive effect (Fig. 3B).

**Anti-hDLL4 inhibits pancreatic tumorsphere formation**

DLL4 is known to play a role in CSC maintenance in colon and breast tumors (29). In addition, it has been reported that CSCs are preferentially resistant to many standard therapies and that CSCs mediate tumor recurrence.
following such treatments (15–20). We hypothesized that the observed growth inhibition and delay in tumor recurrence by anti-DLL4 after gemcitabine treatment was in part because of the effect of anti-DLL4 antibody on CSCs. To test this idea, we conducted in vitro sphere-forming assays. In both normal and cancerous neural tissue, the ability of cells to form spherical colonies in nonadherent culture conditions is reflective of self-renewal capacity (37, 38). This assay has also been used to identify CSCs in breast cancer (39).

Previous results have shown that CD44⁺CD24⁻ESA⁺ pancreatic CSCs make "tumorspheres" in nonadherent culture conditions, whereas CD44⁻CD24⁻ESA⁻ cells do not (13). Treatment of CD44⁺CD24⁻ESA⁺ pancreatic cancer cells from a patient xenograft with anti-hDLL4 significantly inhibited their ability to form tumorspheres, whereas a control antibody had no effect (Fig. 3C and D), suggesting that anti-hDLL4 may have a direct effect on the self-renewal capacity of CSCs.

**Anti-hDLL4 reduces pancreatic tumor initiating cell frequency in vivo**

We next evaluated whether anti-DLL4 may have an effect on CSC function by measuring tumor initiating cell frequency using an in vivo limiting dilution assay. OMP-PN4 or OMP-PN8 tumors were treated with either control mAb, anti-DLL4, gemcitabine, or the combination of anti-DLL4 and gemcitabine for 3 weeks and harvested after the gemcitabine-treated groups had reduced tumor volume by
Figure 3. Effect of anti-DLL4 on pancreatic tumor recurrence. A, time course of OMP-PN4 tumor recurrence following termination of gemcitabine treatment. Established OMP-PN4 tumors were treated with gemcitabine at 50 mg/kg twice per week. After 4 weeks of dosing gemcitabine, treatments were stopped and the mice were treated with either control mAb (filled squares) or anti-DLL4 (open triangles). Anti-DLL4 antibodies (21M18 + 21R30) were dosed at 5 mg/kg, twice per week. B, effect of anti-hDLL4 and anti-mDLL4, or the combination of both on tumor recurrence in OMP-PN8 tumor following gemcitabine treatment. Established OMP-PN8 tumors were treated with gemcitabine at 100 mg/kg once per week. After 3 weeks of dosing gemcitabine, treatments were stopped and the mice were treated with either control mAb (filled squares), anti-hDLL4 (open triangles), anti-mDLL4 (filled circles), or the combination of anti-human and anti-mouse DLL4 (open circles). Both anti-hDLL4 and anti-mDLL4 resulted in a delay in tumor recurrence and the combination of produced an additive effect. Data expressed as mean ± SEM, n = 8–10 animals per group. *, P < 0.005 versus gemcitabine. C and D, effect of anti-hDLL4 on pancreatic tumor sphere formation. Following FACS sorting, single cells suspensions from a patient pancreatic cancer xenograft were resuspended in tumorsphere media. One thousand cells were plated in each well of 6-well ultralow attachment plates. Cells were grown in tumorsphere medium with or without the presence of control antibody or anti-hDLL4 (each at a concentration of 10 μg/mL) for the duration of the culture (5 days). C, tumorspheres in each well were quantified by microscopy. D, the control antibody did not affect the formation of tumorspheres, whereas anti-hDLL4 antibody had a significant effect in reducing tumorsphere formation. Data was expressed as mean ± SEM, n = 3 per treatment. ††, P < 0.0001 versus no antibody.
CSC frequency, about 6-fold lower than the control mAb antibody and gemcitabine produced a further decrease in treatment with anti-DLL4 decreased CSC frequency compared with control in both tumor types. In contrast, tumors had an approximately 2-fold higher CSC frequency and 1/78 in OMP-PN8 tumors. Gemcitabine-treated tumors had a CSC frequency of 1/137 in OMP-PN4 tumors. As seen in Fig. 4A and B, control mAb–treated at the various cell numbers allows the determination of CSC Fig. S3). The frequency of tumor growth (take rate) observed tumor growth was assessed after 90 days (Supplementary measurements). Anti-DLL4 and the combination with gemcitabine decreased CSC frequency and tumorigenic potential of pancreatic xenograft tumors. Data for in vivo studies expressed as mean ± SEM, n = 10 animals per cell dose. *, P < 0.05 versus control mAb; **, P < 0.05 versus gemcitabine by 1-way ANOVA followed by Tukey’s posttest. Effect of anti-DLL4 on gemcitabine-resistant pancreatic tumors. OMP-PN8 resistant tumors were generated by repeated weekly treatment with 100 mg/kg gemcitabine. The resistant tumors were treated with anti-DLL4 or in combination with gemcitabine. At the end of treatment, tumors were harvested for in vivo limiting dilution assay to measure CSC frequency. E, antitumor effect. F, CSC frequency in parental and gemcitabine-resistant OMP-PN8 xenograft tumors. Data are expres?sed as mean ± SEM, n = 9–10 per treatment group. *, P < 0.05 versus control mAb; **, P < 0.05 versus gemcitabine; *** P < 0.05 versus parental OMP-PN8 tumors treated with control mAb.

approximately 50%. The human tumor cells in the xenografts were isolated and equal cell numbers from each of the 4 treatment groups were injected into mice at 3 different cell doses (30, 90, or 270 cells, n = 10 mice per group) and tumor growth was assessed after 90 days (Supplementary Fig. S3). The frequency of tumor growth (take rate) observed at the various cell numbers allows the determination of CSC frequency. As seen in Fig. 4A and B, control mAb–treated tumors had a CSC frequency of 1/137 in OMP-PN4 tumors and 1/78 in OMP-PN8 tumors. Gemcitabine-treated tumors had an approximately 2-fold higher CSC frequency compared with control in both tumor types. In contrast, treatment with anti-DLL4 decreased CSC frequency compared with the control groups in both tumor models (P < 0.01). Importantly, the combination of anti-DLL4 antibody and gemcitabine produced a further decrease in CSC frequency, about 6-fold lower than the control mAb (P < 0.001) and more than 10-fold lower than gemcitabine alone (P < 0.0001) in both OMP-PN4 and OMP-PN8 tumors (Fig 4A and B).

To determine the effect of targeting DLL4 in either tumor or stromal cells, anti-hDLL4 and anti-mDLL4 were dosed separately, and the effect on tumorigenic potential of pancreatic CSCs in vivo was determined in OMP-PN8. Tumors from each treatment group were processed into single cells and 75 cells were implanted into recipient mice (10 mice per group) and allowed to grow for 72 days without further treatment. As seen in Fig. 4C, cells from the control antibody group produced a tumor growth frequency of 9/10. Treatment with gemcitabine alone or anti-mDLL4 plus gemcitabine did not significantly affect the subsequent tumorigenicity of the cells, whereas cells from the anti-hDLL4/ gemcitabine combination reduced the take rate to 4/10 indicative of a reduction in tumor initiating cell frequency...
(Fig. 4C). Similar observations were found in another pancreatic xenograft tumor OMP-PN7 (Fig. 4D). Taken together, the above findings indicated that targeting DLL4 in the neoplastic human tumor cells but not in the stroma/vasculature decreased the tumorigenicity of pancreatic cancer cells. To determine if the lack of effect of anti-mDLL4 on tumor initiating cell frequency was also observed with other antiangiogenic agents, we also tested bevacizumab, anti-VEGF (40). Notably, bevacizumab had no effect on tumorigenicity of pancreatic CSCs when combined with gemcitabine (Fig. 4C).

Anti-DLL4 is efficacious in chemotherapy-resistant tumors and restores drug sensitivity

To further evaluate the role of DLL4-Notch signaling in drug resistance and tumor initiating cell function, we generated a gemcitabine-resistant OMP-PN8 tumor xenograft by repeated in vivo treatment with a high dose of gemcitabine (100 mg/kg, weekly). Tumors were passaged in vivo until they no longer responded to the drug. As a control, OMP-PN8 tumors were passaged in parallel without drug treatment. We treated the gemcitabine-resistant tumors with anti-DLL4, gemcitabine, or the combination and determined the effects on tumor growth. Anti-DLL4 treatment reduced tumor growth of gemcitabine-resistant OMP-PN8 tumors as a single agent, and importantly, combination of anti-DLL4 and gemcitabine sensitized the resistant tumors to the chemotherapeutic agent (Fig. 4E). Limiting dilution analysis showed that the CSC frequency in gemcitabine-resistant tumors was increased 5-fold relative to the non–drug-selected tumors (Fig. 4F). Treatment of the resistant tumors with gemcitabine alone had no effect on CSC frequency, whereas anti-DLL4 treatment alone or in combination with gemcitabine decreased CSC frequency in gemcitabine-resistant tumors (Fig. 4F). The CSC frequency in treated tumors was similar to that of the parental and non–drug-selected xenografts. Collectively, these findings indicated that anti-DLL4 was efficacious against gemcitabine-resistant pancreatic tumors and reestablished the resistant tumors to gemcitabine inhibiting tumor growth and reduced CSC frequency.

Anti-DLL4 modulates expression of EMT markers

EMT is an important process in embryonic development and the transition of epithelial to mesenchymal phenotype plays an important role in tumor progression, metastasis, resistance to chemotherapy, and generation of cancer cells with stem cell–like characteristics in a number of tumor types including pancreatic cancer (41–45). To determine if the efficacy of anti-DLL4 in pancreatic cancer was associated with an effect on inhibiting EMT, we examined the expression of various EMT markers by Q-PCR. We observed that treatment with gemcitabine increased expression of several mesenchymal-related genes (CDH2, FN1, SNAI1, SLUG, TWIST1, TWIST2, ZEB1, ZEB2, and VIM), whereas decreased the epithelial-associated gene E-cadherin (CDH1) in OMP-PN8 xenograft tumors. The induction of expression of these mesenchymal marker genes was suppressed by anti-DLL4 (Fig. 5A). Notably, anti-DLL4 treatment or the combination with gemcitabine produced a 2-fold increase in the epithelial-associated gene CDH1. Similar findings were observed in OMP-PN4 and OMP-PN17 xenograft tumors, where gemcitabine induced mesenchymal gene expression and anti-DLL4 reversed or suppressed these marker genes (Supplementary Fig. S4). Histologic analysis in OMP-PN8 tumors confirmed that gemcitabine increased vimentin (VIM) and ZEB1 expression (Fig. 5B). On the other hand, treatment with anti-DLL4 reversed the gemcitabine-induced increase in VIM expression in the tumor cells, decreased ZEB1 expression in the tumor and stromal cells, whereas increasing CDH1 and cytokeratin expression in the tumor cells (Fig. 5B). To determine the contribution of anti-hDLL4 and anti-mDLL4 to EMT process, in a separate experiment, we examined EMT-related gene expression following anti-human and anti-mouse DLL4 treatment in OMP-PN7 xenograft tumors. We found that while gemcitabine increased mesenchymal-related gene expression, targeting tumor cells by anti-hDLL4 reversed gemcitabine-induced gene changes, suppressed ZEB1 and VIM gene expression, and increased CDH1 expression. On the other hand, targeting mouse stroma/vasculature by anti-mDLL4 had no effect on EMT gene expression in tumor cells compared with control mAb-treated tumors. Treatment with the mixture of both anti-hDLL4 and anti-mDLL4 also showed an effect in reducing expression of EMT markers (Supplementary Fig. S4). Thus, anti-DLL4 as a single agent or in combination with gemcitabine generally repressed expression of EMT markers. These data are consistent with the literature reports suggesting that acquisition of EMT contributes to drug resistance in pancreatic cancer (41, 44, 45). Our findings suggest that anti-DLL4 treatment inhibits EMT, which may contribute to the mechanism underlying the activity of anti-DLL4 in combination with gemcitabine in reducing tumor growth and tumor initiating cell frequency.

Discussion

In this report, we have found that anti-DLL4 has broad spectrum activity in pancreatic xenografts based on testing a panel of patient-derived tumor models. In vivo studies showed that anti-DLL4 was efficacious, alone and in combination with gemcitabine, in reducing tumor growth in all pancreatic tumors tested, including both low- and high-grade tumors. Anti-DLL4 treatment had a potent effect in delaying tumor recurrence after gemcitabine treatment. Furthermore, anti-DLL4 was found to reduce tumor initiating cell frequency as a single agent and in combination with gemcitabine. Agents that reduce CSC frequency have the potential to provide significant clinical benefit by reducing tumor recurrence after therapy and by inhibiting the metastatic spread of the disease. In contrast to anti-DLL4, treatment with gemcitabine alone was ineffective at reducing CSC frequency. While gemcitabine is a standard agent for treatment of pancreatic cancer, the effect of gemcitabine on survival has been disappointing because of low overall
response rates and the lack of durable responses. It has been proposed that tumor initiating cells are relatively more resistant to conventional chemotherapeutic agent and our data are consistent with this hypothesis and with several other reports (15–20). Given the urgent need for more effective therapeutics for pancreatic cancer patients, these findings suggest that targeting DLL4/Notch signaling may provide clinical benefit in this disease setting.

Inhibiting DLL4 is known to have a dual mechanism of action in blocking tumor growth, disrupting productive angiogenesis (29, 30) and targeting tumor cells directly (31, 32). To dissect the mechanism of action of anti-DLL4 in pancreatic cancer, we used species-specific antibodies that we have developed to block DLL4 in either the human tumor cells or in the murine stroma/vasculature cells in our xenograft models. Our results indicated that anti-hDLL4 regulated Notch target genes in the tumor cells but not the stroma, whereas anti-mDLL4 regulated Notch target genes and the endothelial cell marker gene CD31 in the mouse stromal tissues but had no effect on the expression of these genes in tumor cells. The effect of anti-DLL4 on hyperproliferation of endothelial cells in pancreatic tumor was further confirmed by immunofluorescent staining with anti-CD31 (Supplementary Fig. S5). In addition, either anti-mDLL4 or anti-hDLL4 had an effect in reducing tumor regrowth after gemcitabine treatment. Simultaneous treatment with both anti-mDLL4 and anti-hDLL4 resulted in an additive effect, greater than the efficacy observed with either antibody alone. These results indicate that both mechanisms previously ascribed to anti-DLL4 are involved in the antitumor efficacy in pancreatic cancer and with the delay of tumor recurrence. It will be very interesting to study the mechanisms involved in tumor progression during the “maintenance” phase of anti-DLL4 treatment. It is not clear if these tumors have developed “resistance” to anti-DLL4 or gemcitabine and would be insensitive to another round of treatment or if these progressing tumors would respond to further treatment. Future experiments will address this

Figure 5. Effect of anti-DLL4 and gemcitabine on EMT marker expression. OMP-PN8 tumors were harvested after treatment with either a control antibody, anti-DLL4, gemcitabine, or the combination of anti-DLL4 plus gemcitabine. A, mRNA expression. B, histologic analysis. RNA was prepared from tumors and analyzed by Q-PCR for the expression of the indicated genes. Gene expression levels were normalized to the control mAb-treated group. Data are expressed as mean ± SD, n = 4 replicates per treatment group. *, P < 0.05 versus control mAb. Histologic images were taken at ×20 magnification. ZEB1 costained with DAPI and cytokeratin-fluorescein isothiocyanate to show ZEB1 expression in tumor and stromal cells.
important question. To investigate the question of whether targeting DLL4 in tumor cells or in the vasculature had an effect on tumor initiating cell frequency, we carried out serial transplant experiments after treatment with either anti-hDLL4 or anti-mDLL4. We found that that treatment with anti-hDLL4 had an effect on reducing the ability of cells to reinitiate tumor formation after transplantation of cells into new hosts. In contrast, treatment with anti-mDLL4 did not have a significant effect on tumorigenicity. Similarly, we found no significant effect on pancreatic cancer cell tumorigenicity after treatment with the angiogenic agent bevacizumab (anti-VEGF). Bevacizumab has been found to have limited efficacy when combined with gemcitabine in improving survival in pancreatic cancer clinical testing (46). Thus, we envision that the CSC-related mechanism of anti-DLL4 may prove to be critical as to whether this therapy is ultimately found to be efficacious in the clinical management of pancreatic cancer.

The Notch signaling pathway plays a critical role in pancreatic development and maintenance of adult pancreatic tissues (22). Previous studies have suggested the involvement of Notch signaling in regulation of pancreatic progenitor cells to exocrine or endocrine cell fates (23, 24). The Notch target gene HES1 functions to repress the expression of NGN3 and other endocrine genes, thereby impairing the ability of pancreatic precursors to differentiate into the endocrine lineage (22). The observed increase in expression of NGN3, PAX4, and CHGA along with the decrease in HES1 after anti-DLL4 treatment suggests that blockade of DLL4-Notch signaling promotes pancreatic tumor cell differentiation toward cells expressing markers of the endocrine lineage. In our experiments, anti-DLL4 treatment was associated with upregulation of ATOH1 gene. ATOH1 is important in differentiation of intestinal progenitor cells to secretory goblet cells and has recently been shown to have tumor suppressor functions in colorectal cancer (47). Thus, our findings suggest that blocking DLL4-Notch signaling may result in the conversion of pancreatic cancer cells from a stem/progenitor-like state to a more differentiated cell fate. Furthermore, these results suggest that promoting differentiation can be correlated with an increase in sensitivity to cytotoxic chemotherapy and a reduction in tumor initiating cell frequency.

Many lines of evidence link the process of EMT with drug resistance, metastasis, and acquisition of stem-like properties in tumor cells (41–43). Our data indicate that in addition to promoting gene expression indicative of pancreatic cell differentiation, anti-DLL4 represses expression of various markers associated with EMT. The inhibition of EMT by anti-DLL4 may contribute its effects in sensitizing tumor cells to chemotherapy and inhibiting metastasis. The correlation of a reduction in tumor initiating cell frequency and an effect on blocking EMT are consistent with the proposal that EMT plays a significant role in the generation of CSCs (43).

In the present study, we used patient-derived xenograft tumor models for our antibody evaluation. A major advantage of patient-derived xenografts to cell lines is that these xenografts retain much of the cellular heterogeneity of the original tumor. For these experiments, we chose to quantify the effect of anti-DLL4 on tumor initiating cell frequency by carrying out in vivo serial transplantation of cells from treated tumors. This methodology assures a representation of all of the human tumor cells in the xenograft without subdividing the cells. This functional tumorigenicity assay makes no assumptions about the frequency, FACS marker profile, or heterogeneity of the tumor initiating cell population and facilitates the analysis of a diverse panel of tumors. Garrido-Laguna and colleagues have recently described the utility of patient-derived pancreatic cancer tumor models in predicting response to gemcitabine in the clinic (48). It was observed in their study that despite treatment with very high doses of gemcitabine (100 mg/kg, administered 3 times per week) many of the tumors were not sensitive to gemcitabine. In contrast, in our experiments, the tumors were generally sensitive to lower doses of gemcitabine. Although it is difficult to discern the reasons for the discrepancy in 2 independent studies as different sets of patient-derived tumors were used, one potential explanation is the difference in the strain of mice used. Our experiments were carried out in NOD/SCID mice, whereas Garrido-Lugana and colleagues used athymic nude mice. Because of the importance of studying our therapeutics in the context of pancreatic tumors with a poor response to gemcitabine, we developed a gemcitabine resistant xenograft model and found that anti-DLL4 has single agent and combination activity and reduced CSC frequency in this gemcitabine-resistant model (Fig. 4E and 4F).

Our findings provide evidence supporting the rationale for targeting DLL4-Notch signaling as a new therapeutic approach in pancreatic cancer treatment. Our data indicate that DLL4 antagonism inhibits tumor growth through multiple mechanisms including a reduction in tumor initiating cell frequency. Through the use of species-specific antibodies, we can largely attribute the effect on tumor initiating cells in our xenograft experiments to targeting DLL4 expressed on human tumor cells. The evidence for this is based on the fact that anti-hDLL4, but not anti-mDLL4, was found to inhibit in vivo tumorigenicity of pancreatic tumor cells after serial transplantation. Interestingly, bevacizumab also had minimal effects on pancreatic cell tumorigenicity as shown by in vivo serial transplantation. Consistent with the in vivo activity of anti-hDLL4, targeting DLL4 directly with anti-hDLL4 inhibited the proliferation of tumor cells in in vitro tumorsphere assays that are carried out in the absence of any stromal cells.

In addition to targeting Notch ligands and receptors on the cell surface, Notch signaling blockade can be achieved through inhibition of γ-secretase that is involved in proteolytic processing of all 4 Notch receptors. However, a major limitation of the therapeutic utility of these agents is induction of gastrointestinal toxicity because of their simultaneous inhibition of all Notch receptors, particularly Notch1 and Notch2, in normal intestinal stem cells. Gastrointestinal toxicity was not observed in our experiments after anti-DLL4 treatment. It has been shown that DLL4 and
DLL1 have redundant functions in the homeostasis of normal intestinal stem cells (49) so that blockade of DLL4 may be compensated for by the presence of DLL1. Our findings have important implications in treatment of pancreatic cancer. OMP-21M18, an anti-hDLL4 antibody, is currently in clinical testing in multiple settings including pancreatic cancer. Our results suggest that anti-DLL4 can be combined effectively with gemcitabine to block tumor growth through multiple mechanisms. In addition to targeting Notch signaling, we and other investigators have recently shown the feasibility of blocking Wnt, hedgehog, or Nodal/activin to reduce pancreatic CSC frequency (50–52). New therapeutic approaches targeting pathways important for CSCs may improve treatment and, ultimately, overall survival in this very challenging disease.

Disclosure of Potential Conflicts of Interest

W.C. Yen, W.C. Yen, M.M. Fischer, V.P. Yeung, C. A. O'Brien, A. M. Kapoun, J. Lewicki, A. Gurney, and T. Hoey are employees and stock holders of OncoMed Pharmaceuticals, Inc. No potential conflicts of interest were disclosed by the other authors.

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24. Mullendore ME, Koorstra JB, Li YM, Oatheraus DJ, Fan X, Henderson CM, et al. Ligand-dependent Notch signaling is involved in tumor growth through multiple mechanisms. In addition to targeting Notch signaling, we and other investigators have recently shown the feasibility of blocking Wnt, hedgehog, or Nodal/activin to reduce pancreatic CSC frequency (50–52). New therapeutic approaches targeting pathways important for CSCs may improve treatment and, ultimately, overall survival in this very challenging disease.

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Acknowledgments

The authors thank many people at OncoMed Pharmaceuticals, Inc. for their contributions to this work. Special thanks to Inkyung Park and Jim Evans for generation of pancreatic xenograft tumor models, and to Kellie Pickell for vivarium support. The authors also acknowledge the support of the Rich Rogel Pancreatic Research Fund for support of the University of Michigan Primary Tumor Xenograft Program.

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Anti-DLL4 Has Broad Spectrum Activity in Pancreatic Cancer Dependent on Targeting DLL4-Notch Signaling in Both Tumor and Vasculature Cells

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*Clin Cancer Res* 2012;18:5374-5386. Published OnlineFirst September 5, 2012.

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