Anti-DLL4 Inhibits Growth and Reduces Tumor Initiating Cell Frequency in Colorectal Tumors with Oncogenic KRAS Mutations

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Running title: Anti-DLL4 is active in KRAS mutant tumors

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ABSTRACT

KRAS mutations are frequent in colorectal cancer (CRC) and are associated with clinical resistance to treatment with the EGFR-targeted monoclonal antibodies. Delta-like 4 ligand (DLL4) is an important component of the Notch signaling pathway and mediates stem cell self-renewal and vascular development. DLL4 inhibition in colon tumor cells reduces tumor growth and stem cell frequency. Considering the need for new drugs to treat colon cancers with oncogenic KRAS mutations, we examined in this study the efficacy of anti-DLL4 antibodies in KRAS mutant tumors in a panel of early passage colon tumor xenograft models derived from patients. Consistent with clinical findings, mutant KRAS colorectal xenograft tumors were insensitive to the EGFR therapeutic antibody cetuximab, while KRAS wild type tumors responded to cetuximab. In contrast, anti-DLL4 was efficacious against both wild type and mutant KRAS colon tumors as a single agent and in combination with irinotecan. Further analysis of mutant KRAS tumors indicated that the anti-DLL4/irinotecan combination produced a significant decrease in colon cancer stem cell frequency while promoting apoptosis in tumor cells. Our findings provide a rationale for targeting DLL4-Notch signaling for improved treatment of CRC patients with activating KRAS mutations.
INTRODUCTION

Epidermal growth factor receptor (EGFR), a member of the HER-ErbB family of receptor tyrosine kinases, plays an important role in modulating cellular proliferation, adhesion, angiogenesis, migration and survival in colorectal cancer (CRC) (1). Agents that are directed against the EGFR signaling cascade have demonstrated significant clinical benefit in CRC; however, the response rate in the overall population is limited to approximately 10% (2, 3), in part due to the complexity of EGFR signaling pathways and alteration of downstream molecules.

KRAS is an intracellular signaling molecule that functions downstream of EGFR and other receptor tyrosine kinases. Oncogenic mutation of KRAS by amino acid substitution at codons 12, 13 and 61 results in constitutively active protein (4). KRAS mutations are found in approximately 40% of colorectal tumors (5). The presence of mutant KRAS in colon tumors correlates with poor prognosis and is associated with treatment resistance to EGFR-targeted monoclonal antibodies cetuximab or panitumumab (6, 7, 8, 9). These clinical findings have led the European Medical Agency (EMEA) to restrict the use of anti-EGFR antibodies to CRC patients with wild type KRAS. Similarly, based on a comprehensive review of the relevant literature, the American Society of Clinical Oncology released a Provisional Clinical Opinion recommending that metastatic CRC patients with a KRAS mutation in codons 12 or 13 should not receive anti-EGFR antibody therapy (10). Therefore, there is a critical need for new therapies to treat the large segment of CRC patients with activating KRAS mutations.
Accumulating evidence has indicated that epithelial tumors, such as colorectal cancer, are frequently composed of heterogeneous cell types that vary in their ability to initiate new tumor growth and that cancer stem cells (CSCs, also referred to as tumor initiating cells) drive tumor growth and progression and are preferentially resistant to many current therapies (11). The Notch pathway has been shown to be involved in the development of normal tissues and is frequently dysregulated in cancer; in particular, Notch signaling is known to play an important role in normal colon development and in colon cancer (12, 13). Delta-like 4 ligand (DLL4) is an important component of Notch-mediated stem cell self-renewal and vascular development. Inhibition of DLL4 has been shown to result in broad spectrum of anti-tumor activity due to dysfunctional angiogenesis (reviewed in 14). We identified another mechanism of action of anti-DLL4 directly acting on tumor cells and reducing cancer stem cell frequency in colon and breast tumors (15). In the present study, we evaluated the anti-tumor effect of anti-DLL4 on colorectal tumors bearing oncogenic KRAS mutations using colorectal human tumor xenograft models derived from patient samples.
MATERIALS AND METHODS

Antibodies. The generation of anti-human and anti-mouse DLL4 antibodies were described previously (15).

Mutation Analysis. The purified genomic DNA sequence data was obtained by amplifying genomic DNA samples with the Repli-G Mini Kit (Qiagen, Valencia CA) followed by PCR amplification, purification and sequencing with the ABI 3730xL DNA Sequencer (Applied Biosystem, Foster City, CA).

In Vivo Animal Studies. The establishment and characterization of colon xenograft models from patient specimens was described previously (15). All xenograft tumors were generated at OncoMed Pharmaceuticals, Inc. except UM-C4, which was obtained from the University of Michigan. Information on tissue origin and histopathologic diagnosis was evaluated by pathologists. Frozen cells or freshly dissociated single cells (see details in Supplemental Materials and Methods section for preparation of single cell suspension) were injected subcutaneously to NOD/SCID mice for efficacy studies. Treatment started when the mean tumor volumes reached about 100 mm³. Irinotecan (7.5 mg/kg), cetuximab (10 mg/kg) and anti-DLL4 antibody (1:1 mixture of anti-human and anti-mouse DLL4 antibodies at 10 mg/kg final concentration) was administered intraperitoneally once a week throughout the course of study.

In Vivo Limiting Dilution Assay. The procedures for tumorigenicity study were described previously (15) and detailed in Supplemental Materials and Methods section.
Quantitative RT-PCR. Tumor RNAs were isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA) with DNaseI treatment. Total RNA (about 0.5 μg) was reverse transcribed into cDNA. Quantitative real-time PCR was performed in an ABI 7900 HT Fast Real Time PCR System and analyzed using the SDSv2.3 (Applied Biosystems, Foster City, CA). The results were normalized with the housekeeping gene GADPH. All primer and probe sets were obtained from Applied Biosystems (Foster City, CA).

Immunohistochemistry. FFPE Sections were cut 4-5-μm thick. Immunohistochemistry was performed by dewaxing sections, performing HIER using pH6 buffer in a pressure cooker, blocking for endogenous peroxidase and then overnight (PBS, 10% horse serum, 1% BSA, 0.1% Tween-20). The primary antibodies used in this study included anti Ki-67 mAb, clone MIB-1 (Dako, Carpinteria, CA), and anti-cleaved caspase-3, clone FA1E (Cell Signaling Technologies, Beverly, MA). Slides were visualized with HRP conjugated antibodies using Nova Red substrate (Vector Laboratories, Burlingame, CA), counterstained with Hematoxylin.

Flow Cytometric Apoptosis Assays. Single cell suspensions depleted of mouse cells (cell isolation described in Supplemental Materials and Methods) from control and treated tumors were fixed and assayed for active caspase-3 (FITC Active Caspase-3 Apoptosis Kit, BD Biosciences, San Jose, CA), and co-stained with an APC conjugated EpCAM mAb (Miltenyi Biotec, Auburn, CA); or by the TUNEL method, APO-DIRECT Kit (BD Biosciences), per manufacturers protocol. Events were acquired on a BD FACS ARIA II Flow Cytometer and analyzed on BD FACSDiva.
Protein Preparation, ELISA, and Immunofluorescence Assays. See supplemental data.

Data Analysis. In Vivo data is expressed as mean±SEM. Differences in mean values between groups were analyzed by two-way ANOVA with repeated measures followed by Bonferroni post tests. Ex Vivo data is expressed as mean±SD. Differences in mean values between groups were analyzed by one-way ANOVA with repeated measures followed by post tests. Differences of p<0.05 are considered significantly different. Statistical analysis was performed with GraphPad Prism5 (GraphPad Software Inc., San Diego, CA).
RESULTS AND DISCUSSION

Sensitivity of KRAS wild-type and mutant colorectal xenograft tumors to cetuximab. Based on sequence analysis, a number of colorectal xenograft tumors with wild type KRAS, KRAS\textsuperscript{WT} (OMP-C8 and -C40) and mutant KRAS, KRAS\textsuperscript{MT} (UM-C4, OMP-C9, -C12 and -C22) were identified (Table S1). We next evaluated the effect of anti-EGFR antibody cetuximab on the growth of KRAS\textsuperscript{WT} and KRAS\textsuperscript{MT} colorectal xenograft tumors. As seen in Figure 1A and Table 1, cetuximab was efficacious against KRAS\textsuperscript{WT} OMP-C8 and C40 colon tumors, reducing tumor volume to about 50% compared to control mAb-treated tumors. In contrast, cetuximab was ineffective in all four KRAS\textsuperscript{MT} colon tumors examined (Figure 1B and Table 1). There was no correlation between cetuximab sensitivity and EGFR protein expression (data not shown). For example, both KRAS\textsuperscript{WT} OMP-C40 and KRAS\textsuperscript{MT} UM-C4 expressed similar EGFR protein levels; while KRAS\textsuperscript{WT} OMP-C40 tumor was sensitive to cetuximab-mediated growth inhibition, KRAS\textsuperscript{MT} UM-C4 was non-responsive to cetuximab. Our findings were consistent with clinical analysis demonstrating no association between EGFR expression and response to cetuximab (16). The antagonist activity of cetuximab was validated in xenografts, as shown by the decrease of EGFR expression and signaling (Supplemental Figure 1A). The lack of a growth inhibitory effect against KRAS\textsuperscript{MT} colon tumors by cetuximab was likely due to the inability of cetuximab to inhibit EGFR signaling in the presence of a constitutively active RAS protein downstream of the receptor (Supplemental Figure 1B). These data support the current hypothesis that an oncogenic KRAS mutation but not EGFR expression correlates with cetuximab sensitivity. Furthermore, these findings indicate that we were able to recapitulate the
efficacy of anti-EGFR inhibitors observed clinically in pre-clinical models using primary colorectal xenograft tumors.

Irinotecan is a standard-of-care agent for the treatment of CRC. In combination with cetuximab it has demonstrated an improved progression-free survival, and overall survival in some cases, in wild-type but not in mutant KRAS tumors (17). We sought to evaluate the anti-tumor effect of this combination in the primary colorectal xenograft tumors mentioned above. Our data indicated that irinotecan was efficacious as a single agent against both KRAS\textsuperscript{WT} and KRAS\textsuperscript{MT} colorectal xenograft tumors. However, the combination of irinotecan with cetuximab did not demonstrate a significant additive effect compared to single agent irinotecan in all tumors examined except in KRAS\textsuperscript{WT} OMP-C40, where the combination produced a statistically significant greater anti-tumor effect than either agent used alone. In KRAS\textsuperscript{MT} OMP-C12, combining cetuximab with irinotecan resulted in reversing the anti-tumor response of irinotecan alone. Our preclinical findings were consistent with literature reports where the combination of irinotecan and cetuximab provides no clinical benefits for KRAS\textsuperscript{MT} CRC patients (16).

Effect of anti-DLL4 antibody on growth of KRAS wild-type and mutant colorectal xenograft tumors. Based on the above findings, we next evaluated whether there was a therapeutic benefit of combining the anti-DLL4 antibodies with irinotecan in KRAS\textsuperscript{WT} and in KRAS\textsuperscript{MT} tumors. As seen in Table 1 and Figures 1A and 1B, anti-DLL4 was efficacious as a single agent in KRAS\textsuperscript{WT} tumors and two of the four KRAS\textsuperscript{MT} tumors, OMP-C9 and OMP-C12.
In contrast to cetuximab, anti-DLL4 plus irinotecan produced a statistically significant anti-tumor effect greater than irinotecan alone in all six tumors evaluated and this activity was similar in KRAS\textsuperscript{WT} and KRAS\textsuperscript{MT} tumors. Histological analysis indicated that treatment with anti-DLL4 reduced the frequency of proliferative cells and induced hyperproliferation of tumor vasculature in both classes of CRC tumors (Figure S2). Furthermore, the combination of anti-DLL4 and irinotecan resulted in tumor regression at the end of the study in four of these six tumors irrespective of KRAS status. Taken together, the above findings indicated that anti-DLL4, alone and in combination with irinotecan, was efficacious against KRAS\textsuperscript{WT} and KRAS\textsuperscript{MT} CRC xenograft tumors.

**Effect of anti-DLL4 antibody on KRAS mutated colon cancer stem cell frequency.**
It has been reported that CSCs are preferentially resistant to many standard therapies and that CSCs mediate tumor recurrence following such treatments (18). We previously demonstrated that anti-DLL4 in combination with chemotherapeutic agents reduced cancer stem cell frequency in colon and breast xenograft tumors (15). Our previous studies on colon CSCs were carried out with OMP-C8 tumors which are wild type for KRAS. To extend these findings and to determine whether the anti-DLL4-mediated growth inhibitory effect in KRAS\textsuperscript{MT} colon xenograft tumors is associated with an effect on tumor initiating cells, we evaluated the effect of anti-DLL4 on CSC frequency by serial *in vivo* transplant, limiting dilution assay (19). This functional assay measures *in vivo* tumorigenicity and makes no assumptions about the frequency, FACS marker profile, or heterogeneity of the tumor initiating cell population. KRAS\textsuperscript{MT} OMP-C9 tumors were harvested at the end of 4-week treatment from Figure 1B. As seen in Figure S3...
and summarized in Figure 1C, control mAb treated tumors had a CSC frequency of 1/149. Irinotecan treatment had no significant effect on CSC frequency, slightly increasing the frequency to 1/105. On the other hand, anti-DLL4 treatment alone decreased cancer stem cell frequency by 2-fold vs. control mAb- treated tumors. Importantly, the combination of anti-DLL4 and irinotecan produced a further decrease in tumor initiating cell frequency, about 4-fold lower than the control and irinotecan-treated group and 2-fold lower than the anti-DLL4 treated tumors.

To investigate the mechanism of action of anti-DLL4 in KRASMT tumors, gene expression analyses were carried out. These analyses indicated that anti-DLL4 treatment altered gene expression indicative of inhibiting Notch signaling and promoting colon cell differentiation (ATOH1 and CHGA) in OMP-C9 KRASMT tumors in a similar manner as in OMP-C8 KRASWT tumors (15). In UM-C4 tumors where the combination treatment induced tumor shrinkage, irinotecan increased expression of the anti-apoptotic gene HSPA6 while it decreased levels of the pro-apoptotic gene PDCD4 (Figure 2A). The combination of anti-DLL4 and irinotecan down-regulated the induction of HSPA6 and up-regulated the expression of PDCD4 relative to irinotecan alone, potentially leading to sensitization of tumor cells to cell death and tumor regression and providing insight into the mechanism for the synergy of these agents. To further confirm that anti-DLL4/irinotecan-induced apoptotic gene changes lead to cell death, a separate experiment was conducted in UM-C4 xenograft tumors. Apoptosis was analyzed by flow cytometric and immunohistochemical analyses. We observed an increase in caspase-3 activity (Figure 2B and S4A) and a corresponding elevation in late-stage
apoptotic DNA fragmentation by anti-DLL4 and irinotecan combination (Figure 2C). The apoptotic induction by the combination therapy was correlated with a decrease in cell proliferation marker Ki67 (Figure S4B).

In summary, our findings provide a rationale for targeting cancer stem cells and tumor vasculature through inhibiting DLL4 as a new therapeutic approach in the treatment of CRC. In contrast to anti-EGFR, anti-DLL4 was equally efficacious in both KRAS\textsuperscript{WT} and KRAS\textsuperscript{MT} tumors. Our findings suggest that targeting DLL4 may improve the efficacy of current treatments for the large segment of colon cancer patients with oncogenic KRAS mutations.
Disclosure of Potential Conflicts of Interest

The authors are employees and stock holders of OncoMed Pharmaceuticals, Inc.

Acknowledgements

We would like to thank all of our colleagues at OncoMed for their important contributions to this work particularly InKyung Park, Xiaomei Song, Kellie Pickell, Jim Evans, Satyajit Mitra and Jorge Monteon.
LEGENDS TO FIGURES

Figure 1. Effect of cetuximab or anti-DLL4 in combination with irinotecan on colorectal xenograft tumor growth and colon cancer stem cell frequency. Xenograft growth curves from KRASWT OMP-C40 (A), and KRASMT OMP-C9 (B), mean±SEM, n=7 to 10 xenografts per group, *: p<0.05 vs. control mAb; **: p<0.05 vs. single agents by two-way ANOVA followed by Bonferroni post tests. (C) Cancer stem frequency as determined by in vivo limiting dilution assay (See description in Supplemental Materials and Methods and Figure S3), n=10 xenografts per cell dose, four cell doses per treatment group, mean±SEM.

Figure 2. Effect of anti-DLL4 on gene expression and apoptosis induction in KRASMT colorectal xenograft tumors. Gene expression by qRT-PCR in OMP-C9 and UM-C4 (A). Target gene expression was normalized to housekeeping gene GAPDH. The relative quantity of each gene was then compared with and normalized to the control mAb group, mean±SD, n=4 per treatment group, *: p<0.05 vs. control mAb by one-way ANOVA followed by Tukey’s post tests. Flow cytometric analysis of active caspase 3 (B) and DNA fragmentation (TUNEL assay) (C) in UM-C4 xenografts isolated into single cell suspension depleted of mouse cells on day 19. Mean and scatter plot, n=5 xenografts per group, *: p<0.05 vs. control mAb by one-way ANOVA followed by Tukey’s post tests.
Table 1. Effect of anti-DLL4 or cetuximab in combination with irinotecan on growth of CRC xenograft tumors. Data expressed as mean±SEM, n=7-10 xenograft tumors per group. *: p<0.05 vs. control mAb; †: p<0.05 vs. antibody alone; §: p<0.05 vs. irinotecan alone. Two-way ANOVA followed by Bonferroni post tests.

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19. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 1997;89:3919-24.
Figure 1
Figure 2

A

KRAS\textsuperscript{MT} OMP-C9

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KRAS\textsuperscript{MT} UM-C4

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Cancer Res Published OnlineFirst December 30, 2010.

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