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

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Abstract

KRAS mutations are frequent in colorectal cancer (CRC) and are associated with clinical resistance to treatment with the epidermal growth factor receptor (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, whereas *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. *Cancer Res; 71(5); 1–6. ©2010 AACR.*

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; ref. 1). Agents that are directed against the EGFR signaling cascade have showed 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 (6–9). 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 CRC, 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 antitumor activity due to dysfunctional angiogenesis (reviewed in ref. 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

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antitumor 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 was described previously (15).

Mutation analysis

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

In vivo animal studies

The establishment and characterization of colon xenograft models from patient specimens were described previously (15). All xenograft tumors were generated at OncoMed Pharmaceuticals, Inc. except UM-C4, which was obtained from the University of Michigan. Tissue samples were provided by the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. Information on tissue origin and histopathologic diagnosis was evaluated by pathologists. Frozen cells or freshly dissociated single cells (see details in Supplementary 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 mm^3. 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) were 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 Supplementary Materials and Methods section.

Quantitative real-time PCR

Tumor RNAs were isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen) with DNAse I treatment. Total RNA (about 0.5 μg) was reverse transcribed into cDNA. Quantitative real-time (RT) PCR was done in an ABI 7900 HT Fast Real-Time PCR System and analyzed using the SDS software v2.3 (Applied Biosystems). The results were normalized with the housekeeping gene GADPH. All primer and probe sets were obtained from Applied Biosystems.

Immunohistochemistry

Formalin-fixed, paraffin-embedded (FFPE) sections were cut 4–5 μm thick. Immunohistochemistry was done 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, and 0.1% Tween-20).

The primary antibodies used in this study included anti Ki-67 mAb, clone MIB-1 (Dako), and anti-cleaved caspase-3, clone FA1E (Cell Signaling Technologies). Slides were visualized with horseradish peroxidase (HRP)-conjugated antibodies using Nova Red substrate (Vector Laboratories), counterstained with hematoxylin.

Flow cytometric apoptosis assays

Single cell suspensions depleted of mouse cells (cell isolation described in Supplementary Materials and Methods section) from control and treated tumors were fixed and assayed for active caspase-3 (FITC Active Caspase-3 Apoptosis Kit, BD Biosciences), and costained with an APC-conjugated EpCAM mAb (Miltenyi Biotec) or by the TUNEL method, APO-DIRECT Kit (BD Biosciences), per manufacturers protocol. Events were acquired on a BD FACSAria II flow cytometer and analyzed on BD FACSDiva.

Protein preparation, ELISA, and immunofluorescence assays

See Supplementary Data.

Data analysis

In vivo data are 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 are expressed as mean ±SD. Differences in mean values between groups were analyzed by one-way ANOVA with repeated measures followed by Tukey's post tests. Differences of $P < 0.05$ are considered significantly different. Statistical analysis was done with GraphPad Prism5 (GraphPad Software Inc.).

Results and Discussion

Sensitivity of KRAS wild-type and mutant colorectal xenograft tumors to cetuximab

On the basis of sequence analysis, a number of colorectal xenograft tumors with wild-type KRAS, KRAS^{WT} (OMP-C8 and -C40) and mutant KRAS, KRAS^{MT} (UM-C4, OMP-C9, -C12, and -C22) were identified (Supplementary Table S1). We next evaluated the effect of anti-EGFR antibody cetuximab on the growth of KRAS^{WT} and KRAS^{MT} colorectal xenograft tumors. As seen in Fig. 1A and Table 1, cetuximab was efficacious against KRAS^{WT} OMP-C8 and -C40 colon tumors, reducing tumor volume to about 50% compared with control mAb-treated tumors. In contrast, cetuximab was ineffective in all 4 KRAS^{MT} colon tumors examined (Fig. 1B and Table 1). There was no correlation between cetuximab sensitivity and EGFR protein expression (data not shown). For example, both KRAS^{WT} OMP-C40 and KRAS^{MT} UM-C4 expressed similar EGFR protein levels; although KRAS^{WT} OMP-C40 tumor was sensitive to cetuximab-mediated growth inhibition, KRAS^{MT} UM-C4 was nonresponsive 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
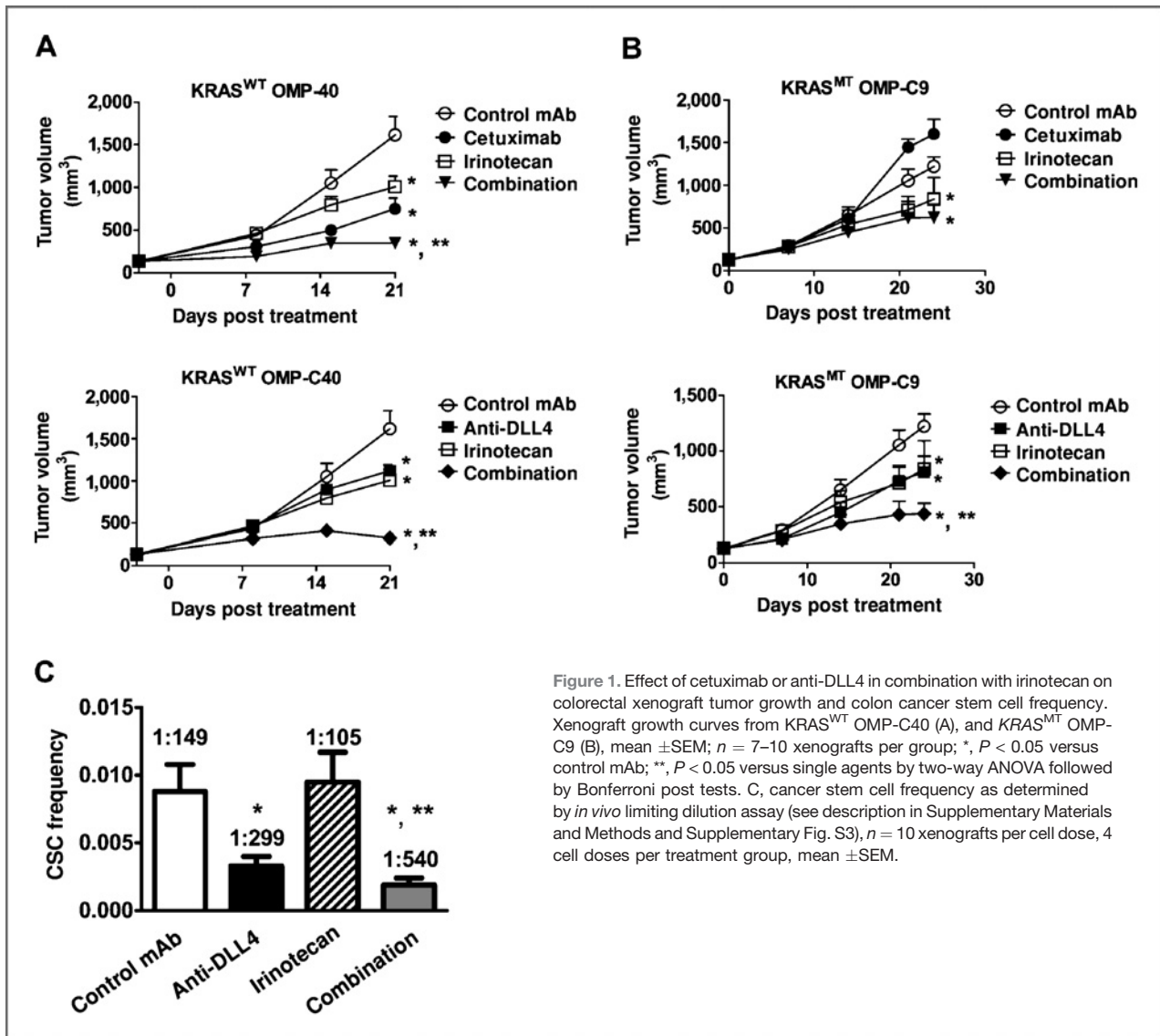
Figure 1. Effect of cetuximab or anti-DLL4 in combination with irinotecan on colorectal xenograft tumor growth and colon cancer stem cell frequency. Xenograft growth curves from KRAS[WT] OMP-C40 (A), and *KRAS*[MT] OMP-C9 (B), mean ±SEM; $n = 7–10$ xenografts per group; *, $P < 0.05$ versus control mAb; **, $P < 0.05$ versus single agents by two-way ANOVA followed by Bonferroni post tests. C, cancer stem cell frequency as determined by *in vivo* limiting dilution assay (see description in Supplementary Materials and Methods and Supplementary Fig. S3), $n = 10$ xenografts per cell dose, 4 cell doses per treatment group, mean ±SEM.

and signaling (Supplementary Fig. 1). The lack of a growth inhibitory effect against KRAS[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 (Supplementary Fig. 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 preclinical models using primary colorectal xenograft tumors.

Irinotecan is a standard-of-care agent for the treatment of CRC. In combination with cetuximab, it has showed 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 antitumor 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[WT] and KRAS[MT] colorectal xenograft tumors. However, the combination of irinotecan with cetuximab did not show a significant additive effect compared with single agent irinotecan in all tumors examined except in KRAS[WT] OMP-C40, where the combination produced a statistically significant greater antitumor effect than either agent used alone. In KRAS[MT] OMP-C12, combining cetuximab with irinotecan resulted in reversing the antitumor 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[MT] CRC patients (16).

Effect of anti-DLL4 antibody on growth of KRAS wild-type and mutant colorectal xenograft tumors

On the basis of the above findings, we next evaluated whether there was a therapeutic benefit of combining the

Table 1. Effect of anti-DLL4 or cetuximab in combination with irinotecan on growth of CRC xenograft tumors.

	Tumor volume reduction,% of control mAb				
KRAS[WT]	**Anti-DLL4**	**Cetuximab**	**Irinotecan**	**Anti-DLL4 + Irinotecan**	**Cetuximab + Irinotecan**
OMP-C8	25 ± 12[a]	43 ± 11[a]	48 ± 8[a]	88 ± 2[a,b,c]	55 ± 6[a]
OMP-C40	31 ± 4[a]	54 ± 8[a]	38 ± 8[a]	79 ± 3[a,b,c]	79 ± 2[a,b,c]
KRAS[MT]	**Anti-DLL4**	**Cetuximab**	**Irinotecan**	**Anti-DLL4+ Irinotecan**	**Cetuximab + Irinotecan**
UM-C4	19 ± 12	18 ± 10	58 ± 5[a]	87 ± 2[a,b,c]	62 ± 8[b]
OMP-C9	34 ± 10[a]	-31 ± 17	31 ± 10[a]	64 ± 9[a,b,c]	49 ± 3[b]
OMP-C12	48 ± 5[a]	2 ± 8	49 ± 3[a]	75 ± 2[a,b,c]	17 ± 8[b,c]
OMP-C22	4 ± 5	-14 ± 16	54 ± 5[a]	90 ± 2[a,b,c]	57 ± 5[b]

NOTE: Data expressed as mean $\pm$SEM, $n = 7–10$ xenograft tumors per group, and Two-way ANOVA followed by Bonferroni post tests.
[a]$P < 0.05$ versus control mAb.
[b]$P < 0.05$ versus antibody alone.
[c]$P < 0.05$ versus irinotecan alone.

anti-DLL4 antibodies with irinotecan in KRAS[WT] and in KRAS[MT] tumors. As seen in Table 1 and Fig. 1A and B, anti-DLL4 was efficacious as a single agent in KRAS[WT] tumors and 2 of the 4 KRAS[MT] tumors, OMP-C9 and OMP-C12.

In contrast to cetuximab, anti-DLL4 plus irinotecan produced a statistically significant antitumor effect greater than irinotecan alone in all 6 tumors evaluated and this activity was similar in KRAS[WT] and KRAS[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 (Supplementary Fig. S2). Furthermore, the combination of anti-DLL4 and irinotecan resulted in tumor regression at the end of the study in 4 of these 6 tumors, irrespective of KRAS status. Taken together, the above findings indicated that anti-DLL4, alone and in combination with irinotecan, was efficacious against KRAS[WT] and KRAS[MT] CRC xenograft tumors.

Effect of anti-DLL4 antibody on *KRAS*-mutated colon cancer stem cell frequency

It has been reported that cancer stem cells (CSCs) are preferentially resistant to many standard therapies and that CSCs mediate tumor recurrence following such treatments (18). We previously showed that anti-DLL4 in combination with chemotherapeutic agents reduced CSC 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[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[MT] OMP-C9 tumors were harvested at the end of 4-week treatment as shown in Fig. 1B. As seen in Supplementary Fig. S3 and summarized in Fig. 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 versus 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 KRAS[MT] 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 KRAS[MT] tumors in a similar manner as in OMP-C8 KRAS[WT] tumors (15). In UM-C4 tumors where the combination treatment induced tumor shrinkage, irinotecan increased expression of the antiapoptotic gene *HSPA6*, whereas it decreased levels of the proapoptotic gene *PDCD4* (Fig. 2A). The combination of anti-DLL4 and irinotecan downregulated the induction of *HSPA6* and upregulated 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 (Fig. 2B and Supplementary Fig. S4A) and a corresponding elevation in late-stage apoptotic DNA fragmentation by anti-DLL4 and irinotecan combination (Fig. 2C). The apoptotic induction by the combi-

Figure 2. Effect of anti-DLL4 on gene expression and apoptosis induction in KRAS^{MT} 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; *, $P < 0.05$ versus 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; *, $P < 0.05$ versus control mAb by one-way ANOVA followed by Tukey's post tests.
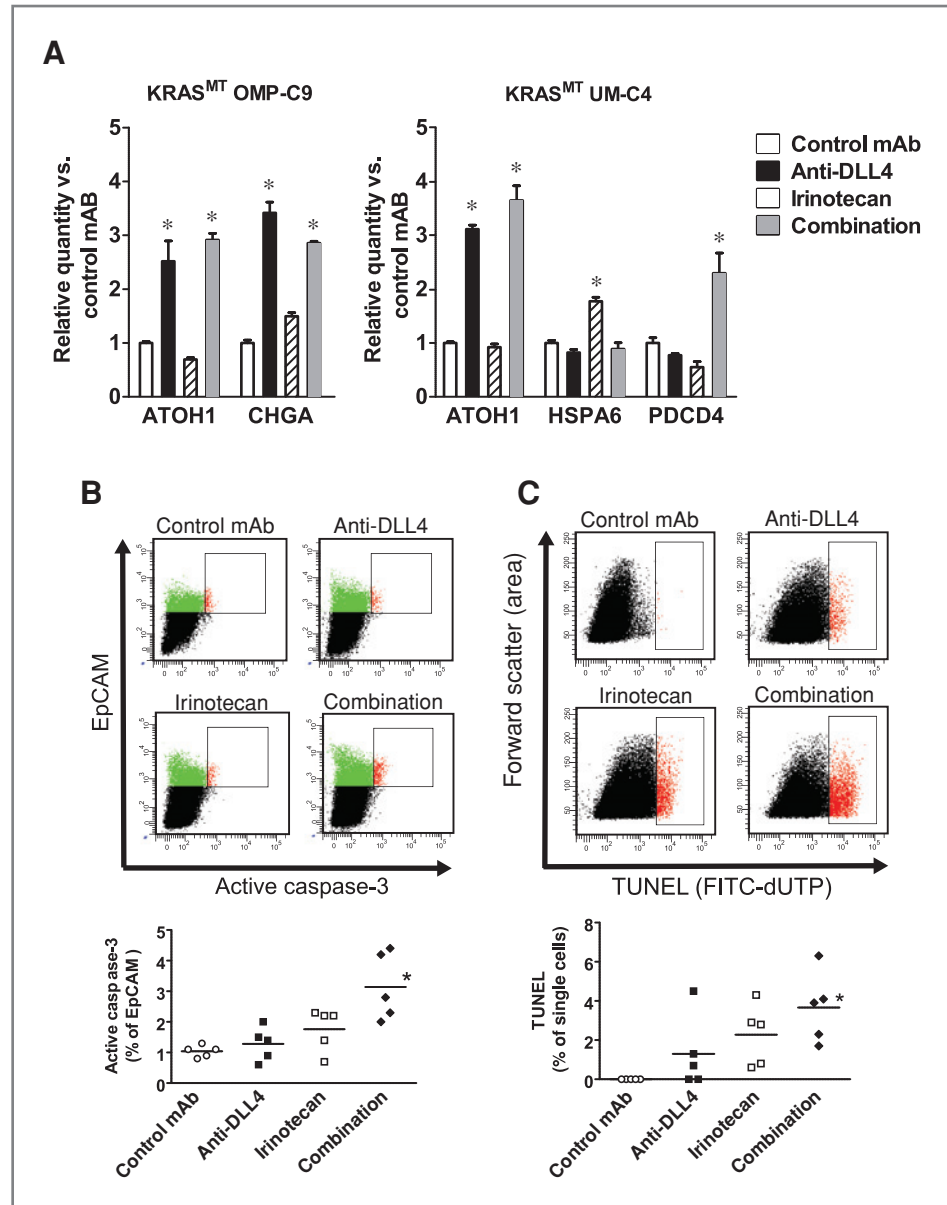
nation therapy was correlated with a decrease in cell proliferation marker Ki67 (Supplementary Fig. S4B).

In summary, our findings provide a rationale for targeting CSCs 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^{WT} and KRAS^{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.

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