EphA2 receptor activation with ephrin-A1 ligand restores cetuximab efficacy in NRAS-mutant colorectal cancer cells

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Abstract. Patients with wild-type KRAS metastatic colorectal cancer (mCRC) that harbors NRAS activating mutations do not benefit from anti-EGFR therapies. Very little is known about oncogenic NRAS signaling driving mCRC unresponsiveness to the EGFR-directed antibody cetuximab. Using a system of paired NRAS-mutant and wild-type isogenic mCRC cell lines to explore signaling pathways engaged by the common oncogenic NRAS Q61K variant upon challenge with cetuximab, we uncovered an unexpected mechanism of resistance to cetuximab involving dysregulation of the ephrin-A1/EphA2 signaling axis. Parental NRAS+/+ cells, but not NRASQ61K/+ cells, activated the ephrin receptor ephA1 in response to cetuximab treatment. Moreover, whereas cetuximab treatment significantly downregulated EPHA2 gene expression in NRAS+/+ cells, EPHA2 expression in NRAS Q61K/+ cells was refractory to cetuximab. Remarkably, pharmacologically mimicked ephrin-A1 engagement to ephA2 receptor converted NRAS-mutant into RAS wild-type mCRC cells in terms of cetuximab efficacy. Accordingly, activation of the ephA2 receptor by bioactive recombinant human ephrin-A1/Fc-fusion protein suppressed the cetuximab-unresponsive hyperactivation of MAPK and AKT and fully restored cetuximab activity in NRAS-mutant colorectal cells. Collectively, these findings reveal that the clinical benefit of cetuximab in mCRC might necessarily involve the suppression of the ligandless oncogenic signaling of the ephA2 receptor. Hence, ligand-dependent tumor suppressor signaling using therapeutic ephA2 agonists might offer new therapeutic opportunities to clinically widen the use of cetuximab in NRAS-mutated and/or ephA2-dependent mCRC tumors.

Introduction

NRAS mutations occur in ~3-5% of metastatic colorectal carcinoma (mCRC) patients and have been associated with lower disease control and response rates to the epidermal growth factor receptor (EGFR)-targeted monoclonal antibody cetuximab (1-8). Because patients with wild-type KRAS mCRC that harbor NRAS activating mutations do not derive benefit from the administration of cetuximab, all major international clinical guidelines recommend restricting its use to mCRC patients with wild-type RAS tumors (6,9,10). Although previous data indicated that NRAS might provide similar or identical oncogenic signals to those of KRAS, as they are not typically found in the same tumor (11,12), accumulating evidence suggests very distinct clinical consequences for the mutually exclusive KRAS- and NRAS-mutant mCRC subsets (13,14). Dissimilar biological consequences for mutations of KRAS and NRAS, which appear to be selected under distinct tumorigenic contexts, underlie their clinical distinction in mCRC patients. Accordingly, NRAS mutations, which appear to arise specifically under settings of continuous exposure to apoptotic stimuli in the context of chronic inflammation, provide a MAPK-related distinct, prosurvival signaling environment that mutational activation of KRAS does not (14).

An important unresolved question arising from the above observation is whether the apparently unique phenotype of mutant NRAS can be exploited as a therapeutic strategy to circumvent the refractoriness to cetuximab. Whereas most studies have focused on investigating the downstream effectors of KRAS signaling for bypassing the response of KRAS-mutant mCRC cells to anti-EGFR therapy, almost nothing is known about the specific pathways employed by NRAS-mutant mCRC cells that render them unresponsive to
cetuximab. Here we used isogenic mCRC cell lines to explore signaling pathways specifically engaged by the most common oncogenic NRAS Q61K variant upon challenge of mCRC cells with cetuximab. We provide evidence for an unexpected deregulation of the erythropoietin-producing hepatocellular (Eph) receptor tyrosine kinase (RTK)/ephrin ligand cell communication system (EphA2/ephrin-A1), which negatively influences cetuximab efficacy in NRAS-mutant mCRC cells.

Materials and methods

Cell lines. The X-MAN™ isogenic cell lines SW48 NRAS-WT (NRAS^wt^) and SW48 NRAS^G61K^ (cat no. HD 103-017), were purchased from Horizon Discovery Ltd. (Cambridge, UK) and maintained following the manufacturer's instructions in RPMI-1640 medium with 2 mmol/l l-glutamine, 25 mmol/l sodium bicarbonate and 10% fetal bovine serum.

Drugs and materials. Cetuximab was provided by the Hospital Universitari de Girona Dr Josep Trueta Pharmacy. Bioactive recombinant human EphrinA1/Fc (EA1-Fc; cat no. 6417-A1) was purchased from R&D Systems (Minneapolis, MN, USA) and dissolved in PBS.

Cell proliferation. Cells were plated in 24-well plates at 5,000 cells/well and incubated for 18 h in a humidified atmosphere containing 5% carbon dioxide at 37°C to allow for attachment, after which a zero-time point was determined. Cells were grown in regular medium with or without 100 µg/ml cetuximab and counted with a Coulter Counter (Coulter Electronics Inc., Hialeah, FL, USA). All assays were performed at least twice in triplicate.

Phospho-proteome profiling. Phospho-receptor screening was performed using Proteome Profiler Human Phospho-RTK array (R&D Systems) according to the manufacturer’s instructions. Densitometry analyses of the scanned phospho-arrays were carried out using Carestream Molecular Imaging Software (Carestream Health, Rochester, NY, USA).

RNA isolation and reverse transcription. Total RNA was extracted from cells using Nucleospin RNA plus kit (Macherey-Nagel GmbH & Co. KG) according to the manufacturer’s instructions. Two micrograms of total RNA was reverse-transcribed into cDNA using High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA concentration and quality were determined in an ND-1000 spectrophotometer (NanoDrop™ ND-1000, NanoDrop Technologies, USA).

Gene expression. cDNA (50 ng) were assayed in triplicate according to established protocols using a QuantStudio™ 7 Flex Real-Time PCR system (Thermo Fisher Scientific) with an automated baseline and threshold cycle detection. GAPDH and ACTB were used as reference genes. Primers and fluorescent probes for EPHA1, EPHA2, EFN1, EFN2, GAPDH, and ACTB were obtained from Thermo Fisher Scientific (TaqMan Gene Expression assays: assay ID Hs00358886-m1, Hs01023290_m1, Hs00178313_m1, Hs00171656-m, Hs99999902_m1, and Hs99999903_m1, respectively). Data were analyzed using the Thermo Fisher Cloud software (Thermo Fisher Scientific).

Real-time cell growth rates. Proliferation was monitored in real time using the xCELLigence RTCA DP Instrument (ACEA Biosciences, San Diego, CA, USA). Cellular growth rate was determined by the slope of the growth curve using the RTCA Software Package 1.2. We conducted the normalization at one-time point before the treatment.

PathScan sandwich immunoassay. The PathScan® Intracellular Signaling array kit (cat no. 7323; Cell Signaling Technology, Danvers, MA, USA) was used as per the manufacturer’s instructions.

Statistical analysis. Data are presented as mean ± SD from at least three independent experiments. Two-group comparisons were performed using Student’s t-test. Comparisons of means of ≥3 groups were performed by ANOVA, and the existence of individual differences tested by Scheffé’s multiple contrasts. P-values <0.01 were considered to be statistically significant. All statistical tests were two-sided.

Results

Heterozygous knock-in of the NRAS activating mutation Q61K is sufficient to allow escape from cetuximab-induced cell growth inhibition. We used an in vitro mCRC model of isogenic pairs of SW48 colon cancer cell lines in which one allele of the endogenous NRAS gene contained a heterozygous knock-in of the c.181C>A activating mutation resulting in an amino acid substitution from glutamine (Q) to lysine (K) at position 61 (NRAS^G61K^). We previously reported that, whereas a strong reduction of cell viability was noted for parental NRAS^wt^ cells cultured in the presence of 100 µg/ml cetuximab, NRAS^G61K^ cells were fully refractory to cetuximab-induced cell viability (15).

NRAS^G61K^ cells fail to activate EphA1 receptor tyrosine kinase in response to cetuximab. We first examined the changes in the phospho-proteome of isogenic NRAS^wt^ and NRAS^G61K^ cells using the commercially available Proteome Profiler Human Phospho-RTK array kit. Phospho-RTK profiling revealed that the SW48-based model of mCRC mostly depends on EGFR signaling to proliferate since EGFR (HER1) was the tyrosine kinase receptor more significantly active among the 42 different phospho-receptor tyrosine kinases included in the array (Fig. 1). Treatment of NRAS^wt^ and NRAS^G61K^ with cetuximab was found to further enhance the phosphorylation/activation status of EGFR (Fig. 1), a phenomenon that was likely due to cetuximab-induced EGFR homodimerization and autophosphorylation as previously reported in non-small-cell lung cancer cells, head and neck squamous carcinoma cells, and triple-negative breast cancer cells (16-18). Closer inspection of the relative levels of tyrosine phosphorylation detected by the array indicated that NRAS^wt^ cells, albeit modestly, activated the endogenous ephrin receptor EphA1 in response to cetuximab, and this was more obvious with a longer exposure of the membrane. By contrast, EphA1
was not activated in cetuximab-refractory NRAS\textsuperscript{Q61K/+} cells in response to cetuximab (Fig. 1).

Cetuximab fails to downregulate EphA2 in NRAS-mutant mCRC cells. The EPH gene family is the largest subfamily of RTKs, including at least 16 receptors and 9 ligands for Eph kinases, termed ephrins (19-22). We performed quantitative real-time RT-PCR in SW48 cells to detect the expression of the transcripts encoding EPHA1 and EPHA2 receptors and EFnA1 (ephrin-A1) and EF nA2 (ephrin-A2) ligands. When NRAS\textsuperscript{+/+} cells were compared with NRAS\textsuperscript{Q61K/+} cells, a trend towards lower expression of the EPHA1 transcript was detected in NRAS\textsuperscript{Q61K/+} cells (Fig. 2A).

A completely different picture emerged when the expression of EPHA1, EPHA2, EFNA1, and EFNA2 transcripts was evaluated following 48-h exposure to cetuximab (Fig. 2B). Whereas the baseline expression of EPHA1 remained unaltered in cetuximab-resistant NRAS\textsuperscript{Q61K/+} cells, a significant 1.5-fold upregulation of the EPHA1 transcript occurred in cetuximab-responsive NRAS\textsuperscript{+/+} cells. Furthermore, cetuximab treatment resulted in a robust and significant \textgreater 3-fold down-regulation of EPHA2 in NRAS\textsuperscript{+/+} cells whereas the expression of EPHA2 remained unaltered in NRAS\textsuperscript{Q61K/+} cells. A trend towards higher expression of EFNA1 ligand accompanied the downregulation of EPHA2 in cetuximab-responsive NRAS\textsuperscript{+/+} cells. By contrast, cetuximab treatment failed to change the expression of EFNA1 and EFNA2 ligands in cetuximab-resistant NRAS\textsuperscript{Q61K/+} cells (Fig. 2C).

Stimulation of ephA2 with a soluble recombinant version of the ephrin-A1 ligand restores cetuximab responsiveness in NRAS-

Figure 1. Cetuximab-induced activation of the EphA1 receptor is unresponsive in NRAS-mutant cells. Phospho-proteome profiling of mCRC cells in response to cetuximab. Total cell lysates (750 µg) from NRAS\textsuperscript{+/+} and NRAS\textsuperscript{Q61K/+} cells before and after treatment with 100 µg/ml cetuximab (48 h) were incubated on membranes of the phospho-proteomics platform as described in Materials and methods. Representative phospho-proteome analyses are shown. Equivalent results were obtained in two independent experiments.

Figure 2. NRAS mutation protects mCRC cells from cetuximab-induced down-regulation of EPHA2. Total RNA from NRAS\textsuperscript{+/+} and NRAS\textsuperscript{Q61K/+} cells cultured in the absence or presence of 100 µg/ml cetuximab (48 h) was characterized in technical triplicates for the relative abundance of the EPHA1, EPHA2, EFNA1, and EFNA2 mRNAs. The transcript abundance was calculated using the \( \Delta \Delta C_t \) method and presented as relative quantification (RQ).
mutant cells. Because ligand-independent cross-talk between EphA2 and other oncogenic pathways (e.g., PI3K/AKT and RAS/ERK) results in tumor promotion (19-22), whereas ligand-induced EphA2 signaling triggers intrinsic tumor suppressive signaling involving the blockade of PI3K/AKT and RAS/ERK pathways (21-24), we hypothesized that the EphA2/ephrin-A1 axis might operate as a molecular switch determining the responsiveness/unresponsiveness of cetuximab in NRAS-mutant mCRC cells. To question whether the loss of ligand-dependent signaling changed the function of EphA2 to a ‘protector’ against cetuximab in NRAS-mutant mCRC cells, we took advantage of the well-documented observation that stimulation of tumor cells with ephrin-A1-Fc (eA1-Fc), a soluble recombinant ephrin-A1 ligand fused to the Fc portion of human immunoglobulin G (IgG), leads to tyrosine phosphorylation of EphA2 and its downregulation (25-28).

Cell proliferation rates of NRAS+/- and NRASQ61K+/- cells cultured with or without cetuximab, eA1-Fc, or cetuximab plus eA1-Fc were dynamically calculated using impedance technology (Fig. 3A). The cell proliferation rate for NRASQ61K+/- cells treated with cetuximab was significantly higher than that for cetuximab-treated NRAS+/- cells, confirming the refractoriness of NRASQ61K+/- cells to the anti-proliferative effects of cetuximab. A small reduction in cell proliferation occurred in NRASQ61K+/- cells treated with eA1-Fc but not in NRAS+/- cells. The addition of eA1-Fc failed to alter the ability of cetuximab to significantly reduce the proliferation rate of NRAS+/- cells. Interestingly, co-treatment with eA1-Fc and cetuximab fully restored the capacity for cetuximab to inhibit the growth of NRASQ61K+/- cells.

Stimulation of EphA2 with eA1-Fc suppresses cetuximab-unresponsive hyperactivation of MAPK and AKT in NRAS-mutant cells. To confirm that engagement of the ligand-dependent...
tumor-suppressive branch of ephA2 signaling synergistically sensitized NRAS-mutant cells to cetuximab via suppression of the pro-oncogenic ligand-independent branch of ephA2 signaling, we used commercially available slide-based antibody arrays to simultaneously assess multiple well-characterized intracellular signaling molecules (Fig. 3B). We confirmed that pharmacological mimicking of ligand-dependent stimulation of ephA2 with eA1-Fc resulted in a significant pan-tyrosine phosphorylation of ephA2. Of note, eA1-Fc-induced activation of ephA2 was stronger in cetuximab-refractory NRASQ61K/+ cells than in cetuximab-responsive NRAS+/+ cells. Co-treatment with eA1-Fc decreased the cetuximab-unresponsive hyperactivation of MAPK in NRAS-mutant cells and decreased also the cetuximab-unresponsive hyperactivation of AKT in NRAS-mutant cells. Thus, co-treatment with eA1-Fc and cetuximab generates a phospho-signaling signature in NRAS-mutant cells reminiscent to that observed in cetuximab-treated NRAS+/+ cells. Indeed, the strong activation of ephA2 with eA1-Fc that occurred in NRAS-mutant cells decreased AKT to levels lower than those observed in NRAS+/+ cells, where no further changes in the deactivation of MAPK and AKT induced by cetuximab occurred when NRAS+/+ cells were co-exposed to eA1-Fc (Fig. 3B).

**Discussion**

In recent years, the ephrin RTKs and ephrin ligands have been established as integral drivers of cancer formation and progression (19-22). Here we provide evidence that dysregulation of the ephrin-A1/ephA2 signaling axis plays an unexpected role...
in determining the refractoriness of NRAS-mutant mCRC cells to the EGFR-targeted monoclonal antibody cetuximab.

Our findings suggest that suppression of the ligand-independent tumor-promoting signaling of ephA2 might be part of the complex molecular mechanism through which cetuximab exerts its growth inhibitory effects against EGFR-dependent RAS wild-type mCRC cells. Because the ephA2 protein can directly interact with EGFR (29-32), and EphA2 is a direct transcriptional target of the Ras-Raf-MAPK pathway (33-35), cetuximab-induced blockade of EGFR signaling and subsequent downregulation of MAPK activity leads to a reduction in EPHA2 expression in wild-type RAS mCRC cells. In NRAS-mutant mCRC cells, however, the incapacity of cetuximab to block MAPK activity impedes the establishment of the feedback loop that negatively regulates EPHA2 expression (Fig. 4), which ultimately translates into the unresponsiveness of NRAS-protected EPHA2 to the downregulatory effects of cetuximab.

Our claim that cetuximab-directed reduction of EPHA2 expression might be part of the mechanism of action of cetuximab is supported by the finding that function-based targeting of ephA2 signaling was sufficient to fully restore the function of cetuximab in NRAS-mutant mCRC cells. Treatment with eA1-Fc, which generates phenotypes similar to those generated by siRNA-mediated or antisense oligonucleotide-mediated genetic knockdown of ephA2 (25-28,36-38), efficiently converted NRAS-mutant cells into RAS wild-type cells in terms of cetuximab functioning and efficacy. Upon restoration of the ligand-dependent tumor-suppressive signaling of ephA2 via stimulation with recombinant ephrin-A1, the constitutively active MAPK signaling of NRAS-mutant mCRC cells was inhibited in the presence of cetuximab. Moreover, supporting and expanding earlier studies attributing ligand-dependent ephA2 activation to suppression of the AKT-mTOR pathway in cancer cells (23,39), treatment with eA1-Fc synergistically interacted with cetuximab to suppress AKT activation in NRAS-mutant cells. Indeed, we observed a significantly stronger phosphorylation of the tyrosine residues of the ephA2 receptor following stimulation with eA1-Fc in NRAS-mutant cells (and a more significant decrease in phosphorylation of AKT) than in RAS wild-type cells, strongly suggesting that NRAS-mutant mCRC cells constitutively exhibit an accelerated phosphorylation/dephosphorylation cross-talk between ephA2 and AKT.

Our findings are in line and expand on recent studies demonstrating the involvement of ephA2 in the resistance to small molecule EGFR tyrosine kinase inhibitors, such as erlotinib and gefitinib, in lung cancer (32,40), vemurafenib in melanoma (41), and the anti-HER2 monoclonal antibody trastuzumab in breast cancer (42-44). It might be argued that the strength of evidence provided by the sole isogenic cell line pair (NRAS\(^{\text{wt}}\) vs NRAS\(^{\text{G12C}}\)) used in our current approach precludes any general extrapolation to mCRC patients. However, it should be noted that, while elucidating new molecular processes contributing to CRC pathogenesis using ephA2\(^{\text{high}}\)-sorted cell subpopulations with stem-like features purified from a chemically-induced model of sporadic colon carcinogenesis, De Robertis et al recently reported that dysregulated expression of the ephA2 receptor accompanied by downregulation of the ligand EFNA1 might operate as a novel mechanism of resistance to cetuximab that can be considered an alternative to KRAS mutations (45). We conclude that, even in the absence of constitutive overexpression of ephA2 in NRAS-mutant cells, dysregulated signaling of the ephrin-A1/epha2 axis suffices to overcome the inhibition of EGFR signaling imposed by cetuximab. Future studies should examine whether the altered functioning of the ephrin-A1/epha2 axis might confer stem-like properties to NRAS-mutant mCRC cells, thus explaining the shortened survival and lack of response to anti-EGFR treatment of NRAS-mutant mCRC patients (1-9).

Because both ephA2 and EFNA1 are recognized as novel biomarkers of benefit from cetuximab-based therapy in mCRC independently of the KRAS mutation status (45-47), our current findings might help to delineate the ephrin-A1/epha2 signaling axis as a common mechanism of cetuximab resistance involving all mCRC patients. Moreover, the fact that cetuximab functioning apparently involves also the upregulation of ephA1, whose reduced expression correlates with poor differentiation, invasion, metastasis and poor overall survival in CRC (48), further underscores the unappreciated relevance of ephrin receptors and ephrin ligands in the clinico-molecular management of mCRC.

In conclusion, our results reveal that: a) the clinical benefit of cetuximab in mCRC might involve the suppression of the ligandless oncogenic state of the ephA2 receptor; b) imparting ligand-dependent tumor suppressing signaling through ephA2 restores the responsiveness of NRAS-mutant mCRC cells to cetuximab. The fact that NRAS-mutant mCRC cells molecularly behave like RAS wild-type cells upon ephrin-A1 signaling to ephA2, in terms of cetuximab efficacy, might open new therapeutic opportunities to clinically widen the usage of cetuximab in mCRC patients.

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