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AUTHOR'S VIEWS

A mechanism for the response of KRAS^{G13D} expressing colorectal cancers to EGFR inhibitors

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ABSTRACT

Previous analysis of Phase 3 clinical trial data for colorectal cancer patients treated with cetuximab revealed that patients harboring a KRAS mutation did not benefit from treatment. This finding set the stage for one of the first examples of cancer personalized medicine. Confusingly, patients with a Glycine to Aspartic Acid mutation at amino acid 13 of KRAS (KRAS^{G13D}) appeared to respond positively to cetuximab, suggesting this mutation is an exception to the rule that KRAS mutations confer resistance to Epidermal Growth Factor Receptor (EGFR) inhibitors. Oncologists have stated that the mechanism that explains why the KRAS^{G13D} mutation is an exception should be identified before KRAS^{G13D} colorectal cancer patients should be treated differently. We have recently elucidated this mechanism using mathematical modeling of the KRAS biochemical system coupled with experimental biology. The mechanism we revealed involves a cetuximab-mediated reduction in HRAS and NRAS signaling within KRAS^{G13D} cancer cells, owing to impaired binding of KRAS^{G13D} to the tumor suppressor, Neurofibromin (NF1).

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Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase and, when activated by one of its ligands, it initiates several signaling cascades that have robust implications in cellular growth, survival, and invasiveness. This pathway is often over-activated in cancers. The EGFR Mitogen Activated Protein Kinase (MAPK) pathway has been at the epicenter of drug development and research programs due to its essential role in tumorigenesis. Many pharmaceuticals that target this pathway are now in clinical use, including the anti-EGFR therapeutic antibody cetuximab that is utilized in colorectal cancer (CRC) treatment regimens.

Resistance has been considered the major obstacle to long-standing benefit from targeted therapies, including cetuximab. Mechanisms of resistance to EGFR inhibitors include the expression of the oncogenic mutant KRAS. Under current clinical guidelines, patients with activating *KRAS* mutations are ineligible for EGFR inhibitors such as cetuximab.

In recent years, however, several studies have demonstrated that there may be an exception to the rule that oncogenic KRAS mutations confer resistance to EGFR inhibitors.[1,2] Originally, a retrospective analysis of the initial clinical trials for cetuximab showed that patients with a Glycine (G) to Aspartic Acid (D) mutation at amino acid residue 13 of KRAS (KRAS^{G13D}) benefitted from cetuximab, in contrast to the population of all other KRAS mutant patients.[1] The finding that patients harboring KRAS^{G13D} responded to EGFR inhibition was quite surprising, given G13D mutations are constitutively active in an EGFR-independent manner, similar to the other oncogenic KRAS mutant proteins.[3,4] Although KRAS^{G13D} has been shown to possess several unique biochemical properties[4-6] and to behave differently for some phenotypes,[3,4] that patients with any constitutively active KRAS mutant could benefit from cetuximab has been considered to run contrary to the well-established principles of RAS biology.[7] The clinical community has effectively stated that until a mechanism that explains why KRAS^{G13D} responds differently to cetuximab is presented, it should be considered a non-responsive mutant.[8]

We had in our hands a unique approach to study this problem: our previously developed mathematical model describes pathological RAS mutant signaling as a function of the inter- and intramolecular reactions that influence the RAS nucleotide binding state.[9] Through the incorporation of experimentally measured biochemical properties for Glycine to Aspartic Acid at amino acid 12 mutant (G12D), Glycine to Valine at amino acid 12 mutant (G12V), and G13D mutant KRAS we can model signaling networks with the three most common KRAS mutants in CRC. We used the model to simulate how EGFR inhibition would impact RAS signaling.[10] The model suggested that it is fully consistent with known principles of RAS biology for different KRAS mutant cancers to respond differently to EGFR inhibition. The model also revealed that the known biochemical properties of these three KRAS mutants are sufficient to suggest cancers with the G13D mutant should be the most sensitive.

The model identified that the key distinction between sensitive and resistant genotypes is that the levels of guanosine triphosphate (GTP) bound wild-type (WT) HRAS and NRAS should fall more substantially within a G13D cancer than in a G12D or G12V cancer (Figure 1). In contrast, the model suggested that EGFR inhibition would have essentially no impact on GTP-bound levels of the KRAS mutant, whether it be G13D, G12D, or G12V. Thus, the model helped redirect focus from the KRAS mutant to the WT RAS proteins that are also present within a cancer cell.

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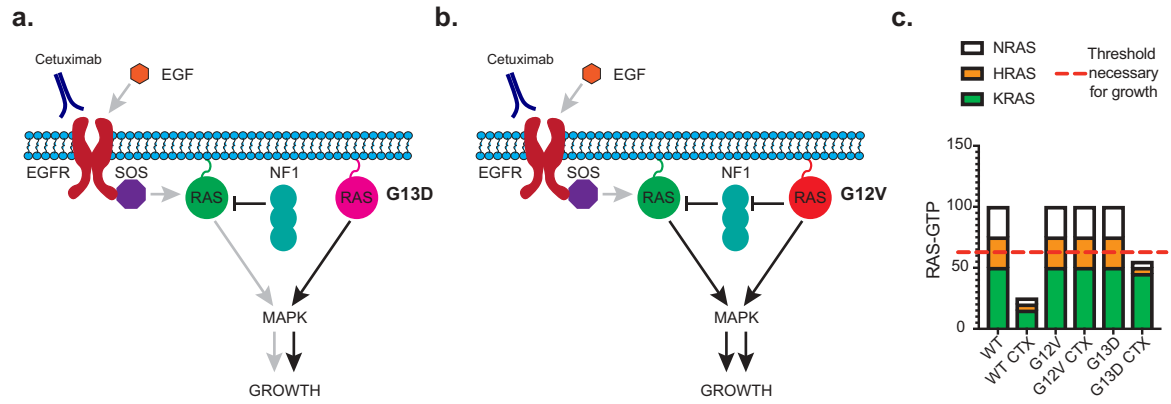

Figure 1. Endogenous RAS activity is depleted in colorectal cancers expressing KRAS^{G13D} but not other KRAS mutants. A. KRAS with a Glycine to Aspartic Acid mutation at amino acid residue 13 (G13D) can activate the mitogen activated protein kinase (MAPK) cascade (black arrows). Additionally, epidermal growth factor receptor (EGFR) can activate wild-type (WT) HRAS and NRAS to further activate the MAPK cascade (gray arrows). Cetuximab treatment blocks WT HRAS and NRAS activation. **B**. KRAS with a Glycine to Valine mutation at residue 12 (G12V), and most other KRAS mutants, activates the MAPK cascade (black arrows). Additionally, these KRAS mutants bind nonproductively to WT RAS negative regulator Neurofibromin (NF1), effectively inhibiting the WT RAS inhibitor and leading to WT HRAS and NRAS activation (black arrows). Cetuximab treatment blocks processes upstream from WT and mutant RAS (gray arrows) but cannot impact this EGFR-independent activation of WT RAS. **C**. Cartoon conceptualizing the levels of HRAS, NRAS, and KRAS activation in the different conditions that we measured in our experiments. Pro-cancer signals are maintained by a high level of active RAS that is comprised of signals from KRAS, NRAS, and HRAS. Cetuximab treatment can inhibit almost all RAS activation in a cancer with no RAS mutation (WT). Cetuximab treatment can only inhibit HRAS and NRAS in a KRAS^{G13D} cancer (G13D). Cetuximab treatment cannot inhibit HRAS and NRAS in a KRAS^{G12V} cancer (G12V) due to the competitive inhibition of NF1 by KRAS G12V.

We experimentally tested and confirmed our hypotheses using CRC cells that were either homozygous WT or hemizygous for G13D or G12V at the KRAS locus. Following cetuximab treatment, or lack thereof, we evaluated levels of HRAS-GTP and NRAS-GTP by performing an active RAS pulldown assay. The resultant active RAS precipitant was then analyzed using three separate methods: Western blot with antibodies specific for each of HRAS, NRAS, and KRAS; isoelectric focusing to separate HRAS, NRAS, and KRAS followed by immunoblotting with a pan-RAS (HRAS, NRAS, and KRAS specific) antibody; and mass spectrometry. In agreement with our mathematical modeling predictions, we found that cetuximab treatment indeed depleted both HRAS-GTP and NRAS-GTP in both WT and G13D cells, when compared to G12V cells. Furthermore, mutant KRAS-GTP did not show any reduction in G12V and G13D cells, again consistent with our model's prediction.

We also determined why WT HRAS-GTP and NRAS-GTP signals decrease only in G13D CRC cells. Analysis of our computational model revealed that the affinity of the KRAS mutant for the tumor suppressor Neurofibromin (NF1) solely determined sensitivity to cetuximab. It has previously been shown that the binding between G13D and NF1 is weaker than that of other RAS mutants.[5] We reproduced a decreased affinity for NF1 experimentally using Bioluminescence Resonance Energy Transfer (BRET) and by co-immunoprecipitation. We also demonstrated that the aspartic acid mutation at residue 13 impairs binding of the G12V mutant to NF1 when we engineered cells containing the two mutations together in cis.[10]

That reduced binding to NF1 might have an impact on sensitivity to upstream inhibition was at first surprising because all three of the common KRAS mutants were modeled to be incapable of having NF1 convert their bound GTP to GDP. GTPase Activating Proteins (GAPs) like NF1 normally maintain a low level of WT RAS-GTP, and loss-of-function NF1 mutations result in increased WT RAS-GTP. Our model previously revealed that the binding of an NF1-insensitive RAS mutant, like G12D and G12V, to NF1 effectively allows the RAS mutant to act as a competitive inhibitor of NF1, thereby promoting increased WT RAS-GTP.[9] In our new work, the model revealed that G13D cannot promote WT RAS-GTP by NF1 competitive inhibition. Thus, we believe that the elevated RAS-GTP in KRAS^{G13D} CRC cells is typically EGFR-dependent, and that targeting EGFR with a drug like cetuximab results in reduced WT RAS-GTP. In contrast, targeting EGFR does not decrease WT RAS-GTP levels in G12V or G12D CRC because the G12V (or G12D) mutant competitively inhibits NF1, resulting in elevated WT RAS-GTP in an EGFR-independent manner.

Overall this work resolves a long-standing problem in cancer personalized medicine and RAS biology. It demonstrates how mathematical approaches that leverage biochemical and biophysical data can play a role in the emerging field of cancer biology and medicine. As a mechanism has now been identified to explain how KRAS G13D CRC patients benefit from cetuximab, we hope this treatment becomes available to these patients, just as it is available to KRAS-WT, NRAS-WT CRC patients.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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