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Clinical and functional characterization of atypical *KRAS*/*NRAS* mutations in metastatic colorectal cancer

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Abstract

Purpose: Mutations in *KRAS/NRAS* (*RAS*) predict lack of anti-EGFR efficacy in metastatic colorectal cancer (mCRC). However, it is unclear if all *RAS* mutations have similar impact and atypical mutations beyond those in standard guidelines exist.

Experimental Design: We reviewed 7 tissue and 1 cfDNA cohorts of 9485 patients to characterize atypical *RAS* variants. Using an *in-vitro* cell-based assay (FACT), Ba/F3 transformation and *in-vivo* xenograft models of transduced isogenic clones, we assessed signaling changes across mutations.

Results: *KRAS* exon 2, extended *RAS*, and atypical *RAS* mutations were noted in 37.8%, 9.5%, and 1.2% of patients, respectively. Among atypical variants, *KRAS* L19F, Q22K and D33E

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occurred at prevalence ≥0.1%, while no *NRAS* codon 117/146 and only one *NRAS* codon 59 mutation was noted. Atypical *RAS* mutations had worse overall survival than *RAS/BRAF* wild-type mCRC (HR 2.90, 95% CI 1.24–6.80, P=0.014). We functionally characterized 114 variants with the FACT assay. All *KRAS* exon 2 and extended *RAS* mutations appeared activating. Of 57 atypical *RAS* variants characterized, 18 (31.6%) had signaling below wild-type, 23 (40.4%) had signaling between wild-type and activating control, and 16 (28.1%) were hyperactive beyond the activating control. Ba/F3 transformation (17/18 variants) and xenograft model (7/8 variants) validation was highly concordant with FACT results and activating atypical variants were those that occurred at highest prevalence in clinical cohorts.

Conclusion: We provide best available evidence to guide treatment when atypical *RAS* variants are identified. *KRAS* L19F, Q22K, D33E and T50I are more prevalent than many guideline included *RAS* variants and functionally relevant.

Keywords

colon; rectal; malignancy; signaling; *RAS*

Introduction:

Mutations in *KRAS/NRAS (RAS)* are important biomarkers in metastatic colorectal cancer (mCRC) that predict lack of benefit from anti-EGFR antibodies and occur in ~60% of mCRCs(1–3). Identification of these alterations is important not only to avoid potential toxicity of ineffective therapy, but *RAS* mutations may also predict worse outcome following anti-EGFR treatment. In the PRIME study, patients with *RAS* mutant mCRC treated with panitumumab + FOLFOX4 had a worse median progression free survival (mPFS) than patients who received FOLFOX4 alone (HR 1.31, 95% CI 1.07–1.60, P=0.008) (4,5). Due to the strength of *RAS* mutations as a predictive biomarker, testing for these alterations has become essential prior to treatment with cetuximab or panitumumab(6).

While the evidence supporting *KRAS* exon 2 mutations as predictive is robust, with a positive interaction test in placebo-controlled trials, the predictive nature of many of the less common variants remains less clear(2–4,7). For example, only 7 patients with codon 59 mutations were identified in the PRIME trial that demonstrated extended *RAS* variants had clinical relevance(4). These mutations were not part of the extended *RAS* mutation analysis, but rather were assessed in a post-hoc analysis that showed removing them from the wild-type population resulted in decreasing the hazard ratio in favor of adding anti-EGFR therapy. Even among guideline cited variants, significant work went into evaluating whether *KRAS* G13D mutations may still benefit from anti-EGFR therapy based on retrospective evidence(8,9). This led to the prospective ICECREAM trial which showed a 0% response rate (RR) following single agent cetuximab among *KRAS* G13D mutant mCRC(10).

Beyond extended *RAS* mutations, other “atypical” variants in *RAS* have been noted and their clinical relevance remains unclear. As we move beyond hot-spot to full gene coverage, these mutations are increasingly observed. Despite G13D being one of the most common *RAS* mutations, the ICECREAM study took over 2 years to recruit 53 patients and demonstrated the difficulty in studying rare variants prospectively. If a similar

prospective strategy is utilized to validate other less common atypical variants, it is unlikely to succeed and alternative strategies are required. With this in mind, we aimed to describe the prevalence of atypical *RAS* variants across a pooled cohort of 9485 patients and explore their functional and clinical significance using *in-vitro* functional data with cross platform validation, *in-vivo* mouse experiments, and retrospective clinical data. We hope this data will provide guidance to clinicians treating patients with uncommon alterations and although this work does not confirm the predictive nature of each individual variant, it provides best available evidence to guide patient care.

Methods:

This study was completed after receiving institutional review board approval and performed according to the Declaration of Helsinki and following institutional guidance for the care of animals. A waiver of consent was obtained for the retrospective review of patient records.

Definitions

KRAS exon 2 mutations were defined as *KRAS* codon 12 & 13 mutations, extended *RAS* mutations were defined as *KRAS* codon 59, 61, 117, 146 and *NRAS* codon 12, 13, 59, 61, 117, 146 mutations, and atypical *RAS* mutations were defined as all other mutations not currently included in standard guidelines in *KRAS*/*NRAS*(6). For patients with more than one mutation in *KRAS*/*NRAS*, variants were considered independently for calculating the specific mutations' prevalence. These patients were categorized into *KRAS* exon 2, extended, or atypical with preference given in descending order from *KRAS* exon 2 to extended to atypical for subsequent analysis.

Patient Population

The 8 cohorts studied are summarized in Table 1 and consisted of 7 tissue-based and 1 cell-free DNA (cfDNA) cohort (Guardant Health, Redwood City, CA). A total of 9485 patients were assessed. The MD Anderson (MDA) CMS 46, MDA T200, a portion (377/1078 patients) of the Mayo cohort, and a portion of the Caris Life Sciences cohort (62/2200 patients) had clinical annotation available for comparison of baseline characteristics and clinical outcomes. Mayo patients with clinical outcomes included all *RAS* mutant cases treated at the Mayo Clinic, Rochester MN. Caris Life Sciences patients with clinical outcomes included patients with extended and atypical *RAS* mutations treated at Georgetown University Medical Center, the West Cancer Center, and Karmanos Cancer Institute.

MSI Testing

Microsatellite instability (MSI) status was retrospectively reviewed from patient's charts where access to charts was available and only evaluated in patients with testing performed as part of their standard care. Testing consisted of a mixture of immunohistochemical (IHC) staining for mismatch repair protein deficiency (MLH1, MSH2, PM2, MSH6) and polymerase chain reaction microsatellite assessment.

Statistical Methods for Clinical Cohorts

Categorical characteristics were compared using χ^2 test or Fisher's exact tests as appropriate, while continuous variables were compared with the Mann-Whitney or Kruskal-Wallis tests when a median is reported and the Student's t-test or ANOVA when averages are shown. P<0.05 was considered significant for all analyses. Right sided tumors were defined based on pathology and surgical reports as those occurring from the cecum up to but not including the splenic flexure. Left sided tumors were defined as those occurring from the splenic flexure to the rectum. Relative variant allele frequency (rVAF) was defined by dividing the allele frequency of a mutation by the maximum allele frequency of any somatic mutation detected in the same sample. OS was defined as the time from diagnosis with stage IV CRC until death or last follow up. Patients alive at the time of last follow up were censored. OS was summarized using Kaplan-Meier curves and compared using the log-rank test and Cox-regression. Where multivariate models were performed, a forward likelihood ratio selection was used. Variables with P<0.05 were included and P>0.1 were excluded during stepwise assessment. All variables met the proportional hazards assumption and were chosen based on differences in baseline characteristics between groups or known prognostic features in CRC. Age was entered as a dichotomy (<60 vs ≥60). Analysis was performed using Graph Pad Prism software version 5.0 (La Jolla, California), SPSS version 22.0 (Armonk, New York) and R studio version 3.30 (Boston, MA).

Functional Validation of Variants

Functional significance was assessed for all *RAS* variants (a) detected at MDA among patients who received a CMS 46 NGS assay for any malignancy, (b) present in a patient with CRC in the CARIS Life Sciences Molecular Diagnostics database, or (C) noted to be of clinical significance or with prior functional annotation in PubMed or COSMIC(11). For example, *KRAS* P34R is associated with cardiofaciocutaneous syndrome (CFC) and *KRAS* T58I is associated with Noonan syndrome. They have both been well characterized to increase cellular proliferation, decrease *KRAS* GTPase activity, and stimulate down-stream phosphorylation of MEK, serving as reasonable controls for activating atypical alterations(12,13)

Novellus Functional Annotation for Cancer Treatment (FACT) Assay—Variants were functionally characterized using an *in-vitro* cell-based assay (FACT) designed to analyze oncogenic activity based on signalling activation (Novellus, Jerusalem, Israel). Variants were generated on a wild-type expression vector then transfected into a live-cell assay with fluorescently tagged ERK2 that is part of the MAPK/ERK pathway and which shuttles from the cytoplasm to the nucleus upon pathway activation(14). Cells were then fixed and scanned by a fluorescent microscope to detect reporter localization that generated nuclear-to-cytoplasmic ratios (NCR) to provide comparisons between signaling activity for each variant. Further details are available in the supplementary methods.

NCR values were normalized and scored according to the activation levels of wild-type and *KRAS* G13D mutations, so that 0% represents wild-type activity and 100% is the activity of the *KRAS* G13D. This was achieved using standard rescaling methods: score = (MT – *KRAS* wild-type)/(*KRAS* G13D- *KRAS* wild-type), where MT is the reported NCR of

the studied mutation and wild-type is the reported NCR of the wild-type condition (15,16). Graphical presentation represents mean and 95% confidence interval.

Analysis comparing OS based on functional activity as defined by the FACT assay was performed using the clinical cohorts and by defining a cut point using histograms demonstrating the distribution of patients with mutations at each activity level (Supplemental Figure 1). This approach was used as there were very few patients with non-activating mutations. As such, the cut points still represent variants that have functional activity significantly above wild-type. Cut point selection and sensitivity analysis are outlined in the results section.

Ba/F3 Transformation Assay—In order to validate findings of the Novellus FACT assay, we utilized a Ba/F3 Transformation Assay. A selection of 17 *RAS* mutations representing *KRAS* exon 2, extended, atypical, activating, and non-activating (per the FACT assay) mutations were assessed using the previously described Ba/F3 transformation assay(17–19). Wild-type cell viability was assessed with 4 technical repeats and each mutation was assessed with 2 technical repeats. Cell viability was compared to wild-type construct using an unpaired t-test. Full details of the methodology for the Ba/F3 Transformation assay are available in the supplemental methods.

***In-vivo* Validation of Functional Significance and Response to Cetuximab—**We created stable *RAS* mutant SW48 cell lines that were used in mouse xenograft models to assess whether functional annotation was concordant with response to cetuximab in animal models. Full details of cell line generation are available in supplemental methods. BALB/c nu/nu 6–8-week old athymic nude mice were maintained in the MDA animal facilities under Institutional Animal Care and Use Committee approved protocols. Wild-type SW48 and mutant *KRAS/NRAS* transduced isogenic clones were used to establish xenografts. Approximately 3×10^6 cells/mouse were injected subcutaneously into right posterior flanks of 5 mice per group. Tumor establishment was monitored twice/week and when tumor volume reached 100–200 mm^3 mice were randomized into treatment groups. The treatment groups received either 250μL saline or cetuximab (0.5 mg/mouse) twice a week via intraperitoneal injection for 21 days at which point mice were euthanized for tumor collection.

Results:

Prevalence of RAS Mutation Classes

The prevalence of missense, nonsense, and indel mutations in *RAS* across the 8 cohorts is demonstrated in Figure 1. *RAS* mutations were noted in 4596/9485 patients (48.5%) and varied between cohorts (P<0.0001) with a range in prevalence from 32.1% to 53.3%. Data from the Project Genie collaboration includes patients with a variety of next generation sequencing (NGS) platforms. Given the heterogeneity in techniques used, data is presented for the entire available version 1 Genie cohort (N=1879) and for only those patients who utilized assays that would cover all exons of *KRAS/NRAS* (depicted with a *, N=1149). There was no statistically significant difference in *RAS* mutation frequency (P=0.65) or distribution of *RAS* mutation class (P=0.93) between versions of the cohort.

A total of 3582/9485 (37.8%) patients had *KRAS* exon 2 mutations, 898/9485 (9.5%) had extended *RAS* mutations and 116/9485 (1.2%) had atypical *RAS* mutations. 13/129 atypical variants (10.1%) occurred in patients who had a co-occurring typical or extended mutation and they were categorized according to their more common variant while only 37/4518 (0.8%) extended or exon 2 variants occurred in patients with co-occurring *RAS* alterations (P<0.0001). Of atypical variants detected, 90 occurred in *KRAS* and 39 in *NRAS*. Prevalence of individual variants is summarized in Supplemental Table 1 and 2, and although most atypical variants occur at low frequencies, certain variants are more common than guideline cited variants. For example, *KRAS* Q22K occurred in 16/9485 (0.2%) patients, and yet not a single *NRAS* codon 117 or 146 variant was detected and only 1 *NRAS* codon 59 variant was detected. Other atypical variants occurring at frequencies ≥0.1% include *KRAS* L19F (7/9485) and *KRAS* D33E (7/9485).

Clonality of RAS Mutations Based on Category of Mutation and Co-Mutations

Data on variant allele frequency was available in the MDA CM 46, MDA T200, Project Genie, and cfDNA cohorts (N=5360). Tissue and cfDNA results are provided separately due to inherent differences in allele frequency distribution (Figure 1B & 1C). In both tissue and cfDNA, we noted that *KRAS* exon 2 and extended *RAS* mutations had significantly higher VAFs than atypical *RAS* mutations (P<0.0001 & P=0.0015, respectively), even after correcting for tumor content using the rVAF (both P<0.0001). Additionally, atypical mutations demonstrated a more diffuse distribution of clonality, while *KRAS* exon 2 and extended *RAS* mutations appeared highly clonal.

Among these same cohorts, we assessed prevalence and clonality of concurrent *RAS*/*BRAF* V600 mutations. We defined mutations as subclonal if they occurred at rVAF<10%. In tissue, there were 8 concurrent *RAS/BRAF* V600 mutations, all of which were clonal, and 3 (37.5%) of which were atypical mutations, a significantly higher prevalence of atypical mutation than in *BRAF* V600 wild-type CRC (P<0.0001). In cfDNA, there were 10 concurrent *RAS*/*BRAF* V600 mutations, of which 7 were subclonal for both the *RAS* and *BRAF* partner. Atypical mutations accounted for 2/10 cases (20%), again significantly more prevalent than in *BRAF* V600 wild type CRC (P=0.037).

Clinical Characteristics and Outcomes of Patients with RAS and BRAF V600 Mutations

We reviewed baseline characteristics and clinical outcomes among 2581 patients with available clinical data. As seen in Table 2, *BRAF* V600 mutations were associated with older age than all other groups, while *KRAS, NRAS*, and wild-type cancers did not differ in age distribution (all pairwise P>0.05). *KRAS, NRAS,* and *BRAF* V600 mutations more commonly associated with female gender (all pairwise P<0.002) and right sided tumors (all pairwise P<0.004), while *BRAF* V600 mutations were more commonly associated with MSI-H status (P<0.0001) but *RAS* mutant tumors did not differ from wild-type tumors regarding MSI status (all pairwise P>0.15). *NRAS* mutations more commonly co-occurred with additional *RAS* alterations than *KRAS* or *BRAF* V600 alterations (P<0.0001).

Patients with *RAS/BRAF* V600 wild-type CRC had better overall survival (OS) than patients with *KRAS* (P<0.0001), *NRAS* (P<0.0001), or *BRAF* V600 mutant tumors

(P<0.0001) (Figure 2A). *KRAS* mutations were associated with a better prognosis than *NRAS* (Hazard Ratio (HR) 0.75, 95% Confidence Interval (CI) 0.58–0.97, P=0.012) or *BRAF* V600 mutations (HR 0.55, 95% CI 0.43–0.70, P<0.0001). *NRAS* mutations were also associated with better prognosis than *BRAF* V600 mutations (HR 0.75, 95% CI 0.57–1.00, P=0.047). In a multivariate model controlling for age, gender, MSI status and primary tumor location, *KRAS* (HR 1.41, 95% CI 1.23–1.62), *NRAS* (HR 1.83, 95% CI 1.40–2.40, P<0.0001), and *BRAF* V600 (HR 2.26, 95% CI 1.75–2.91, P<0.0001) had worse prognosis than wild-type tumors with only right sided location remaining significant in the model for controlled co-variates (HR 1.34, 95% CI 1.17–1.53, P<0.0001). When directly comparing *KRAS* to *NRAS*, there was no statistical difference between genes after controlling for co-variates.

Clinical Characteristics and Outcomes Based on RAS Mutation Class

Extended *RAS* mutations were associated with older age than wild-type tumors (P=0.010), but other *RAS* mutation classes did not differ (P>0.14). *KRAS* exon 2 (P<0.0001) and extended (P=0.0018) *RAS* mutations were more common in women, while atypical mutations did not have different sex distribution from wild-type tumors. *KRAS* exon 2 (P<0.0001), extended (P<0.0001), and atypical (P=0.0022) *RAS* mutations were more likely to be right sided than wild-type tumors. There was no difference in MSI status based on *RAS* mutation class (P=0.25).

KRAS exon 2 (HR 1.48, 95% CI 1.32–1.67, P<0.0001), extended (HR 1.59, 95% CI 1.31–1.93, P<0.0001) and atypical (HR 2.07, 95% CI 0.89–4.85, P=0.014) *RAS* mutations had worse OS than *RAS/BRAF* V600 wild-type tumors (Figure 2B). There was no difference in survival among *RAS* mutation classes (all pairwise P>0.28). In a multivariate model controlling for age, sex, MSI status, and primary tumor location we found that *KRAS* exon 2 (HR 1.44, 95% CI 1.24–1.66, P<0.0001), extended (HR 1.46, 95% CI 1.19–1.78, P<0.0001), and atypical mutations (HR 2.27, 95% CI 1.21–4.27, P<0.0001) all had worse prognosis than wild-type tumors but there was no statistically significant difference between class when assessing prognosis among only patients with *RAS* mutations. In fact, if we included class of mutation and whether the mutation occurred in *KRAS* or *NRAS* in the model as two separate co-variates, we found that class of mutation was not retained in the model and only the presence of the mutation in *KRAS* (HR 1.41, 95% CI 1.23–1.62, P<0.0001) or *NRAS* (HR 1.84, 95% CI 1.40–2.41, P<0.0001) remained in the model. This suggests that differences in prognosis based on mutation class may be defined more by distribution of these variants across the two genes rather than class, however a test of interaction was not significant.

Functional Characterization of RAS Variants Using Novellus FACT Assay

We characterized 62 *KRAS* and 52 *NRAS* variants using the Novellus FACT assay. As seen in Figure 3, *KRAS* and *NRAS* showed a large dynamic range of functional activity. Values represent relative signaling compared to wild-type (0%) and a known activating control mutation for *KRAS* (G13D = 100%) and *NRAS* (Q61R = 100%). Mean activity on this relative scale ranged from 26% below wild-type signaling for a *KRAS* E76G mutation to 313% for a *KRAS* Q22K mutation. All exon 2 and extended mutations were more active

than wild-type *RAS*, indicating the utility of the assay (Figure 3). *KRAS* alterations had a higher median score relative to *NRAS* variants (P=0.0002). Of 57 atypical *RAS* variants, 18 resulted in signaling below wild-type, 23 had signaling between wild-type and activating control, and 16 were more active than the activating control. *KRAS* atypical variants (23/31) and *NRAS* atypical variants (16/26) showed similar ratios of variants that were more activating than wild-type (OR 1.80, 95% CI 0.57–5.07, P=0.39) but atypical *KRAS* variants were more likely to be hyper-activating with signaling above the *KRAS* activating control (15/31) than *NRAS* variants (1/26) (OR 23.44, 95% CI 3.45–255.80, P=0.0002). There was no difference between *KRAS* and *NRAS* in terms of the number of atypical variants with signaling below wild-type (P=0.31).

Orthogonal Validation of Functional Status

Using a Ba/F3 transformation assay, we selected a subset of variants for validation from the FACT assay results(19). Variants chosen for validation focused on atypical mutations that were highly prevalent (*KRAS* L19F, Q22K, and D33E) and a sampling of exon 2, extended, atypical, activating, and inactivating mutations across *KRAS* and *NRAS* as comparators. As seen in Figure 4, 17/18 tested variants were concordant between the FACT and Ba/F3 assay. Only *KRAS* L19F was discordant, lacking transformation activity in the Ba/F3 assay but showing increased signaling in the FACT assay. Importantly, concordance was shown for both the activating alterations in the FACT assay (15/16) and the non-activating alterations (2/2). Using a large library of previously classified *BRAF* variants, we also compared protein expression levels using reverse phase protein arrays (RPPA) and the Ba/F3 viability and demonstrate that there is no correlation between relative protein level and cell viability (r=0.054, P=0.68), suggesting transformation is due to functional alterations rather than potential differences in protein expression (Supplemental Figure 2).

Next, we further evaluated the well characterized *KRAS* G12D and *NRAS* Q61K mutations and atypical variants with prevalence of ≥0.1% in Supplemental Table 1 and 2 using *in-vivo* xenograft models derived from transduced isogenic clones of the SW48 cell line. As none of the atypical variants for *NRAS* were present at this threshold, we randomly selected one atypical alteration (K135N) that was non-activating for characterization. As shown in Figure 5, the SW48 parental and wild-type transduced controls show tumor suppression following cetuximab in xenograft models. Similarly, the well described *KRAS* G12D and *NRAS* Q61K mutant xenograft models show reduced growth suppression with cetuximab. The *KRAS* and *NRAS* mutations fell into three classes when mice were treated with cetuximab, those that completely blocked the effects of cetuximab (*KRAS* G12D, *KRAS* T50I, and *NRAS* Q61K), those that were associated with decreased activity of cetuximab (*KRAS* Q22K and *KRAS* D33E) and those that resulted in response to cetuximab that was similar to wild-type xenografts (*KRAS* L19F, *KRAS* D57N, and *NRAS* K135N). Among atypical variants assessed, only *KRAS* T50I showed a lack of tumor growth suppression with cetuximab while *KRAS* Q22K and D33E showed proliferation that was intermediate between wild-type and known activating mutations. Of tested variants, 7/8 were concordant with the FACT assay if the intermediate category was considered similar to activating in the FACT assay and 8/8 were concordant with the Ba/F3 assay. *KRAS* L19F was inactivating by the Ba/F3 assay and xenograft models but was considered activating with the FACT assay.

When we compared xenografts grown in the absence of cetuximab, we noted no difference in tumor volume change when comparing individual mutations to each other and wild-type transduced controls with a One-Way ANOVA with correction for multiple comparisons. We also performed a Western blot assessing RAS expression and a RAS-GTP pulldown of isogenic lines (Supplemental Figure 3) which demonstrated comparable expression and that all lines were more active than negative control and showed a range of activity that reflected other functional assays except for the *NRAS* K135N line, which was activating in the pulldown but not in any other assay. Mutations with all 4 assays are summarized in Supplemental Table 3.

Impact of Functional Status on Clinical Outcomes

We next assessed the prevalence and clinical impact of FACT assay characterized variants among patients from our epidemiologic cohorts with clinical outcomes. As seen in Supplemental Figure 1, most patients had a functionally active variant with activity similar to the *KRAS* G12D activating mutant. Overall survival did not differ in patients with a mutation occurring above vs below 60% relative activity (HR 1.38, 95% CI 0.84–2.27, P=0.13) but showed trends towards being worse in the less active variants. We reviewed the variants making up this group and noted that all but one of the patients included in the group with *RAS* mutation activity <60% of control were *NRAS*, which likely drove this difference in outcome. We chose 60% based on the histogram in supplemental Figure 1A, but subsequently recreated histograms split by gene and created new cut points specific to *KRAS* (80%) and *NRAS* (60%) and found that neither *KRAS* (HR 0.97, 95% CI 0.83–1.14, P=0.73) nor *NRAS* (HR 1.14, 95% CI 0.68–1.92, P=0.60) signaling activity impacted OS. Unfortunately, the population with minimally active *RAS* variants was small so cut points are quite high and we were unable to determine if atypical variants with functional activity near wild-type had prognosis closer to wild-type patients. When assessing *RAS* activity as a linear prognostic variable in a Cox-regression model, there was no association with OS in univariate (P=0.94) or multivariate models (P=0.41) that controlled for age, sex, primary location, and MSI status.

Discussion:

With increased use of more comprehensive sequencing beyond hot-spot annotation, we increasingly find variants of unknown significance. These mutations pose a challenge for clinicians to interpret. Here we present a landscape of *RAS* mutations in CRC across 9485 patients with tissue and blood sequencing demonstrating a 1.2% prevalence of atypical *RAS* mutations and provide functional characterization across 4 orthogonal platforms. We demonstrate that *NRAS* mutations are associated with worse prognosis than *KRAS* mutations, however atypical *NRAS* variants appear more likely to lack increased functional activity than atypical *KRAS* alterations. Though some atypical *RAS* variants do not appear to increase signaling activity (18/57), many resulted in signaling between wild-type and activating control (23/57) and a subset were more activating than known activating mutations, such as *KRAS* G12D (16/57) (Figure 3). Additionally, the majority of highly recurrent atypical variants do activate *RAS* dependent signaling. Thus, in addition to having functional annotation, the landscape and prevalence data in Supplemental Table 1 and 2

provide further information about which variants may have clinical significance as recurrent alterations are more likely to have functional and clinical relevance.

While we tried to provide a comprehensive annotation of the prevalence and functional significance of as many mutations as possible, there will always be newly discovered uncommon alterations and it was not pragmatic to perform functional validation on all variants. We focused on recurrent alterations as they affect the most patients and by nature of the fact that they are recurrent, are more likely to have oncogenic significance. The atypical variants *KRAS* L19F, Q22K, D33E, and T50I warrant special attention as they occur at frequencies greater than *KRAS* codon 59 or *NRAS* codon 117/146 variants which are currently in standard of care guidelines for clinical testing(6). While *KRAS* L19F increased MAPK signaling in the FACT assay, it did not appear to transform cells in the Ba/F3 assay or xenograft models. Although categorizing variants as “active” or “inactive” is convenient, it overlooks the fact that variants, even in the same gene, may have different effects on cellular signaling and survival that manifest in different ways. Although *KRAS* L19F increased signaling, the presence of this variant alone may not cause resistance to anti-EGFR therapy or cause transformation in the Ba/F3 assay. Our results mirror prior work that showed increased *RAS* pathway signaling with L19F, however limited oncogenic transforming potential(20). This variant has not been previously assessed for effects on anti-EGFR therapy and the observation that our work shows increased signaling but continued response to cetuximab would suggest a potential intermediate phenotype and highlights the benefit of orthogonal functional annotation. Using a single assay in isolation may not detect nuanced responses or differing signaling profiles. The FACT assay is focused on ERK translocation to the nucleus, however this may not be sufficient to determine that a variant is clinically relevant if taken in isolation. Based on this, *KRAS* L19F mutant may represent a functional neomorph that retains the aspects of RAS activity assessed in the FACT assay (translocation of ERK to the nucleus) but does not retain aspects of KRAS signaling that may be required to mediate transformation (or cetuximab resistance). This may explain other discordant findings across assays, such as the discordant RAS-GTP pulldown for *NRAS* K135N suggesting increased signaling, despite the other 3 assays showing the variant does not cause ERK translocation, transformation or cetuximab resistance.

This idea that atypical variants need to be considered within the context of other alterations is also seen in atypical *BRAF* mutations. The *BRAF* D594F mutation is a kinase-dead variant that can hyper-activate ERK phosphorylation in the presence of concurrent *RAS* alterations but lacks this activity in *RAS* wild-type models(21). A recent review of 163 patients with atypical *RAS* mutations by Pietrantonio et al. showed high rates of co-occurring *RAS/BRAF* and *NF1* mutations in 30% and 12% (respectively) of cases with atypical *RAS* variants(22). Although we had a small number of patients with atypical variants in our clinical cohort, we did note a higher rate of atypical *RAS* variants co-occurring with *BRAF* alterations ($P<0.0001$), however this may be spurious due to sample size. We have seen similar findings when reviewing *ERBB2* mutations in mCRC, where concurrent *PIK3CA* mutations were 2–4x as common in tumors with *ERBB2* mutations than in tumors with *ERBB2* wild-type tumors(23). Even among well conserved and highly prevalent mutations in *RAS*, variants may have different evolutionary fitness. Winters et al. introduced a library of mutations in *KRAS* codon 12 and 13 and showed through barcoded

sequencing that variants within the same codon had surprising diversity in fitness based on ability to establish tumors(24). These findings all suggest that both individual mutations and the genomic context within which they occur are important to understand when considering targeted therapeutic strategies.

Separately, our finding of worse outcomes in patients with *NRAS* mutations helps add further support to the prognostic relevance of these mutations in light of mixed prior findings. In the MRC COIN trial, a pooled analysis of five German trials, and an Italian retrospective cohort, there was no difference in outcome between *KRAS* and *NRAS* variants(25–27). There were only 38, 39, and 47 *NRAS* mutant cancers noted in these studies, respectively, limiting statistical power. Contrary to this, Cercek et al showed in a larger study (N=87 *NRAS* mutant tumors) that *NRAS* mutations showed a trend towards worse survival than *KRAS* mutations (P=0.05)(28). Our study was larger than these prior reports and showed a statistically significant better OS in *KRAS* compared to *NRAS* alterations (Figure 2, HR 0.75, 95%CI 0.58–0.97, P=0.012). There are different isoform specific signaling pathways of *KRAS, NRAS,* and *HRAS*, that drive differential expression through Raf-1 and Pi3K and their differing distribution for different cancer histologies suggests these genes impact biology differently(29,30).

Our study must be interpreted in the context of several limitations. While we aimed to provide orthogonal validation across Ba/F3 and xenograft models for a selection of *RAS* variants (Supplemental Table 3), it was not practical to functionally validate all 114 variants assessed with 3 assays as this would have required enormous expenditure beyond our resource capacity. Despite this, positive controls, such as the *KRAS* P34R and T58I which were chosen due to prior characterization in hereditary cancer syndromes showed matched activity in the FACT assay and most atypical variants were highly concordant across platforms. We also acknowledge that while we did see substantial differences in growth following cetuximab noted in the xenograft work performed, the SW48 cell lines using for our isogenic clone generation contains a *MEK* Q56P mutation which may impact sensitivity to EGFR inhibitors (31). Although the gold-standard would be to treat patients with each atypical variant with anti-EGFR agents and assess response in prospective trials, the rarity of these variants makes that impractical. As well, most of our sequencing results came from large tertiary centers which may not be representative. Future efforts to support multi-institutional data-sharing from community and tertiary centers could address this gap while also providing a large population to better describe prevalence and clinical implications of uncommon variants. This would help confirm the prognostic and clinical implications of individual atypical variants which was challenging in our study due to small sample size for individual variants.

Despite these limitations, this work demonstrated atypical variants are present in 1.2% of patients with mCRC, a rate higher than previously reported, and provides best available evidence to guide patient care when one of these variants is found(22). We demonstrate that *NRAS* mutations are associated with a worse prognosis than *KRAS* alterations and identify *KRAS* L19F, Q22K, D33E, and T50I as more prevalent than many guideline included *RAS* variants. These variants should be considered for testing in patients with mCRC as part of

standard care in future testing and alternate non-anti-EGFR antibody treatments should be prioritized where available if a *KRAS* Q22K, D33E, or T50I variant is identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Statement of Translational Relevance:

Mutations in *KRAS/NRAS* (*RAS*) predict lack of benefit from anti-EGFR therapy in metastatic colorectal cancer (mCRC). However, it is unclear if all *RAS* mutations have similar impact and atypical mutations exist beyond those that standard guidelines recommend testing. We reviewed 9485 patients and identified 1.2% of patients with atypical mutations outside standard guidelines. Although most atypical mutations were rare, some occurred more frequently than variants in current guidelines. Atypical variants were associated with survival similar to other *RAS* mutations (worse than wild-type survival) and *NRAS* variants were associated with worse survival than *KRAS*. We functionally characterized 114 variants with an *in-vitro* cell-based assay and provide orthogonal validation using Ba/F3 transformation and mouse xenograft models. Guideline cited variants all increased kinase activity, however there were additional atypical variants including *KRAS* L19F, Q22K, D33E, and T50I that appear both prevalent and relevant variants for consideration as additions to standard guidelines in mCRC.

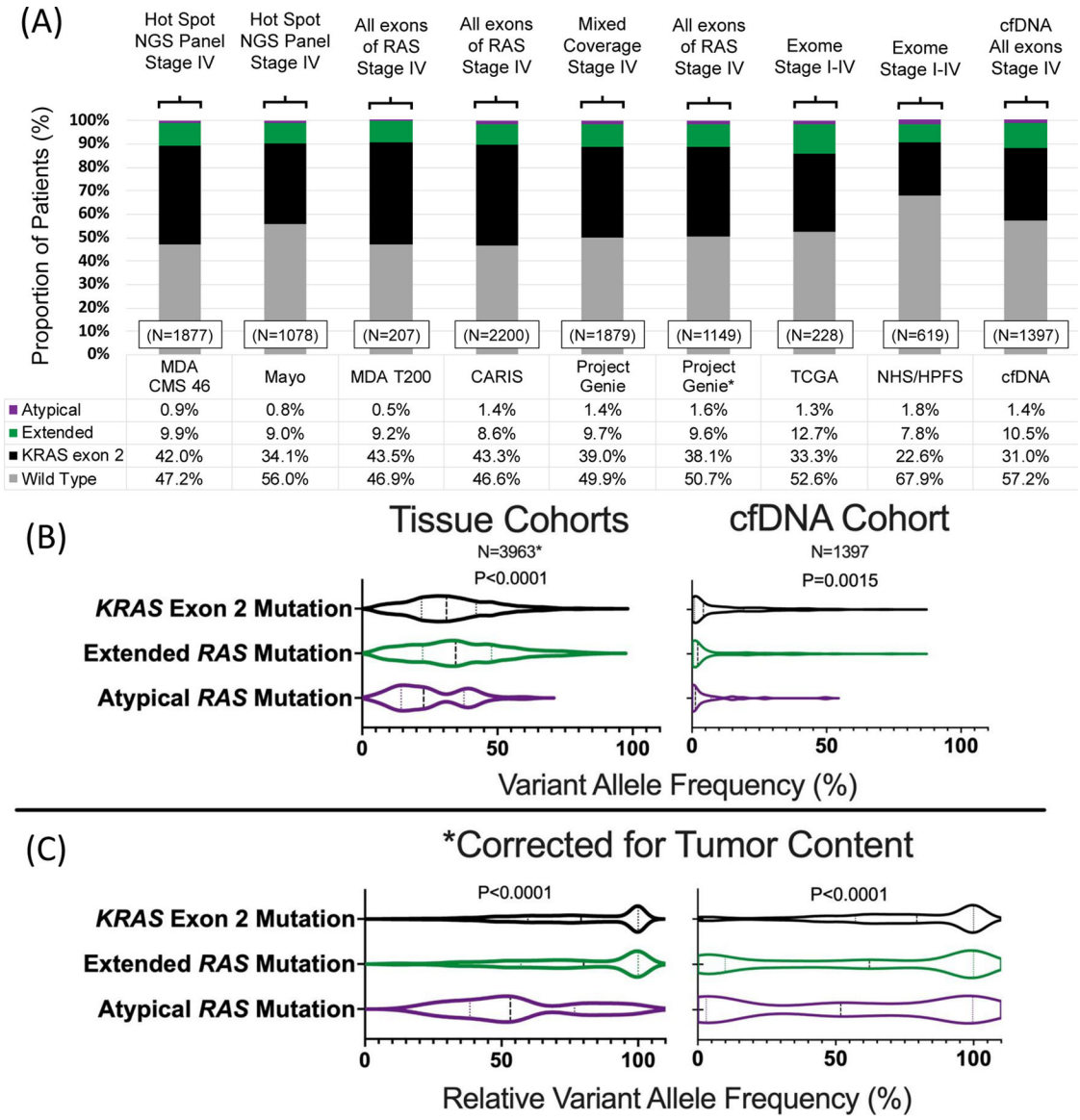

Figure 1.

(A) Prevalence of *RAS* mutations in colorectal cancer across seven tissue and one cfDNA cohort, (B) variant allele frequency of *RAS* mutation by class and (C) relative variant allele frequency of *RAS* variants by class.

*Includes patients with high depth sequencing and known variant allele frequencies from MDA CMS 46, MDA T200 and Project Genie cohorts

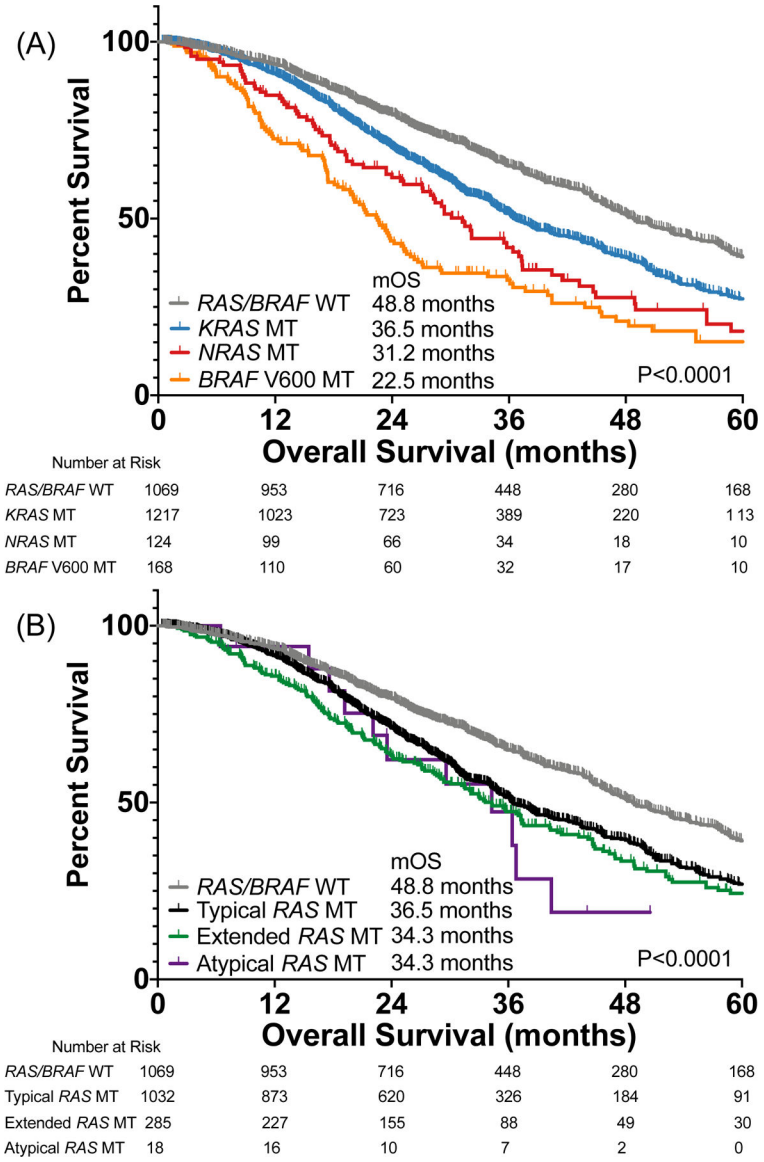

Figure 2.

Impact of (A) *RAS/BRAF* mutations and (B) *RAS* mutation class on overall survival among patients with metastatic colorectal cancer.

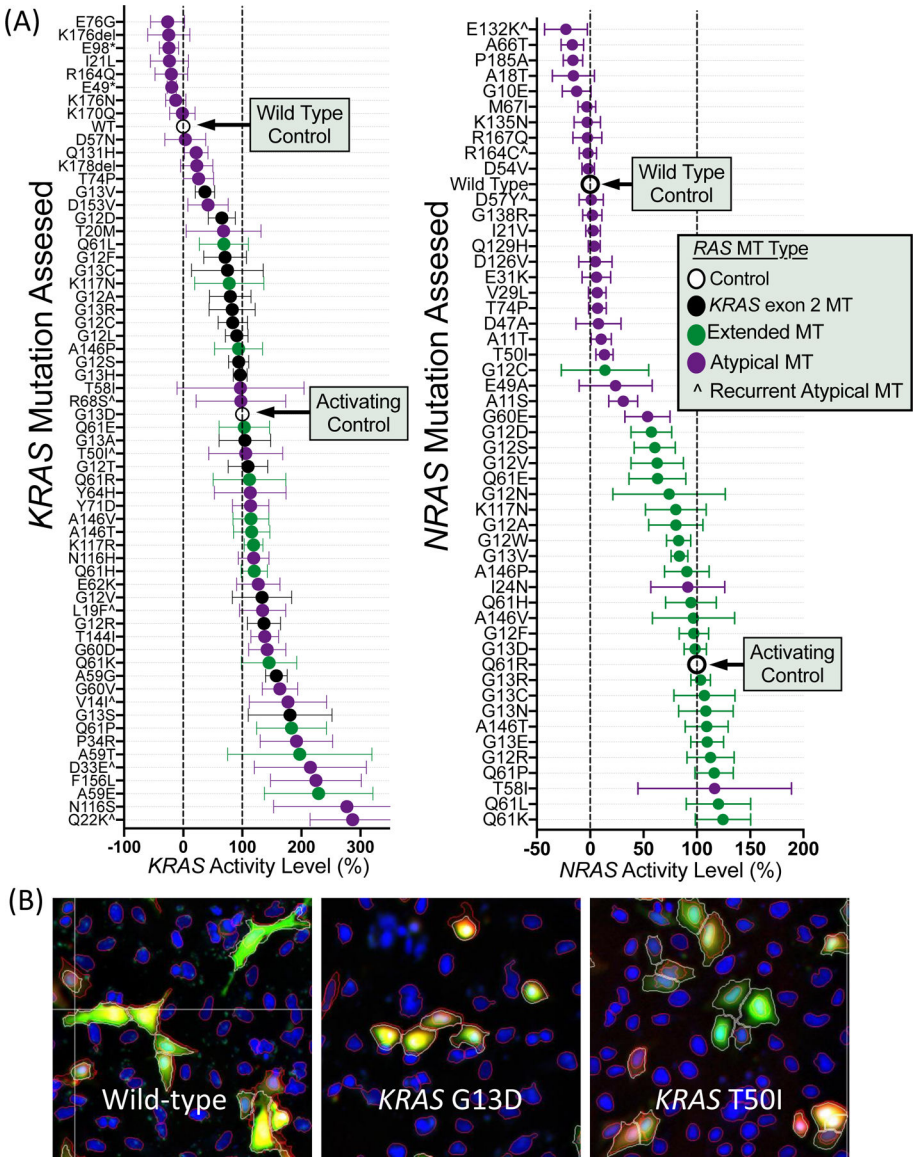

Figure 3.

Functional characterization of MAPK signaling for (A) 114 *RAS* variants assessed using the Novellus FACT assay with (B) representative fluorescent microscopy images from the FACT assay. Values in (A) represent mean +/− 95% confidence interval.

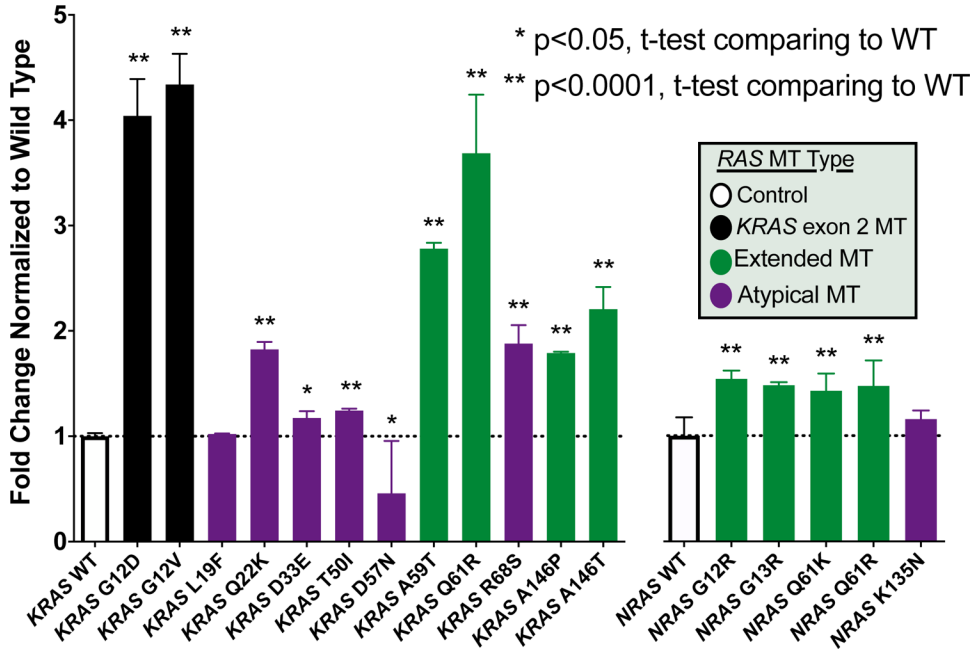

Figure 4.

Functional annotation of *RAS* mutation activation status using the Ba/F3 transformation assay.

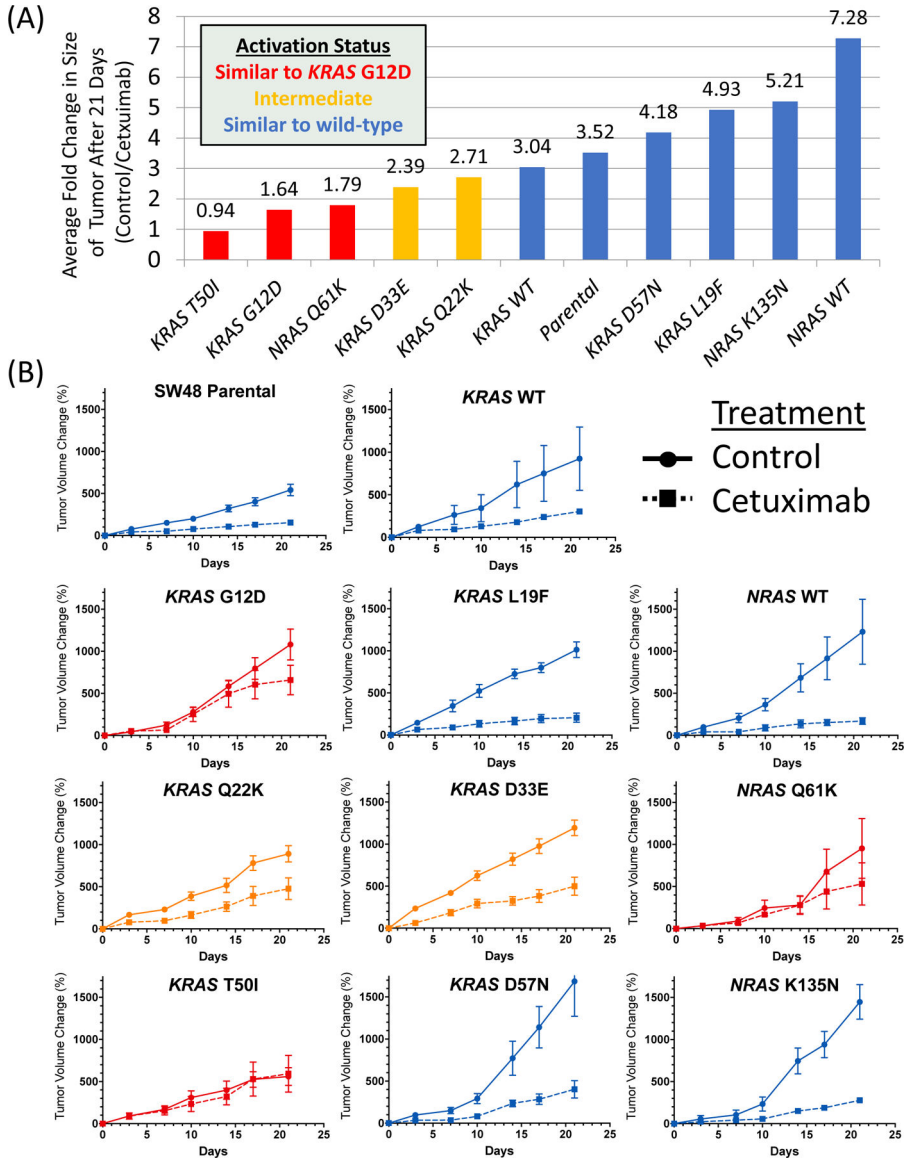

Figure 5.

Impact of cetuximab treatment on tumor volume in mouse xenograft models derived from SW48 isogenic clones carrying select *RAS* mutations. (A) Fold change in size of xenograft at the end of 21 days of cetuximab treatment relative to control and (B) tumor size during 21-day treatment cycle with cetuximab or control (mean +/− standard error) for each tested variant.

Table 1.

Cohorts utilized to characterize relative prevalence of *RAS* mutations in colorectal cancer and their characteristics.

Cohort	MDA CMS 46 (32)	Mayo	MDA T200 (33)	CARIS	Project Genie (34)	TCGA (35)	NHS & HPFS (36)	cfDNA (37)
Patients	1877	1078	207	2200	2081	228	619	1397
RAS Coverage	Hot spot	Hot spot	All exons	All exons	Mixed	All exons	All exons	All exons
Assay Type	46 Gene Multiplex	46 Gene Multiplex	201 Gene; Capture Based	592 Gene; Hybrid Capture Based	Mixed	Exome	Exome	54 to 73 Gene cfDNA
Assay Depth	≥250X	≥250X	Median 906X (tumor)	>750X	Varied by Platform	>20X for 80% of exons	Median 88X (tumor)	8000X
Tumor Cellularity	>20%	>20%	>20%	>20%	>10%	≥60%	Average 45%	n/a
Stage of Patients	Stage IV	Stage IV	Stage IV	Mostly Stage IV	Mostly Stage IV	Stage I-IV	Stage I-IV	Mostly Stage IV
Publicly Available	No	No	No	No	Yes	Yes	Yes	No

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Table 2.

Clinical characteristics associated with class of *RAS* mutation.

	***RAS/BRAF* Wild-type N=1069**	***KRAS* Mutant N=1226**	***NRAS* Mutant N=127**	***BRAF* V600 Mutant N=168**	**P**	***RAS/BRAF* Wild-type N=1069**	**Exon 2 *KRAS* Mutation N=1033**	**Extended *RAS* Mutation N=292**	**Atypical *RAS* Mutation N=21**	**P**
Characteristic										
Median Age (IQR)	54 (45–62)	55 (45–63)	57 (47–65)	60 (54–67)	<0.0001	54 (45–62)	55 (45–63)	57 (47–65)	55 (45–67)	0.018
Sex										
Female	394 (37%)	593 (48%)	65 (51%)	93 (55%)	<0.0001	394 (37%)	505 (49%)	137 (47%)	11 (52%)	<0.0001
Male	675 (63%)	633 (52%)	62 (49%)	75 (45%)		675 (63%)	528 (51%)	155 (53%)	10 (48%)	
Location *										
Right	216 (20%)	477 (39%)	40 (31%)	116 (70%)	<0.0001	216 (20%)	399 (39%)	104 (36%)	10 (48%)	<0.0001
Left	849 (80%)	742 (61%)	87 (69%)	50 (30%)		849 (80%)	631 (61%)	184 (64%)	11 (52%)	
MSI Status *										
MSS	766 (96%)	870 (96%)	99 (99%)	99 (83%)	<0.0001	766 (96%)	735 (97%)	218 (96%)	13 (87%)	0.25
MSI-H	30 (4%)	35 (4%)	1 (1%)	20 (17%)		30 (4%)	26 (3%)	8 (4%)	2 (13%)	
Unknown	273	321	27	49		273	272	66	6	

* Percent represent the proportion of patients with a known result for that variable.

Total n=2581 patients, however patients with multiple variants may be included in multiple categories for comparisons between *KRAS, NRAS,* and *BRAF* V600 but not *RAS* mutation class. All RAS mutation categories (atypical, extended, and KRAS exon 2) are grouped together by gene on the left half of the table.

IQR=interquartile range