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KRAS mutant tumor subpopulations can subvert durable responses to personalized cancer treatments

KRAS mutations in colorectal and lung cancers predict failure to respond to therapies that target the EGFR. Significant percentages of patients with *KRAS* wild-type tumors also fail to respond to these therapies. Relapse occurs in patients with *KRAS* wild-type and mutant tumors, with moderately longer progression-free survival in patients with *KRAS* wild-type tumors. Colon and lung tumors frequently carry *KRAS* mutant tumor subpopulations not detected by DNA sequencing. This suggests detected and undetected *KRAS* mutant subpopulations in colon and lung tumors are undermining the efficacy of anti-EGFR therapies. Therefore, consideration should be given to combining therapies that target *KRAS* mutant cells with those that downregulate EGFR signaling. As tumors are frequently polyclonal in origin and comprised of distinct clonal populations carrying complementing genetic and/or epigenetic lesions, preclinical models that assess the efficacy of combination therapies in the context of heterogeneous tumor cell populations will be essential for progress in this area.

KEYWORDS: ACB-PCR ■ carcinogenesis ■ colorectal cancer ■ epidermal growth factor receptor ■ mutation detection ■ non-small-cell lung cancer ■ oncogene ■ personalized medicine ■ polyclonal tumor origin ■ reactive oxygen species

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Progress has been made toward tailoring a patient's cancer treatment based upon the mutations present in their tumor. Yet, significant obstacles remain. Clinical studies, using therapies that block EGFR signaling in colon and lung cancers (due to *EGFR* mutation and/or overexpression), indicate clinical responses very rarely occur for *KRAS* mutant tumors and are observed in only a subset of patients with *KRAS* wild-type tumors (TABLE 1) [1–5]. Therapies that target EGFR generally fall into one of two categories, anti-EGFR monoclonal antibodies (mAbs; cetuximab and panitumumab) or small molecule tyrosine kinase inhibitors (TKIs; erlotinib and gefitinib). The examples provided in TABLE 1 indicate that the median progression-free survival (PFS) following treatment of advanced colorectal or lung cancers with therapies that target EGFR (also called ERB1 or HER-1) is generally only a few months (see [6,7] for comprehensive meta-analyses of studies on colorectal and lung cancer patients, respectively). Significantly longer PFS has been reported for patients with wild-type as compared with *KRAS* mutant colon and lung tumors (TABLE 1). While virtually all clinical studies investigating anti-EGFR mAbs in colon cancer have found that *KRAS* mutations negatively impact PFS [3,8,9], a number of studies on advanced lung cancer patients did not observe statistically significant differences in response to therapies directed against EGFR

(either mAbs or TKIs) [10–13]. Consequently, *KRAS* testing with the Qiagen Therascreen kit is an US FDA-approved companion diagnostic for the treatment of colorectal cancers (CRCs) with cetuximab, whereas *KRAS* testing is not required in the treatment of non-small-cell lung cancers (NSCLCs) with anti-EGFR inhibitors [14]. Nevertheless, several studies find higher frequencies of responders among *KRAS* wild-type patients treated with anti-EGFR therapies [15,16].

In patients who initially respond, development of acquired resistance to therapy and relapse represents a major challenge for attaining successful personalized cancer treatments. For example, although more than 50% of melanomas with V600E/K *BRAF* mutations respond to *BRAF* inhibitors, virtually all patients develop acquired drug resistance and relapse [17]. For patients treated with EGFR-targeted therapies, remissions are almost always followed by disease progression [18,19]. There are a number of different mechanisms that could result in acquired drug resistance, including secondary mutations in *EGFR*, mutations in downstream molecules of the mitogen-activated protein kinase pathway, and transcriptional activation or amplification of cell surface receptors [20–22]. For example, according to Reungwetwattana *et al.*, the *EGFR* T790M mutation accounts for approximately 50% of EGFR-acquired resistance in

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Table 1. Progression-free survival for patients treated with anti-EGFR therapies, stratified by *KRAS* tumor status.

Cancer	Treatment	Overall response (%) [†]	Overall response among wild-type <i>KRAS</i> (%) [†]	Median PFS for wild-type <i>KRAS</i> (months)	Median PFS for mutant <i>KRAS</i> (months)	Ref.
Metastatic CRC	Panitumumab	10.0	17.0	3.1	1.9	[8]
Metastatic CRC	Cetuximab	7.8	16.0	5.0	1.7	[3]
Metastatic CRC	Cetuximab or panitumumab	19.0	39.0	NR	NR	[9]
Advanced NSCLC	Erlotinib	9.3	10.2	7.0	3.7	[41]
Lung adenocarcinoma	Erlotinib or gefitinib	13.0	16.0	6.0	2.0	[1]

[†]Overall response includes complete and partial responses.

CRC: Colorectal cancer; NR: Not reported (but statistically different); NSCLC: Non-small-cell lung cancer; PFS: Progression-free survival.

TKI-treated NSCLCs, with in-frame duplication and/or insertions in the *EGFR* exon 20 accounting for approximately 5%, cMET overexpression accounting for approximately 15–20%, and unknown mechanisms accounting for approximately 25–30% of acquired resistance [23]. However, this report will focus on the less well-known hypothesis that undetected *KRAS* mutant tumor cell subpopulations drive relapse in patients treated with anti-EGFR therapies, and will summarize evidence suggesting that this mechanism could occur in a majority of colon cancers.

Several lines of evidence support the idea that undetected *KRAS* mutant tumor cell subpopulations are driving relapse in patients treated with anti-EGFR therapies. First, *KRAS* is a central hub or node for a number of different signaling pathways responsible for phenotypes known to drive carcinogenesis [22,24]. Thus, there is an *a priori* expectation that untargeted mutant *KRAS* could effect clinical outcome. In addition, there is evidence that a *KRAS* mutation is present in a significant fraction of colon and lung tumors at higher levels than seen in normal tissue, but below that detectable by standard DNA sequencing [25,26]. There is evidence *KRAS* mutations can be heterogeneously distributed within a given tumor [2,27,28]. Also, there is evidence that *KRAS* mutational status may vary between primary tumors and metastases [2,9,29]. There is support for the idea that the relative abundance of *KRAS* mutant cell populations may decrease in polyclonal colon tumors as they progress [26]. Furthermore, there are several clinical studies that indicate minor *KRAS* mutant subpopulations do, in fact, impact patient response to therapies that target EGFR [1,3,9,18,30].

The highly sensitive and quantitative allele-specific competitive blocker PCR method was used to demonstrate that *KRAS* mutation is

present in normal colonic mucosa [26]. The *KRAS* codon 12 GAT (G12D) mutation was present at a mutant fraction of 1.44×10^{-4} in DNA isolated from normal colonic mucosa, while the *KRAS* codon 12 GTT (G12V) mutation was present at a mutant fraction of 1.15×10^{-5} . These values translate to one heterozygous *KRAS* G12D mutant cell per 3470 wild-type cells and one heterozygous *KRAS* G12V mutant cell per 43,400 wild-type cells. However, *KRAS* mutations are present frequently in colon tumors at levels above that found in normal mucosa, but below that detectable by standard DNA sequencing [26]. It was found that 11 out of 37 (30%) of colon tumors (adenomas and adenocarcinomas) carried *KRAS* codon 12 GAT or GTT mutation at levels of 10^{-1} or above (i.e., $\geq 10\%$ of *KRAS* sequence was mutant) and another 17 out of 37 (46%) of the tumors had one of the two mutations at levels between 10^{-3} and 10^{-1} . Eight of the 37 tumors carried both mutations at levels $\geq 10^{-3}$. Owing to the fact that *KRAS* codon 12 GAT and GTT mutations represent only approximately 57% of the large intestine tumor mutations reported in the COSMIC database [101] and 76% of colon tumors carried one of these two mutations at a level of 10^{-3} or greater (one mutant cell per 500 wild-type cells) [26], it can be concluded that most (if not all) colon tumors possess at least some *KRAS* mutant cells. Furthermore, these measurements are consistent with results reported by Dieterle *et al.* [25]. They showed 11 out of 74 (15%) colon tumors had *KRAS* mutation levels $>10^{-1}$, and 58 out of 74 (78%) had *KRAS* levels between 10^{-3} and 10^{-1} , meaning that 93% of colon tumors possess at least one *KRAS* mutant cell per 500 wild-type cells.

The prevalence of *KRAS* mutant subpopulations is not unique to colon tumors. Allele-specific competitive blocker PCR analyses have established that *KRAS* mutant subpopulations

are prevalent in several tumor types (lung, pancreas and thyroid) [31]. With respect to papillary thyroid carcinomas, 29 and 35% of tumors had *KRAS* codon 12 GAT and GTT mutant fractions above the upper 95% CI for that measured in normal thyroid, respectively, which was surprising given that *KRAS* mutations have been found in only approximately 2% of thyroid cancers by DNA sequencing [32]. The detection of *KRAS* mutations at low frequency indicates the existence of subpopulations of *KRAS* mutant tumor cells. The detection of *KRAS* mutant subpopulations cannot be explained simply as contamination of tumor cell populations with nontumor cells because no significant correlation was observed when *KRAS* mutation levels were correlated with the percentages of tumor cells within tumor tissue samples of papillary thyroid cancers [32] or pancreatic cancers, and an inverse correlation was observed for lung adenocarcinoma [PARSONS BL, MYERS MB, UNPUBLISHED DATA].

Additional support for the presence of *KRAS* mutant subpopulations is derived from studies that demonstrate a *KRAS* mutation is heterogeneously distributed within individual tumors [2,27,28]. Baldus *et al.* compared tumor centers and invasion fronts of primary CRCs and found discordant results in 20% of tumors carrying *KRAS* mutations [2]. Similarly, by testing multiple samples from advanced CRCs, Richman *et al.* were able to detect intratumoral heterogeneity for a *KRAS* mutation in 7.2% of tumors [28].

Tumor heterogeneity has been documented in the form of discordance in *KRAS* mutational status between primary tumors and their metastases [2]. Greater discordance in *KRAS* mutational status was observed between primary CRCs and their metastases when a *KRAS* mutation was characterized by DNA sequencing, as compared with more sensitive methods [9]. A meta-analysis of studies regarding primary NSCLCs and their metastases found discordance in approximately 25% of the paired analyses, with *KRAS* mutation more frequently detected in primary NSCLCs as compared with metastases [29]. Thus, the discordance in *KRAS* mutational status between primary and metastatic tumors reflects the occurrence of *KRAS* mutant cells as tumor subpopulations, and highlights the challenges this creates for *KRAS* mutational testing.

The hypothesis that *KRAS* mutant subpopulations are driving patient relapse following treatment with anti-EGFR therapies is

strengthened by the literature describing the relationships between hypoxia in tumors, Ras signaling, and the role of reactive oxygen species (ROS) in regulating proliferation and senescence [33–35]. To correctly assess the importance and genesis of *KRAS* mutant subpopulations, it is important to recognize that many tumors are polyclonal in origin [36]. Consequently, a clone of cells carrying a *KRAS* mutation may be just one of several preneoplastic clones that together initiate carcinogenesis. To illustrate this, a model for polyclonal colon tumor development and progression is depicted in FIGURE 1. According to the model, polyclonal initiation involves localized interaction between the pre-existing *KRAS* mutations present in the colon [26] and other complementing genetic and/or epigenetic lesions. Following polyclonal initiation, additional genetic lesions may be acquired during tumor progression. The literature indicates hypoxia and/or glucose deprivation occurs at the centers of large advanced tumors [22,37]. Hypoxia and Ras expression can lead to the production of ROS [33–35]. Cellular signaling is altered by prolonged exposure to ROS and oxidative stress, and can lead to cellular senescence or even selective killing of *KRAS* mutant cells, depending on cellular context [38]. Thus, there is a known mechanism by which the relative abundance of a *KRAS* mutation may decrease in advanced cancers. Direct evidence that this occurs was provided by Parsons *et al.*, who demonstrated that a statistically significant decrease in *KRAS* codon 12 GTT mutant fraction (codes for G12V *KRAS*) occurred in colon tumors during adenoma to adenocarcinoma progression [26]. Also, by quantification of *KRAS* codon 12 GTT mutant fraction in colon and thyroid tumors, Parsons and Myers established that significant inverse correlations exist between the mutant fraction and maximum tumor dimension (FIGURE 2) [26,32]. Finally, the senescence-associated secretory phenotype may explain how minor *KRAS* mutant tumor cell populations (present at a frequency of only one mutant cell per 500 wild-type cells) can drive tumor progression [39]. Specifically, senescent *KRAS* mutant cells could potentially induce hyperproliferation, epithelial–mesenchymal transition, and invasiveness through secretion of inflammatory cytokines that alter the tissue microenvironment [39].

Thus, as summarized above, there is evidence that *KRAS* mutant subpopulations exist and there is a theoretical framework explaining why *KRAS* mutations may be present as subpopulations in advanced cancers. Nevertheless,

Figure 1. Model of polyclonal colon tumor initiation and progression. *KRAS* codon 12 GAT mutant cells (G12D) are depicted in green; *KRAS* codon 12 GTT mutant cells (G12V) are depicted in blue; cells containing other oncogenic lesions (i.e., tumor suppressor mutations, or cells with epigenetic silencing of a tumor suppressors gene) are depicted in dark purple. Panels from left to right show: **(A)** *KRAS* mutation pre-exists in normal colon; **(B)** tumor initiation occurs through the colocalization and short-range interactions of two or more clones carrying complementing genetic and/or epigenetic lesions; **(C)** proliferation of colocalized clones increases the tumor mass; **(D)** tumor progression occurs with the accumulation of additional genetic and or epigenetic lesions in the initiating clones; and **(E)** hypoxia-driven production of ROS in large, advanced cancers may cause *KRAS* mutant cell senescence or cell death in a context-dependent manner. In addition, the senescence-associated secretory phenotype of *KRAS* mutant/senescent cells may drive the proliferation of non-*KRAS* mutant clones, leading to relative diminishment of *KRAS* mutant clones while facilitating further progression, invasion and metastasis. ROS: Reactive oxygen species.

it could be argued that minor mutant subpopulations may not have any clinical significance. Therefore, it is important to consider clinical evidence that is consistent and inconsistent with the hypothesis that *KRAS* mutant subpopulations are undermining clinical responses to therapies that target the EGFR. In *EGFR* mutant lung cancer patients treated with erlotinib or gefitinib, the predictive power of *KRAS* mutation was greater when the mutation was characterized by mutant-enriched sequencing (with a reported sensitivity of 10^{-3} – 10^{-4}) than by direct sequencing (with a reported sensitivity of 10–20%) [1]. However, survival outcomes did not differ for patients characterized as having *KRAS* mutant tumors by the different methods. This suggests that *KRAS* mutant clones may eventually lead to relapse, regardless of their initial prevalence before treatment. When *KRAS* characterization was performed using two different methods (DNA sequencing vs amplification refractory mutation system–Scorpion assay) and compared, better concordance between *KRAS* mutational status and patient outcome was observed with the more sensitive method [3]. Molinari *et al.* compared four different methods for characterizing the *KRAS* mutational status of metastatic CRCs: specifically, direct sequencing,

MALDI-TOF MS, mutant-enriched PCR, and engineered mutant-enriched PCR, with sensitivities of 20, 10, 0.1 and 0.1%, respectively [9]. Using the more sensitive methods, they identified 13 out of 68 additional tumors that contained *KRAS* mutations, and all occurred in patients who failed to respond to anti-EGFR mAb treatment [9]. In this study, it was noted that PFS for patients with wild-type and mutant *KRAS* tumors was not significantly different [9]. The strongest evidence that a *KRAS* mutation is a frequent driver of acquired resistance to a therapy that targets the EGFR comes from studies by Misale *et al.* [18] and Diaz *et al.* [30]. The study by Misale *et al.* showed that mutant *KRAS* confers resistance to cetuximab *in vitro* [18]. They analyzed metastases in patients who developed resistance to cetuximab or panitumumab and found amplification or mutation of *KRAS* in six out of ten (60%) of the cases. The study by Diaz *et al.* demonstrated the appearance of *KRAS* mutation in DNA isolated from sera of patients being treated with panitumumab, who were previously found to have *KRAS* wild-type tumors [30]. *KRAS* mutations generally appeared in the sera 5–6 months following initiation of panitumumab treatment. Based on their results, Diaz *et al.* concluded that emergence of *KRAS*

mutations is a mediator of acquired resistance to EGFR blockade [30]. Therefore, there is clinical evidence to support the hypothesis that pre-existing, undetected *KRAS* mutant subpopulations can lead to acquired resistance to anti-EGFR therapies, and it seems likely that such mutations are generally undermining patient outcomes when used as monotherapies.

There are some relatively rare clinical examples of patients with *KRAS* mutant tumors that do respond to therapies directed against the EGFR [27,40]. Zhu *et al.* reported one out of 21 NSCLC patients with *KRAS* mutant tumors responded to erlotinib treatment [41]. A meta-analysis indicated only two out of 124 (~1.6%) metastatic NSCLC patients with *KRAS* mutant tumors exhibited durable responses with erlotinib treatment (24 and 36 months). Both responders were diagnosed with *KRAS* codon 12 TGT tumor mutations (G12C) [40]. One patient with *KRAS* G12D mutation in both a primary colon tumor and its metastases achieved a durable response following second-line chemotherapy with irinotecan plus cetuximab [27]. With respect to metastatic CRC, patients with *KRAS* G13D mutant tumors demonstrate poorer outcomes compared with patients with other tumor *KRAS* mutations when treated with chemotherapy or best supportive care, but show an enhanced response to cetuximab relative to other types of *KRAS* mutations [42]. Consequently, the particular *KRAS* mutation may provide part of the explanation for why these responses are unfortunately rare. It is known that the different *KRAS* mutations have different phenotypic properties and signal through different downstream pathways [43,44]. It may be notable that these durable responses did not occur in patients with *KRAS* G12V mutation, which has been associated with poor patient outcome for both colorectal and NSCLCs [44–46]. It would be valuable to learn which additional genetic and/or epigenetic determinants in these tumors are associated with response to anti-EGFR therapies.

The prevalence of tumor subpopulations in *KRAS* mutant cancers has important implications for development of effective personalized cancer treatments. Monotherapies directed at the non-*KRAS* mutant bulk of polyclonal tumors have the potential to lead to the outgrowth of *KRAS* mutant subclones, and eventually to relapse [14]. The prevalence of *KRAS* mutations detectable by DNA sequencing, along with the much greater frequencies of mutation observed using more sensitive methods, suggests that most of some particular tumor types

may be broadly expected to carry *KRAS* mutations (e.g., colon, lung and pancreatic tumors) [14,26,32]. For some tumor types, therefore, it may be appropriate to routinely treat with drugs that target *KRAS* mutant cells (directly or through downstream effectors, such as MEK, PI3K or AKT) in combination with therapies that target other specific pathways within a patient's tumor.

Conclusion

The relatively small overall response rates and the relatively short PFS data shown in TABLE 1 make it clear that additional improvements in the personalized treatment of colon and lung cancers are needed. There is currently sufficient evidence to conclude that *KRAS* mutant subpopulations are prevalent in advanced CRCs, and that such

Figure 2. *KRAS* mutant fraction is inversely correlated with maximum tumor dimension. (A) colorectal adenomas and carcinomas and (B) papillary thyroid carcinomas. Data taken from [26,32].

subpopulations can impact patient response to anti-EGFR-targeted therapies. What remains to be determined is what level of *KRAS* mutation matters in terms of clinical response. Such information is critical in order to accurately predict which patients are likely to respond to treatment and what sensitivity, therefore, is required in the companion diagnostic.

Now, investigations employing combination therapies are being vigorously pursued [5,19,47–49]. As this investigation unfolds, it will be important to develop therapies that can specifically eliminate *KRAS* mutant cells, perhaps by direct killing based on synthetic lethality or by pharmacologically driving oncogene-induced senescence. However, it needs to be understood that driving *KRAS* mutant cells into senescence will not necessarily eliminate the potential for such cells to stimulate the progression of neighboring cancerous or precancerous cells, as may occur via the release of cytokines in cells with a senescence-associated secretory phenotype [39]. Therefore, specific strategies to target *KRAS* mutant tumor cell subpopulations need to be devised and incorporated into combination approaches [50]. The lack of progress in past attempts to target mutant RAS [51], may reflect (at least in part) the inefficiency of using monotherapies to treat polyclonal tumors.

Future perspective

Although this article has focused on the mechanism of undetected *KRAS* mutant subpopulations leading to relapse following treatment with EGFR-targeted therapies, mutations downstream from *KRAS* in signaling pathways (such as BRAF, MET, ERK, PIK3CA, PTEN, AKT and mTOR) are undoubtedly associated with some portion of the treatment failures [21]. This highlights the need for precise, quantitative information about which genetic lesions do and do not co-occur. Information about the frequency with which genetic lesions do co-occur should be useful in terms of understanding which molecules in signaling pathways could be targeted and what proportion of tumors might be expected to respond to particular targeted therapies. Information about which mutations do not co-occur might similarly be interesting because such information might provide insight about mutations that are not compatible with a cancerous phenotype. Efficient progress in this area will depend on establishing a literature on tumor mutations that is broadly interpretable and comparable across studies. Consequently, mutational analyses need to be performed using

sensitive methods that produce quantitative results, with the sensitivity achieved in particular analyses established and reported along with measurements obtained for particular samples.

Given the focus on combination therapies, going forward, progress toward improving personalized cancer treatment will rely on the development of appropriate models. The task of combining promising monotherapies, which can be given with different relative and absolute doses, as alternating treatments or as simultaneous treatments, grows exponentially with the number of compounds to be tested [49,52]. This means that preclinical models will be needed to prioritize such combined therapies for further clinical investigations. In its 2010 draft ‘Guidance for Industry Co-development of Two or More Unmarketed Investigational Drugs for Use in Combination,’ the FDA indicates preclinical data may be used to demonstrate that two or more investigational drugs are appropriate for codevelopment because they have additive activity when used in combination [53,102]. The fact that tumor subpopulations may lead to relapse highlights the urgent need for preclinical models that can capture the large amount of tumor heterogeneity present in tumors. For these reasons, tumor explant models may be a promising approach [54–56]. In this regard it is interesting to note that testing different treatments on samples of a patient’s tumor is being pursued as an approach to identify appropriate therapies to kill a specific patient’s cancer, much in the same way that appropriate antibiotics are identified to kill a patient’s specific pathogen.

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Executive summary

KRAS predicts failure to respond to some molecularly targeted therapies

- KRAS mutant colorectal and non-small-cell lung cancers fail to respond to therapies that target EGFR.
- These include the tyrosine kinase inhibitors, erlotinib and gefitinib, as well as the antibody therapies, cetuximab and panitumumab.

Many tumors carry KRAS mutations as mutant subpopulations

- KRAS mutant subpopulations have been observed in a number of cancers, including colorectal and non-small-cell lung cancers.
- Hypoxia in large, advanced cancers may result in generation of reactive oxygen species, which can result in KRAS mutant cell death, thereby explaining the occurrence of KRAS mutant cells as tumor subpopulations.

Evidence KRAS mutant subpopulations are subverting EGFR-targeted therapies

- Better concordance between KRAS mutation and patient response are observed when more sensitive mutation detection methods are employed.
- Using more sensitive methods, previously undetected KRAS mutations have been reported to occur in nonresponders.

Strategies to achieve durable responses to personalized cancer treatments

- Drugs that kill KRAS mutant cells or inhibit activated KRAS signaling are needed.
- Such drugs need to be investigated in combination with other therapies directed at the bulk of a patient's tumor.
- Novel preclinical models that incorporate tumor heterogeneity are needed to evaluate and prioritize combination therapies for subsequent clinical investigation.

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