K-RAS mutation in the screening, prognosis and treatment of cancer

The potential use of K-RAS mutation as a cancer screening biomarker has been investigated for many years. Numerous associations between K-RAS mutation and various cancers have been established, but these associations have not been translated into effective, cost-efficient cancer screening strategies. This lack of progress may be due to the existence of K-RAS mutation in nontumor tissues and/or using detection, rather than quantitation, of K-RAS mutation as the endpoint for cancer risk categorization. K-RAS mutation appears to be a useful prognostic biomarker for colon cancer. Recent progress toward sensitive and quantitative mutation characterization and the successful use of K-RAS mutation in a personalized medicine approach to targeted biological therapy selection are likely to re-direct and expand the use of K-RAS mutation as a cancer biomarker in the near future.

KEYWORDS: biomarker ■ carcinogenesis ■ mutation detection ■ oncogene ■ personalized medicine

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Developing informative cancer biomarkers is essential to achieve early detection of tumors, to better stratify patients in terms of prognosis and to optimize therapy for individual cancer patients. Investigation in this area is vigorous and a variety of technologies and end points are being developed. Mutation is one type of biomarker being investigated, with mutation in the K-RAS gene the archetype mutational cancer biomarker. An extensive literature describes the tumor prevalence and the cellular consequences of K-RAS mutation. The K-RAS mutant phenotype provides a strong rationale for using K-RAS mutation as a biomarker of cancer risk.

K-RAS function, mutant phenotype & prevalence

Several lines of reasoning have led to the expectation that somatic mutations should be useful cancer biomarkers. Cancer is considered to be a genetic disease and somatic mutations in oncogenes and tumor suppressor genes are viewed as necessary events in carcinogenesis. Initiating mutations, therefore, are expected to be present at the earliest stages of tumor development and persist during progression. The extent to which K-RAS mutations are found in human tumors is exceeded only by mutations in the *p53* gene (~50%), which are distributed over many codons. For K-RAS, it has been established that a few codons encompass the majority of reported mutations. Specifically, hotspot point mutations in codons 12 and 13 are found in significant percentages of human tumors.

Normal K-RAS protein function and the effects of particular K-RAS base substitution mutations on K-RAS protein function are well described. Accurate post-translational processing of K-RAS (including farnesylation or guanylation of a C-terminal CAAX box) is needed for membrane anchoring [1]. K-RAS is a GTP-binding protein that functions in transducing the signal from ligand-specific receptor binding (including dimeric epidermal growth factor receptor [EGFR]-type molecules: EGFR-EGFR, EGFR-HER2, EGFR-HER3 or EGFR-HER4) through intracellular signaling cascades, eventually impacting nuclear transcription [2]. Mutation at particular sites in K-RAS favors the active, GTP-bound form of RAS, which leads to aberrant intracellular signaling through the RAF-MEK-ERK kinase and the PI3K-Akt pathways. Because the RAS-RAF-MEK-ERK kinase pathway regulates normal cell-cycle control, mutation in K-RAS causes unregulated cell proliferation [3]. Aberrant signaling through the PI3K-Akt pathway activates a cascade of anti-apoptotic and prosurvival signals [2]. In addition, mutant K-RAS increases tissue factor and VEGF₁₈₉ mRNA levels, suggesting it also plays a role in angiogenesis and tumor progression [4].

The K-RAS mutant phenotype is complex, in that different K-RAS mutations confer slightly different selective advantages, which in turn are applicable to particular tissue types [3]. Differences in K-RAS mutant phenotype have been associated with different amino acid substitutions at

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codon 12 [5]. Janssen *et al.* showed that mouse embryo fibroblasts expressing normal levels of K-RAS^{G12D} (codon 12 GGT to GAT) are partially transformed, demonstrate enhanced proliferative properties, lack contact inhibition, and can initiate tumors in animals [6]. A transgenic model specifically expressing K-RAS^{G12D} in a stem cell compartment of mouse colon was found to recapitulate human colon tumor development in terms of low tumor yield and latency time [6]. Furthermore, this occurred in the absence of APC mutation, consistent with the idea that K-RAS is important for colon tumor initiation, just as it has demonstrated importance in pancreatic tumor initiation. The relevance of particular K-RAS mutations in different tissues can be inferred from their prevalence in various tumor/lesion types (TABLE 1). Such information is now easily accessible and searchable through the Catalogue of Somatic Mutations in Cancer (COSMIC) database [7,101].

K-RAS mutation as a biomarker in cancer screening

Development of good cancer screening strategies has been one of the most intractable areas of cancer research, but an area with great potential for public health impact. A successful cancer screening strategy needs to combine several critical elements:

- Analysis of a readily available specimen source;
- Cancer biomarker validation (an established association with disease state);

- A methodology with which to measure the biomarker.

These three elements need to be considered throughout the development of potential biomarkers.

K-RAS mutation has been examined in a variety of clinical specimens. Generally, studies have compared the prevalence of K-RAS mutation in body fluids from cancer patients to the prevalence observed in healthy control subjects as a first step toward validating the use of K-RAS mutation in cancer screening. Clinical samples that have been analyzed for K-RAS mutation include blood/plasma/serum, urine, stool, pancreatic juice, sputum and bronchoalveolar lavage fluid. Using denaturing high-pressure liquid chromatography combined with mutant allele-specific amplification (DHPLC-MASA), Lilleberg *et al.* report 100% concordance in terms of the mutations (including K-RAS) measured in the tumor and plasma samples of colorectal cancer (CRC) patients [8]. K-RAS mutation was detected in ten out of 18 (56%) plasma samples from patients with a K-RAS mutant tumor or polyp [9]. Using a quantitative beaming-based approach, Diehl *et al.* found 50% of plasma samples had the same measurable mutation as the tumor from colon cancer patients [10].

Plasma samples from lung and pancreatic cancer patients also are being analyzed for K-RAS mutant DNA. Using PCR in combination with a restriction fragment length polymorphism (RFLP) methodology, Kimura *et al.* detected K-RAS mutation in the plasma of five out of 25 (20%) non-small-cell lung cancer (NSCLC) patients [11]. A meta-analysis of studies investigating the prevalence of K-RAS mutation in the circulating DNA of lung cancer patients reports that the mutation is detected in 43% of cases but in only 0.8% of healthy controls [12]. Using a peptide nucleic acid-PCR clamp, Luo *et al.* detected K-RAS mutation in the plasma of 19 out of 24 (79.2%) pancreatic cancer patients [13]. Using an enriched-nested PCR, Jiao *et al.* detected K-RAS mutation in the plasma of 25 out of 77 (32.5%) pancreatic cancer patients and concluded the mutation was associated with a history of smoking [14].

K-RAS mutation has been detected in relatively low molecular weight DNA isolated from the urine of CRC patients [15,16]. K-RAS mutation was detected in 15 out of 18 (83.3%) urine samples from patients with a K-RAS mutant tumor or polyp [9]. Su *et al.* characterized the amount of K-RAS mutant DNA, as well as

Table 1. Prevalence of K-RAS mutations in human tumors.

Tumor type	Positive samples per number analyzed*	Percent K-RAS mutant
Pancreas	2842/4773	60%
Biliary	474/1478	32%
Large intestine	5513/17126	32%
Small intestine	61/300	20%
Lung	1981/11401	17%
Endometrium	251/1750	14%
Ovary	243/1696	14%
Prostate	74/919	8%
Cervix	49/645	7%
Stomach	147/2345	6%
Liver	22/365	6%
Testis	11/217	5%

*Data collected from the Catalogue of Somatic Mutations in Cancer database [7,101]. Data current as of April 28, 2009. Tumor types with sample numbers less than 100 or percent mutated less than 5% were not included.

the ratio of mutant to wild-type (WT) K-RAS sequence in urine samples, and reported an 83% concurrence between detection of mutant DNA in urine and within the colon tumor [17].

A few studies have analyzed sputum for K-RAS mutation. Using PCR-RFLP analysis, Destro *et al.* found the same K-RAS mutation in sputum samples as in patients' NSCLCs [18]. Using PCR and single-stranded conformational polymorphism (PCR-SSCP), K-RAS mutation was detected in the epithelial cells collected from the sputum of seven out of 15 (46.7%) lung cancer patients [19].

Despite a large number of retrospective investigational studies, only a few studies have evaluated K-RAS mutation as a prospective marker for tumor development and the results from these studies have been only marginally encouraging. Using a method that could detect mutant DNA at a level of 3–12% of wild-type DNA, K-RAS mutation was measured in the serum of individuals who were then followed for 6.3 years [20]. Serum K-RAS mutations were detected in 13 out of 1098 subjects (1.2%) and six of these developed a cancer during the follow-up period. Significantly, five of the K-RAS-positive cancers were bladder cancers (five out of 137 cases, odds ratio of 4.25) [20]. However, serum K-RAS mutations also were detected in seven out of 713 (0.01%) subjects who did not develop a cancer during the follow-up period. With respect to pancreatic cancer, a large prospective multicenter trial on the frequency of K-RAS in pancreatic juice and bile had a sensitivity of 38% and a specificity of 90% for the diagnosis of pancreatic cancer, with K-RAS mutation also being detected in benign disease, chronic pancreatitis (considered a premalignant condition) and normal pancreas [21].

A significant effort has been directed toward the detection of K-RAS mutation in DNA isolated from stool samples, or in colonocytes isolated from stool. This effort is summarized in a recent review by Loktionov [22]. K-RAS mutations were among the genetic lesions detected by a commercially available stool-based DNA screening test for CRC (the PreGen-Plus™ test released by Labcorp, NC, USA in 2003 and later discontinued). Results obtained using stool DNA-based screening (of which K-RAS mutation was just a part) were compared with those obtained on 4404 asymptomatic patients undergoing colonoscopy [23]. Stool-based screening detected 16 of 31 cancers (51.6% sensitivity) with a specificity of 94.4%. Given its sensitivity and the assay's cost, PreGen-Plus was

not considered sufficiently cost-effective for a general colon cancer screening test [24,25]. Stool DNA-based screening has also been investigated as an approach to confirm positive results in fecal occult blood tests (FOBT) [26]. DNA was successfully amplified from 87.2% of FOBT cards. Combining detection of K-RAS mutation with positive FOBT improved the positive predictive value for malignancy from 17.7 to 60.0%. Using a quantitative beaming-based approach, Diehl *et al.* found 92% of stool DNA samples had the same measurable mutation as the tumor from colon cancer patients [10]. Efforts to develop DNA-based colon cancer screening continue, with a focus on improving DNA isolation from stool or from the mucocellular layer on the surface of the rectal mucosa [22,27,28].

Since mutation in the human KRAS2 proto-oncogene is almost by definition a cancer-relevant marker, rarely is the tumor-relevance of K-RAS mutation discussed. However, it must be recognized that there are numerous reports of K-RAS mutation detection in normal tissues, benign pathological tissues and plasma samples. For example, one study found K-RAS mutation in 30% of plasma samples from non-cancer controls [29]. K-RAS mutation has been detected in normal colonic mucosa of colon cancer patients [30]. Using a sensitive, quantitative allele-specific competitive blocker-PCR (ACB-PCR) approach, K-RAS codon 12 GAT mutation was detected in colonic mucosa samples from individuals without colon cancer and in all mucosa samples from colon cancer patients [31]. K-RAS mutation was detected in the serum of 30% of 40 noncancer hospital controls [32]. Several studies have reported the detection of K-RAS mutation in pancreatic juice from patients with chronic pancreatitis [21]. In fact, when pancreatic juice is tested, K-RAS mutations are found in 20–30% of patients with chronic pancreatitis [33]. Importantly, Shi *et al.* showed that by quantifying levels of K-RAS mutation they could establish a cut-off level above which there was a statistically-significant association between K-RAS mutation and pancreatic cancer, and no association with chronic pancreatitis [34]. Therefore, detection of K-RAS mutation does not necessarily denote the presence of a malignancy. Indeed, the presence of K-RAS mutation in the absence of cancer may explain why removing K-RAS from the PreGen-Plus genetic battery increased the specificity of the test from 91 to 100% [35]. Given the limited progress in applying K-RAS mutation as a cancer screening biomarker and the data indicating

K-*RAS* detection cannot be equated with disease, future research should focus on using sensitive approaches that quantify K-*RAS* mutation in blood or urine and investigate using cut-off values of K-*RAS* mutation as triggers for more rigorous cancer screening.

K-*RAS* mutation as a prognostic biomarker

Prognostic biomarkers are needed for several reasons: to decide whether a more or less aggressive treatment or no treatment is warranted, to aid in patient follow-up and to identify patients who have relapsed or are about to relapse. Because a prognostic biomarker is not applied to very large numbers of healthy individuals, it doesn't need the very high specificity and cost effectiveness that is necessary for a screening biomarker. Compared to the development of a screening biomarker, development of a prognostic biomarker to stratify risk in a cancer patient population is a less demanding task.

A significant body of research indicates K-*RAS* mutational status has prognostic value, whether measured in colorectal tumors or the plasma samples of patients with a K-*RAS*-positive tumor. The large multicenter Kirsten Ras in Colorectal Cancer Collaborative group (RASCAL) studies found no association between K-*RAS* mutation and tumor stage, but showed that detection of a K-*RAS* mutation in a patient's tumor significantly increases the risk of tumor recurrence and death [36–38]. Also, a significant association between K-*RAS* codon 12 mutation and poor 5-year survival has been demonstrated [39]. In particular, the K-*RAS*^{G12V} mutation (codon 12 GGT to GTT) has been associated with poor prognosis, including a 50% increased risk of relapse or death [37,40]. This base substitution apparently causes significant changes in Ras protein function, in terms of its GTP-binding affinity and GTPase activity [40]. In addition, transversion mutations in K-*RAS* codon 12 (including TGT and GTT) are over-represented in smokers as compared with never smokers [41]. Interestingly, the recent study by Winder *et al.* suggests for the first time that K-*RAS* mutations (other than codon 12 valine) in colorectal carcinoma are associated with increased patient survival relative to patients with wild-type tumors [40].

More recent studies have addressed the question of whether detection of K-*RAS* mutation in free circulating DNA can predict tumor recurrence. Using a PCR-single strand conformation polymorphism analysis, Wang *et al.* showed

serum K-*RAS* mutation, in conjunction with *APC* and *p53* mutation, was associated with a higher postoperative metastasis/recurrence rate [42]. Cancer-related, 24-month survival was reached by five out of nine (56%) Dukes' stage D patients without circulating K-*RAS* mutation but none out of seven (0%) Dukes' stage D patients with circulating K-*RAS* mutation [43]. In a multicenter prospective study, K-*RAS* mutation (but not *p53* mutation or p16^{INK4A} methylation) in plasma DNA was significantly associated with accelerated patient relapse [44]. After 3 years, approximately 78% of patients who had a preoperative blood sample characterized as K-*RAS* wild-type survived, whereas only approximately 15% of patients who had a preoperative blood sample characterized as K-*RAS* mutant survived. One study combined detection of K-*RAS* mutation with quantitative measurement of circulating plasma DNA [45]. They concluded that this approach could "confirm the presence of CRC, define disease-free status, and indicate the presence of relapse." Also, in a relatively small study examining pancreatic cancer patients treated with gefitinib, paclitaxel and radiation, patients demonstrating clearance of plasma K-*RAS* mutant DNA had longer survival times than patient with detectable K-*RAS* mutant plasma DNA following treatment [46].

The prognostic value of K-*RAS* mutational status with respect to NSCLC is much less clear. A meta-analysis of studies investigating this question found K-*RAS* mutation negatively impacts survival from NSCLC [47]. Gautschi *et al.* reported that survival of patients with mutant plasma K-*RAS* was significantly worse than that of patients with WT K-*RAS* [48]. By contrast, Camps *et al.* found no significant differences in NSCLC patient survival attributable to serum K-*RAS* mutation [49]. Part of the controversy regarding the use of K-*RAS* mutation as a prognostic biomarker for NSCLC or pancreatic cancer relates to finding different K-*RAS* mutations in circulating DNA and in tumor DNA [50,51].

K-*RAS* mutation as a biomarker for targeted biological therapy selection

Detection of mutation in the various molecular entities comprising the RAS–RAF–MEK–ERK kinase pathway, including K-*RAS* and EGFR, spurred development of therapies directed against these targets [2,3]. These included inhibitors of RAS farnesylation, antisense oligonucleotides against kinase signaling cascade molecules, small molecule kinase inhibitors directed against vascular endothelial growth

factor, the extracellular domain of EGFR, or RAF isoforms (bevacizumab, erlotinib, gefitinib, sorafenib) and antibodies designed to block EGFR dimerization (cetuximab, panitumumab). A major turning point in the application of anti-EGFR therapies came when a number of studies observed that K-RAS mutational status was correlated with therapeutic response, specifically, a K-RAS mutant status could predict failure to respond to these therapies.

The importance of K-RAS mutational status was demonstrated in lung cancer patient response to EGFR-targeted therapy, where the response of lung adenocarcinoma patients to the EGFR thymidine kinase inhibitors, gefitinib and erlotinib, was stratified with respect to K-RAS mutation [52]. K-RAS mutation was detected in none of 12 tumors of patients sensitive to gefitinib therapy and in five out of 12 tumors of patients refractory to gefitinib therapy. K-RAS mutation was detected in none of the nine tumors of patients sensitive to erlotinib therapy and in four out of 26 tumors of patients refractory to erlotinib therapy. In a clinical trial examining the efficacy of erlotinib for advanced NSCLC, none of the five subjects showing a partial response had a K-RAS mutant tumor [53]. In a similar study, none of the 17 patients with a stage IIIB or IV K-RAS mutant NSCLC responded to erlotinib therapy [54]. In patients diagnosed with bronchioloalveolar carcinoma or adenocarcinoma (bronchioloalveolar carcinoma subtype), none of 18 patients with a K-RAS mutant tumor responded to erlotinib [55]. A meta-analysis of studies reporting responses of NSCLC patients to EGFR-targeted agents, as stratified by detection of K-RAS tumor mutation, concluded that K-RAS mutations are highly-specific negative predictors of response to single-agent EGFR thymidine kinase inhibitors [56].

The utility of cetuximab, a mouse-human chimeric monoclonal antibody directed against the EGFR, was investigated in patient populations with advanced CRC. In 2006, Lievre *et al.* stratified cetuximab responders and nonresponders by K-RAS mutational status [57]. Remarkably, they found that none of the 11 responders (0%) had K-RAS mutant tumors, whereas 13 out of 19 (68.4%) nonresponders had K-RAS mutant tumors. This was confirmed in a second study where none of the 12 (0%) responders had K-RAS mutant tumors, but 17 out of 28 (60.7%) patients whose disease progressed had a K-RAS mutant tumor [58]. De Roock *et al.* observed an objective response to cetuximab therapy in 27 out of 66 (40.9%) metastatic colorectal cancer (mCRC)

patients with WT K-RAS, whereas no response was seen in any (0%) of the 42 patients with a K-RAS mutant tumor [59]. Frattini *et al.* observed a partial response to cetuximab therapy in nine out of 17 (52.9%) mCRC patients with WT K-RAS, but a partial response was observed in only one of ten (10%) patients with a K-RAS mutant tumor [60]. Khambata-Ford *et al.* reported K-RAS mutation in tumors of three out of 27 subjects (11%) who responded to cetuximab or had stable disease, whereas K-RAS mutation was detected in 27 out of 53 (51%) tumors from non-responders [61]. Compared with best supportive care alone in a randomized clinical trial, cetuximab treatment significantly improved overall survival and progression-free survival of advanced CRC patients with WT K-RAS tumors [62]. There was no significant difference in overall survival or progression-free survival in an otherwise similar patient population with K-RAS mutant tumors. Cetuximab's use as a first-line treatment, in combination with irinotecan, fluorouracil and leucovorin (FOLFIRI) has also been investigated and, again, the benefit from cetuximab therapy was limited to the K-RAS WT patient population [63].

Panitumumab, a fully-humanized monoclonal antibody directed against the EGFR, was used in several clinical trials for the treatment of patients with mCRC [2]. A meta-analysis of three Phase II clinical trials of panitumumab found 37% of WT patients had progressive disease compared with 79% of patients with a K-RAS mutant tumor [64]. In a Phase III clinical trial comparing panitumumab treatment to best supportive care for mCRC, Amado *et al.* observed the median progression-free survival with panitumumab treatment in the WT K-RAS group was 12.3 weeks, but only 7.3 weeks in the K-RAS mutant group [65]. Benvenuti *et al.* reported K-RAS mutation in one out of 11 responders (9%) and 15 out of 37 nonresponders (40.5%) to panitumumab or cetuximab therapies for CRCs [66].

Antibody-mediated EGFR blockade has become a major research focus and a number of review articles have described the clinical research in this area [38,67,68]. Several clinical trials investigating the efficacy of anti-EGFR antibody therapies in patients with advanced CRC are summarized in TABLE 2. Based on these and the additional studies mentioned above, the American Society of Clinical Oncology (ASCO) issued a provisional clinical opinion stating that these therapies should not be used in patients with K-RAS mutant tumors [69]. In addition, a label change for cetuximab, indicating that it has not been shown to be effective in colon

Table 2. Impact of K-RAS mutational status on efficacy of antibody-mediated EGFR blockade in the treatment of advanced colorectal cancer.

Author (year)	Treatment arms	K-RAS status (sample size) [method]	Response rate (%)		Median progression-free survival (months)		Ref.
			+Ab	-Ab	+Ab	-Ab	
Amado <i>et al.</i> (2008)	Panitumumab versus best supportive care for mCRC	WT (n = 243) MUT (n = 184) [allele-specific real-time PCR]	17 0	0 0	3.1 1.8	1.8 1.8	[65]
Karapetis <i>et al.</i> (2008)	Cetuximab versus best supportive care for advanced CRC	WT (n = 215) MUT (n = 151) [PCR + DNA sequencing]	12.8 1.2	0 0	3.7 1.8	1.9 1.8	[62]
Tol <i>et al.</i> (2009)	Capecitabine + oxaliplatin + bevacizumab ± cetuximab for mCRC	WT (n = 314) MUT (n = 206) [allele-specific real-time PCR]	61.4 45.9	50.0 59.2	10.5 8.1	10.6 12.5	[98]
Bokemeyer <i>et al.</i> (2009)	oxaliplatin + leucovorin + fluorouracil ± cetuximab for mCRC (first-line)	WT (n = 134) MUT (n = 99) [PCR + melting curve analysis]	61 33	37 49	7.7 5.5	7.2 8.6	[99]
Hecht <i>et al.</i> (2009)	Oxaliplatin + bevacizumab ± panitumumab for mCRC (first-line)	WT (n = 404) MUT (n = 260) [allele-specific real-time PCR]	50 47	56 44	9.8 10.4	11.5 11.0	[100]
Hecht <i>et al.</i> (2009)	Irinotecan + bevacizumab ± panitumumab for mCRC (first-line)	WT (n = 115) MUT (n = 86) [allele-specific real-time PCR]	54 30	48 38	10.0 8.3	12.5 11.9	[100]
Van Cutsem <i>et al.</i> (2009)	Irinotecan + fluorouracil + leucovorin ± cetuximab for mCRC (first-line)	WT (n = 348) MUT (n = 192) [PCR + melting curve analysis]	59.3 36.2	43.2 40.2	9.9 7.6	8.7 8.1	[63]

Ab: Antibody; CRC: Colorectal cancer; mCRC: Metastatic colorectal cancer; MUT: Mutation; WT: Wild-type.

cancer patients with K-RAS mutant tumors, was recently approved by the US FDA. Clearly, these studies demonstrate the importance of characterizing tumors for K-RAS mutation as part of the routine management of cancer patients. A brief summary of the College of American Pathologists (CAP) Perspectives on Emerging Technology (POET) report is included in the ASCO provisional clinical opinion [69], whereas the complete report entitled 'KRAS mutation testing for colorectal cancer, February 2009 (Rev 2)' can be accessed from the CAP website [102]. By accurately characterizing patient tumors in terms of K-RAS mutation and applying the therapies described above only to patients with K-RAS WT tumors, responses will be optimized and patients with K-RAS mutant tumors will not be burdened unnecessarily by ineffective and expensive therapies. Furthermore, given the evidence that different K-RAS mutations are associated with different clinical outcomes [36,37,40], the specific K-RAS base substitution in a patient's tumor, rather than just the presence or absence

of a K-RAS mutation, may need to be considered in the selection of therapeutic approach. Also, the fact that patients with K-RAS mutant tumors fail to respond to some of the targeted biological therapies highlights the need for effective therapies appropriate for use in patients with K-RAS mutant tumors [38,70].

Theoretically, therapies that specifically target RAS or signaling molecules downstream from RAS would be appropriate for the treatment of K-RAS mutant tumors. Considerable effort has been directed toward developing targeted biological therapies that abrogate the post-translational processing of RAS, with the idea that mutant K-RAS will not function in signal transduction if it loses its membrane localization [71,72]. Vaccination of cancer patients with mutant K-RAS peptide was performed with the goal of developing tumor-specific immunity [73]. The use of small interfering RNA molecules to knock-down mutant K-RAS expression is being investigated [74]. Nevertheless, there is a need for additional research in this area.

Nature of tumor-associated mutations, methodologies for mutation characterization & data interpretation

Now that K-RAS mutational status has demonstrable clinical significance in the care of cancer patients, understanding the complexities involved in characterizing K-RAS mutational status and the strengths and weaknesses of the methodologies used to characterize K-RAS mutational status has become quite important. Some issues that should be considered are:

- The need to detect tumor-associated mutations, such as K-RAS, as subpopulations within a tumor tissue sample;
- The tissue sampling approach most appropriate for detecting subpopulations of tumor cells within a patient's tissue sample;
- The sensitivity required;
- The feasibility of achieving that sensitivity in the context of patient care;
- Determining the best metric with which to describe K-RAS mutation.

Monoclonal tumor origin persists as a default assumption and significantly influences interpretation of tumor mutation research. Over the years considerable evidence has been collected and published in a number of review articles indicating that many, perhaps most, tumors are polyclonal in origin [75–77]. The clearest example is colon tumors, for which there is definitive evidence of polyclonal tumor origin. Polyclonal tumor origin complicates expectations regarding how K-RAS mutations will persist during tumor development. Most importantly, polyclonal tumor origin predicts K-RAS mutations will be present as subpopulations in many or most tumors, and using sensitive and quantitative methodologies, this has proven to be the case [31,78,79]. Dieterle *et al.* reported ratios of K-RAS mutant:WT DNA ranged over four orders of magnitude (10^0 – 10^{-4}), with the median at approximately 10^{-2} [78]. Additional indirect evidence that tumors contain K-RAS mutant subpopulations comes from studies that compare a sensitive mutation detection approach to standard DNA sequencing and report higher numbers of K-RAS-positive tumors using the more sensitive methodology [80,81]. For example, Beau-Faller *et al.* detected a subset of K-RAS-positive tumors using a peptide nucleic acid-mediated PCR clamping technique that were K-RAS negative by DNA sequencing, even though the samples were characterized as having

a sufficient percentage of tumor cells for DNA sequencing analysis [79]. They concluded these samples had mutant tumor subclones.

Mutant subpopulations of tumor cells can be explained in two different ways, an initial heterogeneity due to polyclonal tumor origin, or heterogeneity that arises during tumor progression. Even when K-RAS is an initiating mutation, additional heterogeneity may occur during polyclonal tumor outgrowth. For example, a K-RAS mutant clone might be outgrown by a more aggressive, initially complementing clone carrying a tumor suppressor gene mutation. There is considerable evidence in the literature of initial heterogeneity in preneoplastic lesions that is lost in the carcinoma stage, suggesting a single clone frequently predominates as tumors progress and metastasize [77]. Indeed, it has been concluded that “considering the genetic heterogeneity of colorectal cancers, the absence of detectable K-RAS mutations in the primary tumor cannot formally exclude the presence of KRAS mutation in metastases” [82]. There is evidence that K-RAS codon 12 GTT mutations are lost during colonic adenoma to adenocarcinoma progression [31]. This quantitative data is consistent with the qualitative colon tumor data in the COSMIC database, where 36 out of 352 adenomas (10.2%) but only 146 out of 1976 adenocarcinomas (7.4%) carry the K-RAS codon 12 GTT mutation [101]. Conversely, K-RAS codon 12 GAT mutation becomes more prevalent during progression; 39 out of 352 adenomas (11.1%) and 262 out of 1976 adenocarcinomas (13.3%) carry the K-RAS codon 12 GAT mutation [101]. This aspect of plasticity in tumor-associated mutations may explain why numerous studies report a fraction of serum, plasma, or urine samples contain a different K-RAS mutation than that found in the patient's tumor [29,83,84]. Regardless of their origin or plasticity during tumor progression, the fact that K-RAS mutations are present as subpopulations in tumors means that methodologies more sensitive than standard DNA sequencing are needed to accurately interpret their significance.

The impact of tumor subpopulations on the results of clinical trials is an open question. It is clear that patients with K-RAS mutant tumors fail to respond to therapies directed against the EGFR. What is unclear, however, is to what extent patients characterized as K-RAS WT have undetected K-RAS mutant tumor subpopulations that affect their response to therapy. In this regard, it is important to note that clinical trials find significant fractions of K-RAS WT patients who also do not respond to anti-EGFR therapies.

Failure to respond may be due to a genetic lesion in a different molecular entity in the RAF–MEK–ERK kinase pathway. However, until K-RAS mutant:WT levels are determined and associated with particular therapeutic responses, the possibility that failure of anti-EGFR therapies is due to undetected K-RAS mutant tumor subpopulations cannot be excluded. One study, however, suggests that small K-RAS subpopulations may not be clinically important [79]. A weak association was detected between poor event-free survival of NSCLC patients and K-RAS mutation (as measured by DNA sequencing); however, the association was not apparent using mutational data collected with a more sensitive method.

Because K-RAS mutation has become important to the practice of oncology, clinicians will need to work closely with pathologists to select a molecular diagnostic approach and appropriate samples to analyze [85]. K-RAS mutation testing may be performed in house at many oncology centers. Although there is currently no US FDA-approved test for K-RAS mutation, several companies currently provide K-RAS testing services and their numbers are likely to increase in the future [69,85]. A variety of methods have been developed for the analysis of K-RAS mutation and have been reviewed [85–87]. Reliable approaches for K-RAS mutation detection include methods based on separation of electrophoretic variant sequence, including single-strand conformation polymorphism (SSCP), heteroduplex analysis (HA), thermal gradient capillary electrophoresis (TGCE) and constant denaturation capillary electrophoresis. TABLE 3 describes a sampling of assays and technologies recently used to characterize the K-RAS mutation in clinical specimens. TABLE 3 also illustrates that assay sensitivity may not be established in the context of clinical sample analyses and that most of the approaches for the analysis of clinical samples are not quantitative. As additional personalized oncology therapies are developed, the use of companion diagnostics may be evaluated as part of clinical trials, thereby providing efficacy information for a drug in the context of its use with a companion diagnostic [88,103]. It is likely that the clinical need for personalized patient information will drive development of more sensitive and quantitative methods to characterize tumor-associated mutations. Because of the need to detect mutant subpopulations of tumor cells, sensitivity is a critical issue in companion diagnostic development. A recent review describes a proposal for a European quality assurance program as it relates to clinical K-RAS mutation testing [86]. The recommendation put forward in this

review is that “a lower detection limit of mutant signal should be set at 1% of tumor cells for allele-specific PCR and 25–30% for direct sequencing.” However, as mentioned above, when a sensitive methodology was applied, colon tumors were shown to have a median K-RAS mutant fraction of approximately 10^{-2} [78]. Therefore, a cut-off of 1% would fail to detect K-RAS mutations in approximately half of all samples, and a majority of K-RAS mutations would be missed if samples were analyzed by standard DNA sequencing. Presumably, the 1% cut-off is based on the idea that low levels of K-RAS mutation will not be clinically relevant. However, the clinical significance of low levels of K-RAS mutation cannot be known until they are quantified and particular levels of mutation are associated with clinical response. The possibility that therapies directed against predominantly WT K-RAS tumors could provide an opportunity for the clonal outgrowth of small subpopulations of K-RAS mutant tumor cells should be considered.

Several aspects of clinical sample analysis require additional investigation and optimization. Methods for circulating tumor DNA isolation from plasma or urine samples need to be optimized because different DNA isolation approaches have been shown to produce different results [84,89]. Optimization of sample collection techniques also needs to be considered. The proposal for the European quality assurance program suggests that the optimal material is to evaluate for K-RAS mutation are preserved tissue blocks from the primary tumor, if available, or its metastases [86]. If K-RAS mutational testing is to become a routine part of clinical care, when appropriate, the storage of a representative portion of a patient's tumor as unfixed frozen tissue is much preferable for sensitive mutational analyses. If K-RAS mutation is present as small subpopulations within tumors, then complete exclusion of nontumor cells becomes less important than analyzing a fairly large representative sample of tumor tissue and avoiding artifacts due to fixation. Methodologies used to characterize K-RAS mutation need to be quantitative. The use of any end point as a cancer biomarker in the absence of quantification is unlikely to produce consistently useful results. While sensitive and quantitative research methods to measure K-RAS mutation exist, many are too cumbersome to be appropriate in a clinical setting [78,90]. Fortunately, there are a few technologies on the horizon that may prove useful in this regard. One promising approach described by Diehl *et al.* uses real-time PCR to quantify the amount of circulating tumor DNA

Table 3. Recent approaches to K-RAS mutational analysis*.

Method name	Method description	Sensitivity	Quantitative	Ref.
RFLP-PCR	Measures mutations that alter a restriction endonuclease recognition sequence; digestion by a restriction enzyme reduces the amount of wild-type sequence, PCR with primers flanking the restriction site amplifies the uncut mutant sequence	10^{-6} – 10^{-8} †	No	[11]
Mutant allele-specific amplification with denaturing high-pressure liquid chromatography (MASA/DHPLC)	An allele-specific PCR (3' end of primer matches mutation being detected) is followed by heteroduplex formation and identification of heteroduplexes by DHPLC	10^{-3} †	No	[8]
Peptide nucleic acid-mediated PCR clamping and melting curve analysis	Capillary PCR performed using a wild-type peptide nucleic acid clamp and melting temperature analysis performed using mutant-specific probes with different fluorophores	$\sim 10^{-3}$ – 10^{-4}	No	[92]
PCR, mutant allele enrichment, and denaturing gradient gel electrophoresis (PCR/MAE/DGGE)	PCR amplification followed by restriction digestion to enrich for mutant sequence, then analyzed by DGGE	$\sim 10^{-4}$ – 10^{-5} †	No	[19]
Isothermal multiple displacement amplification (IMDA)	IMDA provides uniform amplification of clinical sample DNA, K-RAS mutation was analyzed by PCR and DNA sequencing	NSR	No	[93]
Membrane array	Poly(A)+ RNA isolated from blood is used to synthesize a cDNA and probe membranes containing 22 genes up-regulated by mutant K-RAS	5 mutant cells/1 ml of blood	No	[94]
Real-Time PCR and DNA melting curve analysis	Real-time PCR performed on a LightCycler with specific anchor probes. Melting of PCR product and anchor probe is monitored as fluorescence, with specific K-RAS mutations identified by Tm	10^{-3}	No	[81]
Capillary PCR with peptide nucleic acid clamp/sensor probe	Capillary PCR performed with a peptide nucleic acid complementary to the wild-type sequence and spanning codons 12 and 13 of K-RAS reduces wild-type amplification and acts as sensor probe in melting temperature analysis	10^{-4}	No	[13]
Enriched-PCR/RFLP	Multiple rounds of PCR and digestion of WT K-RAS codon 12, followed by direct sequencing	NSR	No	[14]
Real-time PCR with peptide nucleic acid clamp	Real-time PCR performed in the presence of a peptide nucleic acid sequence specific for wild-type K-RAS codons 12 and 13	NSR	No	[82]
COLD-PCR/sequencing	Preferentially enriches 'minority alleles' by converting mutant into heteroduplex and performing PCR using a temperature that will denature heteroduplex but not abundant homoduplex, mutations are identified by DNA sequencing	10^{-2}	No	[95]
Real-time PCR with bead, emulsion, amplification and magnetics (BEAMing)	Sequence of interest is amplified in a PCR adding tag sequences, the tag sequences are used to amplify and attached DNA to beads isolated in emulsions, specific sequences are identified by fluorophore-specific oligonucleotide hybridization analyzed by flow cytometry	10^{-4}	Yes	[10,91]
LigAmp assay	Two oligonucleotides are hybridized adjacent to the basepair being interrogated, if a base substitution is present in the target DNA the oligonucleotides are ligated and then detected by real-time, quantitative PCR	10^{-4}	Yes	[34]
Single-strand conformation polymorphism-capillary electrophoresis (SSCP-CE)	Combined a universal fluorescent primer with PCR products mutant in codons 12 or 13 separated and identified by CE	NSR	No	[96]
Mutant allele-specific amplification with electrochemiluminescence (MASA/ECL)	An allele-specific PCR (3' end of primer matches mutation being detected) conducted on magnetic beads with detection based on ECL emission of $\text{Ru}(\text{bpy})_3^{2+}$	10^{-4}	No	[97]

*Quantification is rare and studies do not always document the sensitivity of applied methods.

†Sensitivity cited by authors, but not demonstrated/evaluated as part of the study. CE: Capillary electrophoresis; COLD: Co-amplification at lower denaturation temperature; DHPLC: Denaturing high-pressure liquid chromatography; ECL: Electrochemiluminescence; NSR: No sensitivity reported; RFLP: Restriction fragment length polymorphism.

in plasma, along with an approach called 'beaming' (TABLE 3) to determine the ratio of mutant:WT DNA in the circulating tumor DNA [10,91]. The 'LigAmp' assay is another promising approach that has been used successfully to establish clinically relevant levels of K-RAS mutation [34].

Conclusion

Detection of K-RAS mutation in various bodily fluids cannot be equated with cancer and is unlikely to lead to a successful cancer screening strategy. Approaches that quantify K-RAS mutation in plasma or urine and use a level of K-RAS mutation as a trigger for additional cancer screening have potential and should be investigated. The presence of K-RAS mutation in a patient's tumor does have clinical significance in terms of patient prognosis and therapy selection.

Future perspective

As the archetype mutational biomarker, past experiences with K-RAS mutation in the clinical setting should inform future efforts toward personalized medicine. K-RAS mutation may be present in a subpopulation of tumor cells and K-RAS mutant fraction may change during progression and metastasis, making the

measurement of K-RAS more challenging than is generally recognized. Determining if K-RAS mutation is present or absent from patient samples is not a sufficiently rigorous characterization of mutational status. Progress in understanding the significance of particular levels of K-RAS mutations with respect to therapeutic outcomes will only be achieved when quantitative data begin to be collected and analyzed.

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Executive summary

Significance of K-RAS as a mutational biomarker

- K-RAS mutations are detected in significant fractions of many human cancers.
- K-RAS mutation disrupts normal cell proliferation and induces prosurvival factors, thus the K-RAS mutant phenotype drives carcinogenesis.
- Mutations are clustered at codon 12, making K-RAS codon 12 the most cancer-prevalent mutational target.

K-RAS mutation in cancer screening

- Studies have investigated K-RAS mutation in blood/plasma/serum, urine, stool, pancreatic juice, sputum and bronchoalveolar lavage fluid.
- To date, no cost-effective cancer screening strategy has been developed based on K-RAS mutation.

K-RAS mutation in cancer prognosis

- In colorectal cancer patients, K-RAS mutation, whether measured in tumor or plasma/serum, has been associated with poor prognosis.
- In non-small-cell lung cancer patients, the value of plasma/serum K-RAS as a prognostic biomarker remains an open question.

K-RAS mutation in personalized medicine

- K-RAS mutation predicts failure to respond to targeted biological therapies directed against the EGFR.
- Better approaches for treating patients with K-RAS mutant tumors are needed.

Nature of tumor-associated mutations & methods for mutation characterization

- K-RAS mutation can be present in subpopulations of tumor cells.
- Sensitive mutation detection methods frequently detect mutations in tumors that are missed by standard DNA sequencing.
- For personalized use of targeted biological therapies to be successful, sensitive and quantitative measurement of tumor-associated mutations will be required.

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- 202 College of American Pathologists Perspectives on Emerging Technology (POET) report. www.cap.org/apps/docs/committees/technology/KRAS.pdf
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