



RAS mutations and oncogenesis: not all RAS mutations are created equally

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Mutation in RAS proteins is one of the most common genetic alterations observed in human and experimentally induced rodent cancers. *In vivo*, oncogenic mutations have been shown to occur at exons 12, 13, and 61, resulting in any 1 of 19 possible point mutations in a given tumor for a specific RAS isoform. While some studies have suggested a possible role of different mutant alleles in determining tumor severity and phenotype, no general consensus has emerged on the oncogenicity of different mutant alleles in tumor formation and progression. Part of this may be due to a lack of a single, signature pathway that shows significant alterations between different mutations. Rather, it is likely that subtle differences in the activation, or lack thereof, of downstream effectors by different RAS mutant alleles may determine the eventual outcome in terms of tumor phenotype. This paper reviews our current understanding of the potential role of different RAS mutations on tumorigenesis, highlights studies in model cell culture and *in vivo* systems, and discusses the potential of expression array and computational network modeling to dissect out differences in activated RAS genes in conferring a transforming phenotype.

Keywords: ras, cancer, mutation, tumorigenesis

INTRODUCTION

The RAS gene family consists of three small G proteins – Ha-, N-, and Ki-*ras* (with Ki-*ras* existing as the predominant Ki-*ras*4B and the alternatively spliced Ki-*ras*4A isoforms) – that play a central role in cell signaling (Barbacid, 1987; Malumbres and Barbacid, 2003). RAS proteins are anchored on the cytoplasmic side of the cell membrane, where they mediate signal transduction downstream from tyrosine kinase membrane receptors to a variety of effector molecules, stimulating a cascade of parallel phosphorylation reaction pathways that ultimately culminate with the activation of nuclear transcription factors. The three main effector pathways that are activated downstream of RAS – RAF/MEK/MAPK, PI3K/AKT, and RAL-GDS – play major roles in mediating signals relating to cell proliferation, cell survival, cell adhesion, and cell motility (Fan and Bertino, 1997; Campbell et al., 1998; Gille and Downward, 1999; Zuber et al., 2000; Bounacer et al., 2004). Each of the RAS isoforms appears to differentially regulate its downstream effectors *in vivo*, resulting in marked differences in the strength and type of signal produced (Hancock, 2003; Ehrhardt et al., 2004; Moon, 2006; Omerovic et al., 2008). This differential signaling appears to be mediated partly by the trafficking pathways used by each RAS isoform to reach the plasma membrane, as well as the location of each isoform in the plasma membrane itself (Chiu et al., 2002; Hancock, 2003; Moon, 2006); N- and Ha-*ras* associate with lipid rafts in the plasma membrane, whereas Ki-*ras* appears to be located in non-raft domains.

The RAS pathway is one of the most prevalent oncogenic alterations in both human and experimentally induced animal tumors (Bos, 1989; Conti, 1992; Malumbres and Barbacid, 2003). *In vivo*, oncogenic mutations have been shown to occur at exons 12, 13, and 61, resulting in any 1 of 19 possible point mutations for each RAS isoform. When stimulated by upstream signaling molecules, wild type RAS proteins interact with guanine nucleotide exchange factors to replace GDP with GTP, resulting in an activated protein conformation. RAS activity is terminated by interaction with GTPase activating protein, which stimulates the GTPase activity of the protein and converts GTP back to GDP, thereby restoring the inactive form of RAS. Mutations in RAS inhibit the GTPase activity and lock the protein in the active GTP bound conformation (Barbacid, 1987; Bos, 1989; Malumbres and Barbacid, 2003). In particular, mutations in the Ki-*ras* gene have been shown to play a key role in the pathogenesis of a variety of human tumors, with mutations occurring in 95% of pancreatic tumors, 50% of colon tumors, and 30% of lung adenocarcinomas (Barbacid, 1987; Bos et al., 1987; Conti, 1992; Malumbres and Barbacid, 2003). Of these cancers, lung and colon cancer are the first and second leading cause of cancer-related deaths in the U.S., respectively (Jemal et al., 2010). Among the candidate genes implicated in the initiation of these cancers, Ki-*ras* has received considerable attention as mutations in Ki-*ras* appear in early neoplastic lesions in both human and experimentally induced murine lung and colon tumors, and influence both tumor progression and drug resistance (Cerny et al., 1992; Reynolds et al., 1992; Hruban et al., 1993; Westra et al., 1993, 1996; Li et al., 1994a; Miller, 1994; Gryfe et al., 1997; Grady and Markowitz, 2002; Agbunag and Bar-Sagi, 2004; Fleming et al., 2005). The spectrum of RAS mutations differs by organ site and allele frequency,

Abbreviations: AC, adenocarcinomas; AD, adenomas; CCSP, Clara cell secretory protein; DOX, doxycycline; IHC, immunohistochemistry; RT-PCR, reverse transcription-polymerase chain reaction; rtTA, reverse tetracycline *trans*-activator; tet, tetracycline.

probably as a result of different environmental exposures and tissue specific differences in RAS expression. The Sanger Institute's COSMIC database (Catalog of Somatic Mutations in Cancer; http://www.sanger.ac.uk/genetics/CGP/cosmic/add_info/) integrates data from the published literature on type and frequency of somatic mutations in human cancers. Using the database search tools, we examined the spectrum and frequency of *Ki-ras* mutations (Table 1). Consistent with the literature, *Ki-ras* mutations were observed most frequently in cancers of the lung, large intestine (including colon, rectal, and anal), pancreas, and biliary tract (including bile duct and gall bladder). Based on a collective mutational analysis involving > 15,000 tumors, the most frequent alterations observed were point mutations at codons 12, 13, and 61. A spectrum of predominant mutant alleles were observed, and their relative frequencies are shown in Table 1 as the percentage of all mutant alleles observed for a given tumor type. Consistent with historical observations, ASP¹², VAL¹², and CYS¹² emerged as the predominant mutant *Ki-ras* alleles. However, that the alleles distributed with large variation within each cancer type may reflect the non-redundant functions of the different alleles in tumorigenesis. Large variation across cancer types was also observed for some alleles, such as CYS¹², ASP¹², and ASP¹³, suggesting that mutant allele functionality may also depend, to some extent, on the tumor tissue of origin.

Although some studies have provided evidence for mutation specific effects of different mutant RAS alleles, most studies and therapeutic approaches have treated RAS mutations as a single entity – the gene is either mutated or wild type. We believe that the different RAS mutations exhibit subtle differences in their ability to signal to their downstream effectors, which may impact

their relative contribution to the carcinogenic process, their role as driver mutations, and tumor responsiveness to novel therapeutic agents that target RAS or its downstream effectors. Thus, this manuscript reviews the evidence obtained from biochemical, cell culture, and animal model data, as well as the limited number of human studies available, documenting the differential response of cells to different mutant RAS alleles.

IN VITRO EVIDENCE FOR DIFFERENTIAL EFFECTS OF DIFFERENT MUTANT RAS ALLELES

Studies on the potential differences in the mutagenicity/oncogenicity of different RAS mutant alleles began shortly after the identification of RAS as a transforming oncogene. Initial studies demonstrated that different *Ha-ras* mutant alleles exhibited differences in their ability to transform mouse fibroblasts (Fasano et al., 1984; Seeburg et al., 1984; Der et al., 1986). Focusing on the *Ha-ras* gene, Fasano et al. (1984) found that the VAL¹² mutation was the most potent in terms of the induction of focus formation in the NIH3T3 assay, with ARG¹², ASP¹², SER¹², ASP¹³, and SER¹³ exhibiting transforming efficiencies that were 60, 50, 40, 20, and 0.1% relative to the VAL¹² mutant. Seeburg et al. (1984), found somewhat similar results in Rat-1 cells with mutants to both *Ki-ras* and *Ha-ras*. Although not as quantitative as Fasano et al. (1984), these authors found that alleles of both *Ki-* and *Ha-ras* containing the VAL¹² and ARG¹² mutations exhibited greater transforming activity than alleles with the CYS¹², ASP¹², ASN¹², and SER¹² mutations. All of the mutant alleles exhibited growth in soft agar. Der et al. (1986) examined 17 different codon 61 mutations in the *Ha-ras* gene and found that the transforming activity in NIH 3T3 cells varied by more than 300-fold between the different mutant alleles.

Several groups (Gibbs et al., 1984a,b; McGrath et al., 1984; Sweet et al., 1984; Manne et al., 1985), using purified *Ha-ras* produced in *E. coli*, demonstrated that the valine 12 mutant exhibited 5 to 10-fold lower GTPase activity than the normal wild type allele. It was initially thought that the relative level of GTPase activity might account for the differences in transforming potential between the different mutant alleles. However, the studies by Der et al. (1986) and Colby et al. (1986) did not find any correlation between the transforming potency and GTPase activity of the different alleles. Subsequent to these early studies, the biochemical activity of RAS and its interactions with its downstream receptors has continued to be the focus of intensive investigations. Several laboratories have demonstrated that mutations in specific amino acids have very significant effects on both guanine nucleotide exchange factors and RAS GTPase activity (Nielsen et al., 2001; Zhang et al., 2005; Filchtinski et al., 2010; Lukman et al., 2010). However, many of these mutational analyses have been limited to examining amino acids that cause conformational changes in regions of the RAS genes associated with GTPase activity, such as the A59G variant (Lukman et al., 2010), rather than the common mutagenic variant alleles associated with tumorigenesis. More recently, redox agents such as reactive oxygen species and reactive nitrogen species have been shown to enhance the rate of guanine nucleotide exchange as a result of the formation of a thiyl radical on CYS¹¹⁸ *Ha-ras* (Del Villar et al., 1996; Kjeldgaard et al., 1996; Reuther and Der, 2000; Ford et al., 2002; Jourdeuil et al., 2003; Schrammel et al., 2003;

Table 1 | Relative frequencies of the major *Ki-ras* mutations by cancer type: analysis of the Sanger COSMIC database.

<i>Ki-ras</i> mutation [#]	Lung	Large intestine [*]	Pancreas	Biliary tract [^]
CYS ¹²	41.77	8.62	3.25	8.33
ASP ¹²	17.23	34.56	49.12	48.06
VAL ¹²	20.25	22.59	29.85	18.02
ALA ¹²	6.35	6.26	2.05	4.26
SER ¹²	4.49	6.14	2.63	11.43
ARG ¹²	2.21	1.34	12.02	4.07
PHE ¹²	0.74	0.10	0.06	0.00
ASP ¹³	2.39	18.70	0.61	3.10
CYS ¹³	3.19	0.48	0.09	0.97
ARG ¹³	0.11	0.23	0.00	0.78
SER ¹³	0.14	0.11	0.00	0.58
VAL ¹³	0.04	0.11	0.03	0.00
ALA ¹³	0.04	0.10	0.00	0.00
HIS ⁶¹	0.32	0.29	0.29	0.19
LEU ⁶¹	0.42	0.21	0.00	0.19
ARG ⁶¹	0.32	0.14	0.00	0.00
ASP ⁶¹	0.00	0.01	0.00	0.00

[#]The relative percentage of each mutant allele within a cancer type (i.e., of the four predominant *Ki-ras* mutation-bearing cancer types) is shown; ^{*}includes colon, rectal, and anal cancer; [^]includes cancers of the bile duct and gallbladder.

Williams et al., 2003; Heo et al., 2005, 2006; Davis et al., 2011). This raises the possibility that codon 12, 13, and 61 mutations could affect RAS GTPase activity by either (1) increasing oxidative stress and thereby increasing thiylation of CYS¹¹⁸ or (2) altering the accessibility of the CYS¹¹⁸ residue to modification by reactive oxygen species. As will be discussed below, the CYS¹² allele has been shown in a variety of experimental systems to exhibit less potent tumorigenic activity than other mutations in codon 12. It is thus possible that the CYS¹² variant could provide a new redox active cysteine motif in the RAS protein that differentially responds to the increased oxidative stress of the cancer cell environment in a different manner than other mutant RAS alleles. Clearly, none of these potential mechanisms are mutually exclusive and suggest new areas of inquiry into the mechanisms of the observed differential effects of RAS mutants on tumorigenicity.

IN VIVO EVIDENCE FOR DIFFERENTIAL EFFECTS OF DIFFERENT MUTANT RAS ALLELES

The initial studies utilizing the NIH3T3 focus assay were confirmed using *in vivo* models. Studies from this and other laboratories have demonstrated an association between the histological stage of both murine and human lung tumors and the presence of specific mutant RAS alleles in tumor tissue. Early studies by Nuzum et al. (1990) demonstrated that, following treatment of adult A/J mice with urethane, mouse lung adenocarcinomas (ACs) had a high incidence of GLU⁶¹ → ARG⁶¹ (CAA → CGA) mutations relative to the smaller adenomas (ADs), which preferentially exhibited GLU⁶¹ → LEU⁶¹ (CAA → CTA) mutations. Li et al. (1994b) also demonstrated that lung ACs showed a higher incidence of mutations at the second base of codon 61 following treatment of newborn mice with nitrochrysene and its metabolites. Utilizing a transplacental treatment protocol, whereby mice are exposed *in utero* to a single dose of the chemical carcinogen 3-methylcholanthrene, we have demonstrated in three independent studies using different strains of mice that different RAS mutations are associated with tumor stage (Leone-Kabler et al., 1997; Gressani et al., 1999; Jennings-Gee et al., 2006). Treatment of pregnant mice with 3-methylcholanthrene resulted in a high incidence of lung tumors in the offspring 6–12 months after birth. Mice harboring a VAL¹², ARG¹², ASP¹², or ARG¹³ mutant *Ki-ras* gene were more likely to contain later stage tumors than mice with the CYS¹² or wild type allele, which exhibited mostly benign ADs and hyperplasias. Further work with this model also suggested that the type of mutation induced in *Ki-ras* following *in utero* exposure to the chemical carcinogen was associated with specific types of damage (hypermethylation vs. base pair mutations) at the *Ink4a* gene locus (Mizesko et al., 2001). Interestingly, the mutant *Ki-ras* alleles associated with progression to later stage tumors in our transplacental mouse studies were the same ones associated with a trend for poorer patient outcomes in a clinical study of human lung cancer (Keohavong et al., 1996). A clinical study examining the prognostic significance of *Ki-ras* mutations in lung cancer patients found that patients containing CYS, ARG, and ASP mutations at codon 12 appeared to have a poorer prognosis than those containing hydrophobic amino acid substitutions such as VAL or ALA (Siegfried et al., 1997). However, the sample sizes for this analysis were small and the authors did not find an overall association of

Ki-ras mutations and poorer patient survival, as has been noted in several other studies (reviewed in Rodenhuis and Slebos, 1992). Similar observations have been made in colon cancer, as Finkelstein et al. (1993a,b) reported that the *Ki-ras* ASP¹² mutant allele was associated with the metastatic properties of colon tumors.

In addition to the ability of different RAS mutant alleles to initiate tumor formation, the above mentioned results obtained *in vivo* also suggest differences in tumor progression imparted by the different RAS variant alleles. In the *in vivo* studies cited above, a consistent finding was that more aggressive tumors (i.e., ACs) were more prevalent with one type of RAS mutation, often the VAL¹² allele, whereas benign lesions such as ADs or hyperplasias were more prevalent with other types of RAS variants, most notably the CYS¹² mutation. These results suggest that specific RAS mutant alleles can impart a greater growth advantage than other alleles, and the often disparate results may be due to context and organ dependent effects of the different alleles (Guerra et al., 2003). Studies by Cespedes et al. (2006) have extended these findings and also confirmed the relatively weak transforming activity of the *Ki-ras* CYS¹² allele observed in our studies. These authors transfected NIH3T3 cells with the ASP¹², VAL¹², and CYS¹² alleles of *Ki-ras*. When the transforming properties of the alleles were assessed, the *Ki-ras* VAL¹² allele exhibited a more aggressive tumorigenic phenotype than the *Ki-ras* ASP¹² allele, which was attributed to the inability of the ASP¹² allele to signal through the RAF/MEK/ERK pathway. When the transfected cells were injected into nude mice, the *Ki-ras* CYS¹² containing cells failed to establish tumors.

Along the same lines, studies by this group utilizing NIH3T3 cells found that the *Ki-ras* VAL¹² allele, in contrast to the wild type and *Ki-ras* VAL¹³ allele, exhibited increased glycolysis (Vizan et al., 2005). In addition, cells transfected with the VAL¹² mutant allele were resistant to the induction of apoptosis induced by confluence and exhibited a much greater ability to grow in soft agar. The VAL¹² mutant clones exhibited elevated levels of p-AKT, increased expression of Bcl-2, E-cadherin, β -catenin, and focal adhesion kinase, and decreased expression of RhoA (Guerrero et al., 2000). Similarly, Recktenwald et al. (2008) found that the VAL¹² and ASP¹³ variants of *Ki-ras* enhanced cell survival and resistance to oxidative stress. Although they did not specifically address this in their paper, the VAL¹² allele exhibited greater protection against formaldehyde- and H₂O₂-mediated toxicity and reduced caspase 3/7 activity relative to the ASP¹³ allele. Thus, a generally consistent finding across the animal studies, cell culture experiments, and human patient samples suggests that the CYS¹² allele is associated with a relatively weak or no transforming activity, while VAL¹² and ASP¹² alleles were associated with more aggressive oncogenic properties. These transforming properties were not only associated with increased tumor formation, but seemed to also play a role in tumor progression.

The somewhat conflicting studies with human samples most likely result from the multitude of alterations that occur in human tumors by the time they are diagnosed. It is very likely that tumors with weakly transforming RAS alleles may require additional alterations at other oncogenic loci in order to drive tumorigenesis. Thus, in some cases, a RAS mutation such as the VAL¹² allele could act as a driver mutation whereas in other cases, such as the CYS¹² allele, mutations in other genes may be the key drivers of

tumorigenesis. A major limitation of studies utilizing human tissue is the fact that human tumors contain several mutations in a variety of oncogenic loci. If a weakly oncogenic *Ki-ras* mutation occurs in a particular patient's tumor, it is likely that by the time the tumor is isolated, mutations in other critical driver genes would have occurred. To our knowledge, no studies have been published which attempt to correlate specific mutations in *Ki-ras* with the presence or absence of other known driver mutations. Thus, attempts to associate specific RAS mutations with patient outcome or survival may not be able to distinguish the relative contribution of a specific *Ki-ras* mutation to the tumorigenic process.

In order to characterize the role of *Ki-ras* mutations in the initiation of lung tumorigenesis, several investigators have utilized inducible expression systems in transgenic mice. These included: (1) regulation of a murine *Ki-ras* ASP¹² cDNA transgene via a doxycycline (DOX) regulated promoter (Fisher et al., 2001); (2) sporadic activation of a human *Ki-ras* VAL¹² cDNA transgene via a *Cre-lox* mediated recombination construct (Meuwissen et al., 2001; Guerra et al., 2003); (3) spontaneous activation of the murine *Ki-ras* ASP¹² gene via a somatic recombination system (Johnson et al., 2001); and (4) sporadic activation of the same murine *Ki-ras* ASP¹² gene mutation via a *Cre* based *lox-stop-lox* construct (Jackson et al., 2001). Expression of these mutant *Ki-ras* alleles were shown to be a strong oncogenic stimulus for lung epithelial cells, resulting in significant increases in lung tumorigenicity. In each of these models, 100% of the mice developed aggressive ACs and died within 2–10 months from their lung tumor burden.

In contrast to these results our laboratory, utilizing a transgenic mouse in which the mutant human *Ki-ras* CYS¹² allele is regulated in a DOX-inducible and lung-specific manner (Floyd et al., 2005), found that these mice developed hyperplasias and small, benign adenomas (ADs) after 12 months of DOX treatment that rarely progressed to the carcinoma stage. The CYS¹² transgene appeared to signal to a subset of its downstream effectors, exhibiting increases in proliferative and both anti- and pro-apoptotic signals, as well as up-regulation of cell cycle inhibitory molecules. Mice harboring this mutant *Ki-ras* allele exhibited increased signaling through the RAS/RAF/ERK/cyclin D1, p38, and RAL/GDS pathways, but no alterations in signaling through the JNK and PI3K/AKT pathways (Floyd et al., 2006; Dance-Barnes et al., 2008). Interestingly, the relatively benign tumor phenotype observed in *Ki-ras* CYS¹² mice was also observed in mice with activated and wild type RAF genes (Kerkhoff et al., 2000). These authors developed a strain of mice expressing an activated human *c-raf-1* transgene specifically in the lung (SPC-*c-raf-1*-BXB mice) that also exhibited a relatively benign tumor phenotype, despite increased phosphorylation of downstream effector molecules in the ERK pathway. Because mutant RAF is downstream of RAS and thus signals to a specific subset of RAS effector molecules, these experiments provide further *in vivo* confirmation of the need for the activation of multiple RAS downstream effector molecules to mediate the full transformation of lung epithelial cells and provide the most definitive evidence to date of the potential for different *Ki-ras* mutations to exhibit differential effects on the carcinogenic process. With the limitations that these studies often involved different inducible gene systems, different strains of mice, and the

unknowns of the potential effects of integration of the transgene in the mouse genome, future studies will need to take advantage of targeted knock-in technologies to create a series of transgenic mouse strains that replace the endogenous mouse *Ki-ras* gene with various mutated alleles and allow the mutants to be expressed from the natural murine *Ki-ras* promoter. These types of studies will be critical in dissecting out the effect of different RAS mutations on downstream signaling pathways and tumorigenicity.

DIFFERENTIAL EXPRESSION OF *Ki-ras* MUTANTS IN HUMAN LUNG TUMORS

Several studies using human tissue samples and cell lines (Bhattacharjee et al., 2001; Beer et al., 2002; Miura et al., 2002; Virtanen et al., 2002; Wikman et al., 2002; Creighton et al., 2005) have utilized RNA and cDNA microarray analyses to document the complex array of alterations in signaling pathways that accompany lung tumorigenesis. Yao et al. (2002) isolated lung tumors 6 and 14 months following treatment of A/J mice with a single injection of *N*-methylnitrosourea at 6 weeks of age. Using the Affymetrix Atlas MouseTM cDNA Expression Array, consisting of 588 known mouse genes, they were able to identify 19 genes that showed differential expression between ADs and ACs, as well as 10 genes that exhibited similar alterations in expression levels between the two tumor types. Subsequent studies from the same group, using the more extensive Affymetrix Mu74Av2 chip, which can interrogate 36,000 full-length mouse genes and EST clusters from the Unigene Database, identified 50 genes that were either up- or down-regulated in ADs vs. ACs, including genes involved in cell cycle control, differentiation, and apoptosis (Bonner et al., 2004). In addition, when the murine tumors were compared with lung ACs isolated from human patients, the murine ADs and ACs clustered with two groups of human ACs that differed in their differentiation status, with murine ADs clustering with the well differentiated human ACs and murine ACs clustering with the less differentiated tumors. These investigators also identified 39 genes that were similarly regulated in murine and human lung ACs, further emphasizing the appropriateness of the mouse as a model for human lung cancer. Similarly, Jacks' group (Sweet-Cordero et al., 2005) used the data obtained from this study in combination with their own analysis of mouse tumors from transgenic *Ki-ras*^{LA} mice, which express the *Ki-ras* ASP¹² transgene sporadically as a result of spontaneous recombination (Johnson et al., 2001), and compared the results obtained with the murine lung tumors with human arrays from a variety of sources, including their own analyses as well as those from Beer et al. (2002) and Bhattacharjee et al. (2001). These investigators were able to demonstrate patterns of gene expression that were common to both human and murine lung ACs (Sweet-Cordero et al., 2005). Most interesting, these researchers demonstrated that a *Ki-ras* expression signature in human ACs that was not identifiable by statistical analysis unless the mouse data was included in the integrated analyses, due to the high degree of variability inherent in human lung tumor tissues, results which have been confirmed by Creighton et al. (2005).

Interestingly, in all of these studies mutated RAS genes were treated as a single entity, as the comparison that was made was samples containing mutated RAS genes vs. those that did not. Thus, to date, it is unclear to what extent these signatures reflect

the differential effects of different RAS mutant alleles on downstream signaling pathways. Accounting for this added complexity may allow a further refinement of the signatures down to the subset of RAS stimulated genes that are the most critical for tumor initiation and maintenance. Clearly, different RAS mutant alleles may engage different subsets of RAS downstream effector pathways, which may influence the gene expression profiles. This could be the reason that statistical significance for RAS mutated vs. wild type tumors could not be reached until the mouse data, which had a more homogenous *Ki-ras* mutational spectrum, were added.

Recent studies have utilized synthetic lethal and genome wide inhibitory RNA approaches to identify RAS effector molecules that are critical for tumorigenesis (Kassie et al., 2008; Barbie et al., 2009; Luo et al., 2009; Scholl et al., 2009; Singh et al., 2009; Vicent et al., 2010). These studies have clearly shown the dependency of tumor cells on a subset of RAS downstream effector pathways when mutated RAS genes are present. All of these studies have been conducted with the alleles that animal and *in vitro* studies have identified as the most oncogenic RAS mutations. Future studies will thus need to compare the use of synthetic lethal approaches with different RAS mutants. This will allow an understanding of which mutant RAS alleles signal to these critical pathways and will be an important approach in developing novel therapeutic agents.

EMERGING RESEARCH

A number of studies have shown that mutations in *Ki-ras* are prognostic factors for poor patient outcome in lung cancer (Mitsudomi et al., 1991; Rodenhuis and Slebos, 1992; Rosell et al., 1993, 1996; Keohavong et al., 1996; Siegfried et al., 1997; Huncharek et al., 1999). Patients whose tumors contained *Ki-ras* mutations often exhibited poorer overall survival and reduced time to disease progression. As noted above, one study found that lung cancer patients whose tumors contained the CYS, ARG, or ASP mutations at codon 12 appeared to have a poorer prognosis than those containing hydrophobic amino acid substitutions such as VAL or ALA (Siegfried et al., 1997). However, a recent study in Stage III colon cancer patients failed to find an association with disease-free or overall survival (Ogino et al., 2009). A limitation of both of these studies was the fact that mutations in *Ki-ras* were treated as a single entity, so that all patients with mutations were compared to patients with wild type *Ki-ras*.

More recent studies have examined the effects of RAS mutations on drug sensitivity. Garassino et al. (2011) found that lung tumors of patients harboring the CYS¹² mutations were less sensitive to cisplatin therapy but exhibited increased sensitivity to taxol and pemetrexed relative to the VAL¹² and ASP¹² mutant alleles. The ASP¹² mutation demonstrated increased resistance to taxol and enhanced sensitivity to sorafenib. While none of the RAS mutants exhibited differential sensitivity to the tyrosine kinase inhibitors erlotinib, a study examining colorectal cancer patients with chemotherapy-refractory metastatic disease who harbored the *Ki-ras* ASP¹³ mutation found a small but statistically significant increase in overall survival and progression-free survival relative to patients harboring other *Ki-ras* mutations (De et al., 2010). These results clearly suggest that different RAS mutations may have a significant impact on patient response to therapeutic interventions, and that the RAS mutational profile may need to

be considered in future clinical trials assessing the effects of novel agents in tumors harboring RAS mutations.

Recent studies have identified novel mutations in RAS genes, whose influence on tumorigenicity are first being assessed. Mutations in exon 4 of *Ki-ras* coding for LYS¹¹⁷ and ALA¹⁴⁶ were associated with an increased probability of disease-free survival despite increases in RAS-GTP (Janakiraman et al., 2010). In addition, recent studies by To et al. (2008) demonstrated that the minor *Ki-ras4A* isoform may be the critical form of *Ki-ras* that is responsible for lung carcinogenesis. Smith et al. (2010) recently identified four additional *Ki-ras* mutations. The ASN¹¹⁷ and THR¹⁴⁶ mutations, when transfected into NIH3T3 cells, produced a similar number of transformed foci as the ASP¹³ mutation but fewer colonies than the VAL¹² and ASP¹² mutant alleles. The PHE¹⁹ and GLN¹⁶⁴ mutant alleles, similar to the wild type allele, had little transforming activity. When expression arrays were performed for cell lines harboring the different alleles, differential expression of a number of genes associated with tumor proliferation and cell survival were observed, consistent with the different alleles stimulating different gene expression programs. This work is among the first to document mutant-specific alterations in gene transcriptional programming, and may yield further insights into the effector pathways of allele-specific signaling.

Finally, a *Ki-ras* single nucleotide polymorphism in the 3' untranslated region of the gene that disrupts binding of the *let7* microRNA has been shown to be a potential prognostic marker of ovarian cancer risk, as 25% of unselected ovarian cancer cases and 61% of hereditary ovarian cancer patients lacking mutations in the *BRCA* genes harbored this RAS variant allele (Ratner et al., 2010). Interestingly, Hwang and Cohen (1997) found that deletion or inversion of a splicing enhancer region located in the 3' untranslated region of the *Ha-ras* gene reduced the transcription of RAS mRNA and thus decreased the transforming activity of this variant allele. Thus, the list of mutant RAS alleles with the differential ability to influence tumorigenicity continues to grow. How each of these mutant alleles influences carcinogenicity and the mechanisms by which they do so are an area of research that should be a major emphasis for future studies.

CONCLUSION AND FUTURE PERSPECTIVES

Since the identification of mutant RAS genes as transforming oncogenes, investigators have attempted to identify a mechanistic basis for the allele-specific differences that could account for the observed differences in transforming ability observed *in vitro* and *in vivo*. These studies have so far failed to identify a specific gene pathway or pathways that account for the differential effects of different RAS mutants. The majority of evidence obtained to date and described above suggests that, in particular, the CYS¹² mutation may exhibit less potent transforming properties than the VAL¹² and ASP¹² mutants, but a rigorous comparison of all possible RAS mutations of biological significance in the same experimental system has not been conducted. Most studies have examined only a limited number of mutations. In addition, the list of clinically relevant and biologically active RAS mutations continues to grow.

Some of the reason for the lack of a clear mechanistic basis for the observed phenotypic differences imparted by different mutant RAS alleles may be due to very subtle effects of the mutant RAS

alleles on multiple gene pathways (either at the transcriptome or proteome level) and context as well as cell and tissue specific effects of mutant RAS genes. Recent advances in technology should enable us to determine the basis of these differences going forward. Biochemical and kinetic studies demonstrating the redox sensitivity of the CYS¹¹⁸ residue (Heo et al., 2005; Heo and Campbell, 2006; Raines et al., 2007) suggest that specific mutant forms of RAS could exhibit differential sensitivity to the redox state of the cell, which could influence tumor progression by either direct effects on RAS GTPase activity or indirect effects via alterations of protein interactions between RAS and its downstream effectors. As illustrated by the expression profiling studies described above (Smith et al., 2010), applications of transcriptomic and proteomic expression profiling hold much promise for delineating the allele-specific effects of different RAS mutant alleles. These studies need to be done in both human tumor samples as well as novel transgenic models.

The development of knock-in mouse models for each of the RAS mutant alleles would be a critically important advance in the field. Because of the tissue specific and dose related effects of RAS gene expression, it will be important to test these alleles in the same genetic background, driven from the endogenous RAS promoters, to eliminate some of the uncertainties associated with traditional transgenic constructs. While these studies should initially be done with *Ki-ras*, which is mutated more frequently than other RAS

family members in human cancer, extending this research to other RAS family members will allow the development of a comprehensive understanding of RAS signaling networks. This will provide important information in terms of developing targeted therapies to the alleles most responsible for driving tumorigenesis as opposed to those that have much less significant contributions to maintenance of the tumorigenic phenotype.

It is likely that the answer will not be simple and that more than one mechanism will influence the behavior of different mutant RAS alleles. Since RAS is the most commonly mutated gene in human cancers and signals to a wide variety of molecules involved in proliferation, cell death, cell survival, oxidative stress, angiogenesis, inflammation, and drug resistance (Campbell et al., 1998; Hancock, 2003; Malumbres and Barbacid, 2003; Young et al., 2009), it is likely that patients whose tumors harbor specific RAS mutations will exhibit differences in survival, tumor aggressiveness, and response to chemotherapy agents. An understanding of the properties of each of the RAS mutants in specific tissues will aid in future attempts to personalize cancer treatment regimens and assure the best possible outcomes for patients.

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