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Recurrent *KRAS* Codon 146 Mutations in Human Colorectal Cancer

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Abstract

An activating point mutation in codon 12 of the *HRAS* gene was the first somatic point mutation identified in a human cancer and established the role of somatic mutations as the common driver of oncogenesis. Since then, there have been over 11,000 mutations in the three RAS (*HRAS, KRAS* and *NRAS*) genes in codons 12, 13 and 61 reported in the literature. We report here the identification of recurrent somatic missense mutations at alanine 146, a highly conserved residue in the guanine nucleotide binding domain. In two independent series of colorectal cancers from Hong Kong and the United States we detected *KRAS* A146 mutations in 7/126 and 2/94 cases, respectively, giving a combined frequency of 4%. We also detected *KRAS* A146 mutations in 2/40 (5%) colorectal cell lines, including the NCI-60 colorectal cancer line HCC2998. Codon 146 mutations thus are likely to make an equal or greater contribution to colorectal cancer than codon 61 mutations (4.2% in our combined series, 1% in the literature). Lung adenocarcinomas and large cell carcinomas did not show codon 146 mutations. We did, however, identify a *KRAS* A146 mutation in the ML-2 acute myeloid leukemia cell line and an *NRAS* A146 mutation in the NALM-6 B-cell acute lymphoblastic leukemia line, suggesting that the contribution of codon 146 mutations is not entirely restricted to colorectal cancers or to *KRAS*.

Keywords

KRAS; colorectal; cancer; mutation; A146

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INTRODUCTION

Somatic mutations in critical target genes are the basic driving force in the development of cancer. The first somatic point mutation described in human cancer was an activating codon 12 glycine to valine mutation in the *HRAS* gene present in the EJ/T24 bladder carcinoma cell line.1,2 The further identification and characterisation of somatic mutations from the full spectrum of human cancers has been a major focus of cancer research in the ensuing 25 years.

To date, over 11,000 point mutations in codons 12, 13 and 61 have been reported in the *HRAS, NRAS* and *KRAS* genes combined (www.sanger.ac.uk/genetics/CGP/cosmic). The three RAS genes encode 21 kilodalton proteins with intrinsic GTPase activity which cycle between a GDP-bound “off” form and a GTP-bound “on” form. These alternate forms are regulated by GAPs (GTPase activating proteins) and GEFs (guanine nucleotide exchange factors), respectively. RAS proteins are involved in coupling signal transduction from cell surface receptors to cytoplasmic targets and mediating a variety of cellular responses including proliferation, cytoskeletal reorganisation and survival pathways. Activating mutations stabilise or otherwise promote a preponderance of the GTP-bound “on” form, thus inappropriately affecting downstream activities.

The *KRAS* gene is mutated in over 30% of colorectal cancers. There have been approximately 3000 *KRAS* point mutations in colorectal cancer reported in the literature (www.sanger.ac.uk/genetics/CGP/cosmic/). The majority (~82%) of reported mutations are in codon 12. Mutations at codons 13 and 61 contribute to a lesser degree, accounting for ~17% and ~1% respectively. We report here the identification of recurrent mutations at codon 146 in *KRAS* in colorectal cancers, indicating that mutations at this codon are making a heretofore unappreciated contribution to human neoplasia.

MATERIALS AND METHODS

The collection of all patient materials and their use in the current study were approved by appropriate local Institutional/ethical Review Board. Genomic DNA was extracted from tumour and paired normal tissues as previously described.3 The Johns Hopkins University colorectal cancer sample series was carried as early passage cell lines or xenografts as described previously.4 PCR amplification and direct sequencing of *KRAS* using fluorescent dideoxy sequencing on ABI-3730 sequencers was done as previously described5 using the following primers flanking all four coding exons of *KRAS* (isoform b, accession GI: 34485723 NM_004985): exon 2:F-GTGTGACATGTTCTAATATAGTCA, R-GAATGGTCCTGCACCAGTAA; exon 3:F-TCAAGTCCTTTGCCCATTTT, R-TGCATGGCATTAGCAAAGAC exon 4: F-GAAACCAAAGCCAAAAGCAG, R-AGTAGAAGAAGGAAGGAAAATTTGG; exon 5: F-TGGGAATACTGGCACTTAGAGG, R-TTGACAAAACACCTATGCGG.

Sequence data was analysed using semi-automated analysis using Mutation Surveyor and in-house software coupled with manual inspection of potentially mutant traces followed by a manual rescoring of all sequence traces.

STATISTICAL METHODS

Differences in the relative incidences of *KRAS* mutations in codons 12, 13, 61 and 146, between tissue types, were assessed by first computing a chi-squared statistic based on the contingency table of mutation counts by codon and type. The significance of this statistic was evaluated by Monte Carlo simulation, using 10,000 simulations of the data under the null hypothesis of no difference in the relative incidence of codons, conditional on the

number of mutations in each codon group and the total number of mutations of each type. This provides an exact statistic, necessary for contingency tables containing low counts.

RESULTS

Coding exons of the *KRAS* gene were resequenced in a series of 126 primary colorectal cancer cases (CRC) from Hong Kong. Recurrent point mutations resulting in amino acid substitutions of alanine 146 were identified in seven cases (Table 1 and Fig. 1). Further screening in an additional series of 94 colorectal cancer samples from the United States yielded two further codon 146 mutations. All of these codon 146 mutations from cancers were demonstrated to be somatic by analysis of normal DNA from the same individuals. Two additional codon 146 mutations were detected in 40 colorectal cancer cell lines (Tables 1 and 2). However, normal DNAs from these individuals were not available to confirm their somatic origin. Combining colorectal cancer primary tumor, xenograft and cell line data, 11 mutations (Table 1) were identified: 8 c.436G>A p.A146T, 2 c.437C>T p.A146V and 1 c. 436G>C p.A146P. A set of 94 sporadic colorectal adenomas were screened in addition and no instances of A146 mutation were identified. Full frequency and distribution data for *KRAS* mutations in the colorectal screens are shown in Table 3. In addition, a series of 63 adenomas from familial adenomatous polyposis patients and 34 hyperplastic colorectal polyps where both *KRAS* and *BRAF* have previously6 been shown to play a role were also screened but no A146 mutations were identified (data not shown). Further screening of all three RAS genes in series of ~700 cancer cell lines revealed two additional A146 mutations: a *KRAS* c.436G>A p.A146T in the ML-2 acute myeloid leukemia cell line and a c.436G>A p.A146T in *NRAS* in the NALM-6 B-cell acute lymphoblastic leukemia cell line (Table 1).

The distribution of *KRAS* mutant alleles was different between CRC from Hong Kong and United States ($p = 0.0306$). This was primarily due to a difference in relative mutation prevalence between codons 12 and 13 which has been noted previously.7 However, a higher proportion of A146 mutations in Hong Kong compared to US CRC also contributed to the difference between the two series and larger genetic epidemiological studies are warranted to determine if this A146 trend is reproducible. Overall, *KRAS* A146 mutations were detected in 4% (11/260) of CRC and accounted for 8% (11/135) of all observed *KRAS* mutations. These data suggest that A146 mutations make a larger contribution to colorectal cancer than Q61 mutations, which accounted for 2% (5/260) of cases in this study and 1% in the literature (www.sanger.ac.uk/genetics/CGP/cosmic/). Screening for mutations in *BRAF* revealed no cases with both A146 and *BRAF* mutations (data not shown). One primary tumor (PD1532) had both a heterozygous p.A146V mutation as well as a heterozygous p.G12V mutation in *KRAS*. Whether this reflects intratumoral heterogeneity or truly coincident, presumably biallelic, *KRAS* activation in the same clone is not known. As this case is a primary tumour, single-cell cloning experiments are not possible to differentiate the two possibilities. Further work to determine if biallelic RAS mutations have greater transforming potential would be of interest.

The contribution of A146 mutations to another cancer type that is driven in large part by *KRAS* mutations was investigated by analysing a series of non-small-cell lung cancers (NSCLC) comprised of adenocarcinomas, bronchioalveolar and large cell undifferentiated carcinomas in which *KRAS* mutations are known to be prevalent. In total, 99 primary tumor and 66 cell lines were sequenced for *KRAS*. While KRAS codon 12, 13 and 61 mutations were detected at the expected combined frequency of 33% (55/165), no instances of A146 mutations were detected ($p = 0.0305$ compared to CRC).

DISCUSSION

We have identified A146 missense substitutions as a new class of recurrent somatic mutation in the KRAS gene in colorectal cancers. Data from independent series indicate that A146 mutations are involved in approximately 4% of colorectal cancers. Extrapolating from a worldwide CRC incidence of approximately 950,000 cases/year,8 these data suggest there are approximately 30,000 cases/year of CRC that are *KRAS* A146 mutant. Additional A146 (both *KRAS* and *NRAS*) mutations were detected in leukaemia cell lines, suggesting a role for A146 alleles in other tumour types. However, we did not detect A146 mutations in a series of NSCLC where KRAS mutations are also prevlaent. It is possible that the sequence context of codon 146 (tca GCA^{146} aag) renders the guanine at codon 146 less susceptible to adduction by tobacco smoke carcinogens than guanines at codons 12 and 13 (gct GGT^{12} GGC^{13} gta). Alternatively, it may be that substitution of serine for alanine at codon 146, which would require a G>T transversion typical of cigarette smoke polycyclic aromatic hydrocarbon mutagenesis,9 may not confer transforming activity on *KRAS*.

To our knowledge there has been no report of recurrent A146 mutations to date and only two reports of single A146 mutations in human cancer in the literature from over 15 years ago.10-12 It is remarkable that, despite ~70,000 cancer samples analysed for mutations in RAS genes over the last 25 years (www.sanger.ac.uk/genetics/CGP/cosmic/), the role of codon 146 mutations essentially has been overlooked. This reflects a persistent bias in mutation screening which has been almost exclusively directed at the first two coding exons of the RAS genes (encoding codons 12,13 and 61). Mutation screening should be extended to include A146 for all three RAS genes in future.

RAS mutations at alanine 146 have been identified and characterised in experimental systems twice in the literature. A transforming *Kras* p.A146T allele was detected in a thymic lymphoma induced by exposure of mice to an acute whole-body dose of neutron radiation. 13 NIH 3T3 focus-forming assays with tumour DNA from the lymphoma-derived nude mouse tumours demonstrated transforming capability of the A146T allele, albeit at lower efficiency than *Kras* codon 12 and 13 alleles. In an in vitro mutagenesis screen for mutations that increased guanine nucleotide exchange rates, GTPase activity and transforming potential in *HRAS*, a p.A146V mutation was found to have partial transforming activity which was attributable to >1000-fold increase in the GDP → GTP exchange rate without affecting the GTPase activity.14 Alanine 146 is within the highly conserved G-5 (aa144-146) region of the protein (Fig. 2) and forms a hydrogen bond with the guanine ring of GTP.15 Mutations of this residue presumably alter the local structure such that the GTP-bound state is much favoured over GDP-bound form. Whether this is accomplished by increased GEF binding or through some other mechanism is unknown. It is likely however that increased exchange is accounting, at least in part, for the oncogenic properties of A146 mutant alleles.

Various studies in colorectal cancer have suggested that *KRAS* codon 12 and 13 mutations are generally predicitve of a poorer prognosis, with evidence being presented for mutation-specific prognostic as well as histopathology correlates.16-19 The influence of A146 alleles on clinical outcome and tumour characteristics therefore requires investigation. As well, the extent to which colorectal cancers harbouring this class of *KRAS* mutation are more or less responsive to current therapies needs to be investigated. It has recently been shown that *KRAS* mutations are predictive of resistance to treatment with cetuximab in colorectal cancer.20 This study analysed only mutations in codons 12 and 13 and it is therefore plausible that a further subset of patients are resistant due to A146 mutations. It will be also be interesting to assess response of the A146 mutant cancer cell lines detailed here (one of which, HCC2998 is a component of the NCI-60 series) to various anticancer agents and

compare these responses to cancer cells that have other RAS and *BRAF* mutations, as exemplified by the recently described work on MEK inhibitors.21

In summary, we have demonstrated that *KRAS* A146 mutations are recurrent in human colorectal cancers, are more prevalent than Q61 mutations in this cancer type and are thus making a substantial contribution to colorectal cancer in the population. These findings will further empower the molecular pathology and clinical investigation of this common tumor type.

Acknowledgments

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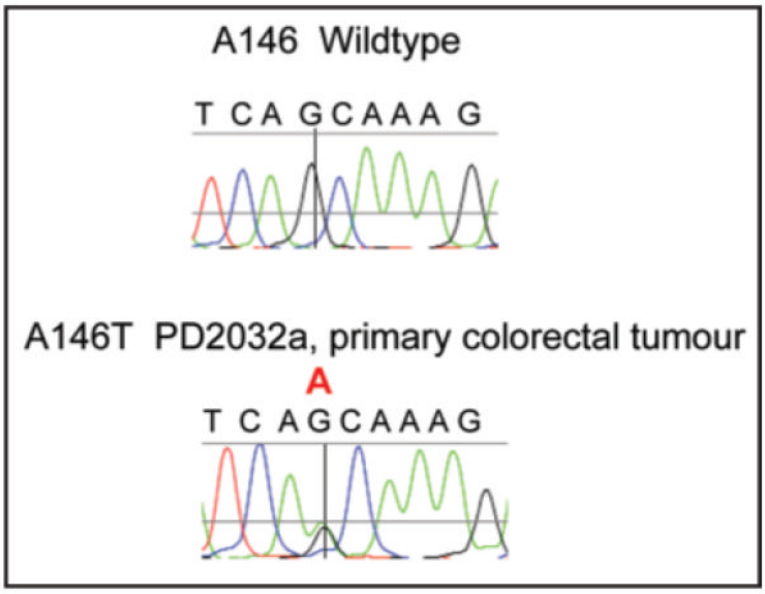

Figure 1.
Representative sequencing chromatogram of c.436G>A p.A146T *KRAS* mutation from primary colorectal tumour PD2032a. The postion of the G>A substitution is indicated by the crosshairs and red A above the trace.

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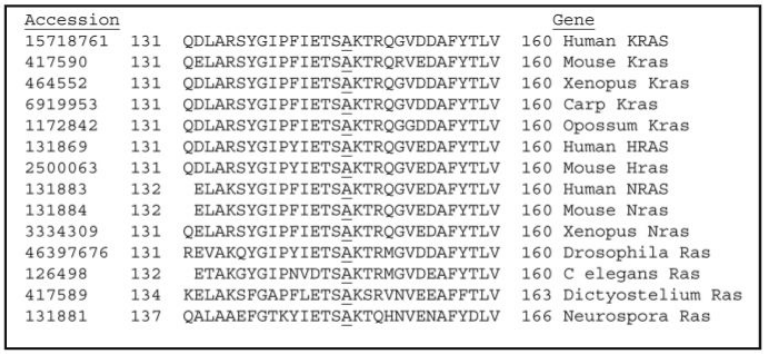

Accession				Gene
15718761	131	QDLARSYGIPFIETSAKTRQGVDDAFYTLV	160	Human KRAS
417590	131	QELARSYGIPFIETSAKTRQRVEDAFYTLV	160	Mouse Kras
464552	131	QDLARSYGIPFIETSAKTRQGVDDAFYTLV	160	Xenopus Kras
6919953	131	QDLARSYGIPFIETSAKTRQGVDDAFYTLV	160	Carp Kras
1172842	131	QDLARSYGIPFIETSAKTRQGGDDAFYTLV	160	Opossum Kras
131869	131	QDLARSYGIPYIETSAKTRQGVEDAFYTLV	160	Human HRAS
2500063	131	QDLARSYGIPYIETSAKTRQGVEDAFYTLV	160	Mouse Hras
131883	132	ELAKSYGIPFIETSAKTRQGVEDAFYTLV	160	Human NRAS
131884	132	ELAKSYGIPFIETSAKTRQGVEDAFYTLV	160	Mouse Nras
3334309	131	QELARSYGIPFIETSAKTRQGVEDAFYTLV	160	Xenopus Nras
46397676	131	REVAKQYGIPYIETSAKTRMGVDDAFYTLV	160	Drosophila Ras
126498	132	ETAKGYGIPNVDTSAKTRMGVDEAFYTLV	160	C elegans Ras
417589	134	KELAKSFGAPFLETSAKSRVNVEEAFFTLV	163	Dictyostelium Ras
131881	137	QALAAEFGTKYIETSAKTQHNVENAFYDLV	166	Neurospora Ras

Figure 2.

Conservation of alanine 146 in RAS genes. Accession number of sequence and species of origin/gene are given the left and right of the alignment, respectively. The position of A146 is underlined.

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Table 1

Codon A146 mutations detected

Colorectal Cancers		
Gene	**Sample**	**Mutation**
KRAS	PD2032a	c.436G>A p.A146T
KRAS	PD2048a	c.436G>A p.A146T
KRAS	PD2070a	c.436G>A p.A146T
KRAS	PD2019a	c.436G>A p.A146T
KRAS	PD1978a	c.436G>C p.A146P
KRAS	PD2068a	c.437C>T p.A146V
KRAS	PD1532a	c.437C>T p.A146V
KRAS	JHU-1	c.436G>A p.A146T
KRAS	JHU-2	c.436G>A p.A146T

Cancer Cell Lines		
Gene	**Sample/Cancer Type**	**Mutation**
KRAS	HCC2998, CRC	c.436G>A p.A146T
KRAS	LS1034, CRC	c.436G>A p.A146T
KRAS	ML-2, AML	c.436G>A p.A146T
NRAS	NALM-6, B-ALL	c.436G>A p.A146T

CRC, colorectal cancer; AML, acute myeloid leukemia; B-ALL- B-cell acute lymphoblastic leukemia.

Table 2

***KRAS* mutations in CRC cell lines**

CRC Cell Line	*KRAS* mutation	Zygosity
C2BBe1	ND	-
Car-1	ND	-
CoCM-1	ND	-
COLO-205	ND	-
COLO-320-HSR	ND	-
COLO-678	c.35G>A p.G12D	Het
COLO-741	ND	-
CW-2	ND	-
DLD-1-JCRB	c.38G>A p.G13D	Het
ECC4	c.A182A>G p.Q61R	Hom
Gp2D	c.35G>A p.G12D	Het
GP5d	c.35G>A p.G12D	Het
HCC2998	c.436G>A p.A146T	Het
HCT-116	c.38G>A, p.G13D	Het
HCT-15	c.38G>A, p.G13D	Het
HT-29	ND	-
HT55	ND	-
KM12	ND	-
LoVo	c.38G>A p.G13D	Het
LS1034	c.436G>A p.A146T	Hom
LS-123	c.34G>A p.G12S	Het
LS-174T	c.35G>A p.G12D	Het
LS-411N	ND	
LS-513	c.35G>A p.G12D	Het
NCI-H508	ND	-
NCI-H630	ND	-
NCI-H716	ND	-
NCI-H747	c.38G>A p.G13D	Het
RCM-1	c.35G>T p.G12V	Hom
RKO	ND	-
SK-CO1	c.35G>T p.G12V	Hom
SNU-C1	ND	-
SNU-C2B	c.35G>A p.G12D	Hom
SW1116	c.G35G>C p.G12A	Het
SW1417	ND	-
SW1463	c.35G>A p.G12D	Hom
SW403	c.35G>T p.G12V	Hom
SW48	ND	-
SW480	c.35G>T p.G12V	Hom

CRC Cell Line	*KRAS* mutation	Zygosity
SW620	c.35G>T p.G12V	Hom
SW837	c.G34G>T p.G12C	Het
SW948	c.A182A>T p.Q61L	Het
T84	c.38G>A p.G13D	Het

Het, heterozygous; Hom, homozygous; ND, none detected

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Table 3

KRAS mutation distribution in study sets

HK_CGP CRC		**JHU CRC**	
WT	76	WT	29
Codon 12 MUT	24	Codon 12 MUT	46
Codon 13 MUT	18	Codon 13 MUT	15
Codon 61 MUT	1	Codon 61 MUT	2
Codon 146 MUT	7	Codon 146 MUT	2
Total	126	Total	94

CRC Cell Lines		**HK_CGP sporadic adenoma**	
WT	19	WT	68
Codon 12 MUT	14	Codon 12 MUT	18
Codon 13 MUT	3	Codon 13 MUT	6
Codon 61 MUT	2	Codon 61 MUT	2
Codon 146 MUT	2	Codon 146 MUT	0
Total	40	Total	94

HK_CGP, Hong Kong, Cancer Genome Project; JHU, Johns Hopkins University; CRC, colorectal cancer.