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Characterization of rare transforming *KRAS* mutations in sporadic colorectal cancer

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Abbreviations: mCRC, metastatic colorectal carcinoma; mAbs, monoclonal antibodies; EGFR, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded

KRAS mutational status has been shown to be a predictive biomarker of resistance to anti-EGFR monoclonal antibody (mAb) therapy in patients with metastatic colorectal cancer. We report the spectrum of *KRAS* mutation in 1506 patients with colorectal cancer and the identification and characterization of rare insertion mutations within the functional domain of KRAS. *KRAS* mutations are found in 44.5% (670/1506) of the patients. Two cases are found to harbor double mutations involving both codons 12 and 13. The frequencies of *KRAS* mutations at its codons 12, 13, 61, and 146 are 75.1%, 19.3%, 2.5%, and 2.7%, respectively. The most abundant mutation of codon 12 is G12D, followed by G12V and G12C while G13D is the predominant mutation in codon 13. Mutations in other codons are rare. The *KRAS* mutation rate is significantly higher in women (48%, 296/617) than in men (42.1%, 374/889, $P = 0.023$). Tumors on the right colon have a higher frequency of *KRAS* mutations than those on the left (57.3% vs. 40.4%, $P < 0.0001$). Two in-frame insertion mutations affect the phosphate-binding loop (codon 10–16) of *KRAS* are identified. One of them has never been reported before. Compared with wild-type protein, the insertion variants enhance the cellular accumulation of active RAS (RAS-GTP) and constitutively activate the downstream signaling pathway. NIH3T3 cells transfected with the insertion variants show enhanced anchorage-independent growth and in vivo tumorigenicity. Potentially these mutations contribute to primary resistance to anti-EGFR mAb therapy but the clinical implication requires further validation.

Introduction

Colorectal cancer (CRC) is one of the most common lethal cancers worldwide. In 2008, more than 1.2 million new cases were diagnosed, with approximately 608 700 deaths estimated to have occurred.[1] Epidermal growth factor receptor (EGFR), a critical molecule in CRC initiation and progression, is frequently overexpressed in metastatic CRC (mCRC) tumors.[2,3] The phenomena lead to the development of molecular targeting therapy to inhibit the EGFR signaling pathway. Using anti-EGFR monoclonal antibodies (mAbs) such as cetuximab and panitumumab, have been approved in treating mCRC to inhibit EGFR activity and hence switching off downstream pathways.[2,3]

However, anti-EGFR therapy does not work on all CRCs, largely due to the resistance to the anti-EGFR mAbs.[4] Different studies have reported the response and outcome of CRCs to the anti-EGFR mAbs was poor with *KRAS* mutation which accounting for 30–40% of non-responsive cases.[4-7] *KRAS* mutation status is now considered to be a predictive biomarker of resistance to anti-EGFR mAbs treatment for mCRC patients. KRAS is one of the RAS superfamily of proto-oncoproteins which is small signal switch molecule called GTPase, cycling between inactive GDP-bound (RAS-GDP) and active GTP-bound (RAS-GTP) forms, to regulate cellular growth and differentiation.[8] Activating mutations of RAS proto-oncogenes continuously elevate the cytoplasmic RAS-GTP level. Oncogenic signaling pathways, such as Raf-MEK-ERK and PI3K/AKT cascades, are then constitutively activated in an EGFR activation-independent manner and therefore promote cell cycle progression.[6,8] *KRAS* mutation is found in 40% of CRCs and missense point mutation is the most common mutation. The majority of the point mutation sites of *KRAS* in CRC patients are located at codons 12 and 13 (~80% and ~17%, respectively), together with rare mutations at codons 61 and 146 (~1–4%).[3,9-11]

Most clinical studies of *KRAS* mutation in CRC were conducted in western countries. However, *KRAS* mutation rate or spectrum in CRCs may partially depends on the population studied.[12] It has been reported that *KRAS* mutations were

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identified in CRC patients from the UK, Switzerland, and Spain, for 27.4%, 38%, and 41% respectively.[12] This epidemiological variation indicates the essence of establishment of a local CRC *KRAS* mutation data in different populations. There has been a dramatic increase in reported incidence of colorectal cancer in Asian.[12] It is of paramount importance to investigate the *KRAS* mutation spectrum in our locality in view of the implication in using anti-EGFR targeting therapy. We aim to analyze the *KRAS* mutation status and the clinical correlation in Chinese patients with CRC in Hong Kong. Here we report the spectrum of *KRAS* mutation in a large cohort of colorectal cancer and the identification and characterization of a novel insertion mutation within the function domain of KRAS.

Results

Clinical characteristics of the patients

We tested a total of 1506 patients with colorectal cancer. Of them 889 (59%) were males and 617 (41%) were females. The median age at presentation was 61 ± 11.3 y (range 21–89 y). The clinical characteristics were in keeping with other reported populations of colorectal cancer.[11] The age of female patients were slightly younger than males (59 ± 12.1 vs 61 ± 11.2, $P = 0.014$). There was significantly higher frequency of left colon tumor (75.8%) than the right side (24.2%, $P < 0.0001$). However, the right side tumors were more common in females (28.7%) compared with males (21.1%, $P = 0.001$). When rectal tumor was considered a separate entity, female patients had a higher frequency of right side tumor whereas the rectal tumors were more commonly found in male patients ($P < 0.0001$). The clinical characteristics of the patients tested were summarized in **Table 1**.

Status of *KRAS* mutation

KRAS mutations on codons 12, 13, 61 and 146 were analyzed by PCR-direct sequencing using microdissected FFPE tumor tissues from 1506 patients. A total of 672 *KRAS* mutations were identified from 670 patients (44.5%, 670 out of 1506, **Table 2**). Two cases were found to harbor double mutations. Both cases involved codon 12 and codon 13 of *KRAS* gene. One case harbored concomitant G12C and G13D, while the other had both G12V and G13D. Within 672 *KRAS* mutations identified, the frequencies of mutations at codons 12, 13, 61, and 146 were 75.1%, 19.3%, 2.5%, and 2.7%, respectively. Majority of the mutations occurred at codons 12 and 13 which accounted for more than 94% of all mutations identified. The most common mutation was glycine to aspartate on codon 12 (G12D), which accounted for 37.5% of all mutations (252 out of 672). Mutation from glycine to valine (G12V) was the second most common of all specified mutations (20.1%; 135 of 672). Mutation from glycine to aspartate on codon 13 (G13D) accounted for 19.0% (128 of 672) of specified mutations.

The *KRAS* mutation rate was significantly higher in women (48%, 296 of 617, **Table 3**) than in men (42.1%, 374 of 889, $P = 0.023$). The mutation rate did not differ according to the primary tumor site if the tumor location was classified as either ascending, hepatic flexure, transverse, splenic flexure, descending, sigmoid, or rectum. If the tumors on the right side of the colon (ascending and transverse colon) were group together and compared with those on the left (splenic flexure to rectum), the frequency of *KRAS* mutations were significantly higher in the right colon (57.3% vs. 40.4%, $P < 0.0001$). The *KRAS* mutation was not associated with the age of the patient. In comparison of the most frequently mutated codons between left and right colon, codon 12

Table 1. Clinical characteristics of 1506 patients tested for *KRAS* status

	Total	Female	Male	*P* value
n =	1506	617 (41%)	889 (59%)	
Age	61 ± 11.3	59 ± 12.1	61 ± 11.2	0.014
Tumor site (right vs left)				0.001
Right	365 (24.2%)	177 (28.7%)	188 (21.1%)	
Left	1141 (75.8%)	440 (71.3%)	701 (78.9%)	
Tumor site (right vs left vs rectum)				< 0.0001
Right	365 (24.2%)	177 (28.7%)	188 (21.1%)	
Left	538 (35.7%)	228 (40.0%)	310 (34.9%)	
Rectum	603 (40.1%)	212 (34.3%)	391 (44.0%)	

Table 2. KRAS mutations spectrum in 670 colorectal cancers

Mutation	Frequency	Percentage
Codon 12	505	75.1%
G12D	252	37.5%
G12V	135	20.1%
G12C	46	6.8%
G12S	33	4.9%
G12A	29	4.3%
G12R	10	1.5%
Codon13	130	19.3%
G13D	128	19.0%
G13C	2	0.3%
Codon 61	17	2.5%
Q61H	9	1.3%
Q61L	5	0.7%
Q61K	1	0.1%
Q61R	2	0.3%
Codon 146	18	2.7%
A146T	18	2.7%
Others	2	0.3%
c.30_31insGGA, p.G10_A11insG	1	0.1%
c.33_34insGGAGCT:p.A11_G12insGA	1	0.1%
Total	672[a]	100%

[a]A total of 672 KRAS mutations were detected from 670 colorectal tumors. Two tumors harbored double mutations.

Table 3. Correlation of *KRAS* mutation status with clinical features

Characteristics	*KRAS* mutation		Total	*P* value
	+	–		
n = 1506				
No. cases	670 (44.5%)	836 (55.5%)		
Age	61.3 ± 11.3	60.5 ± 11.3		NS
Gender				0.023
F	296 (48%)	321 (52%)	617	
M	374 (42.1%)	515 (57.9%)	889	
Age	62.1 ± 10.1	60.4 ± 9.0		NS
Tumor site (right vs left)				
Right	209 (57.3%)	156 (42.7%)	365	< 0.0001
Left	461 (40.4%)	680 (59.6%)	1141	
Total	670	836	1506	
Tumor site (right vs left vs rectum)				< 0.0001
Right	209 (57.3%)	156 (42.7%)	365	
Left	198 (36.8%)	340 (63.2%)	538	
Rectum	263 (43.6%)	340 (56.4%)	603	
Total	670	836	1506	

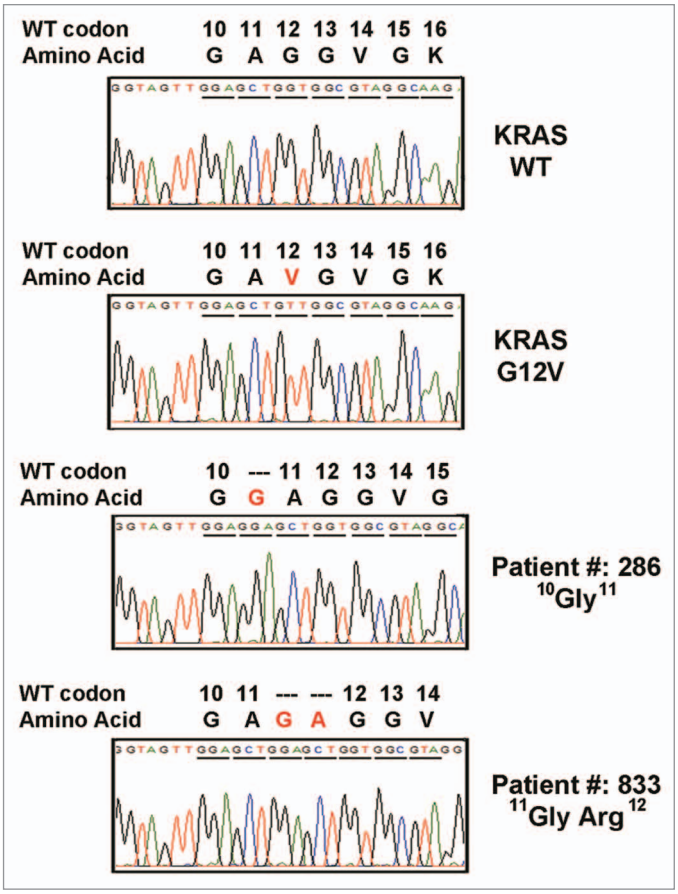

Figure 1. Electropherogram for *KRAS* mutants. Tissue DNA from the patient with colorectal cancer were amplified and cloned for sequencing analysis. Two novel in-flame insertions ($^{10}G^{11}$ and $^{11}GA^{12}$) in exon 2 of *KRAS* gene were identified.

mutations were significantly more likely to occur in rectum (right colon 28.8%, left colon 29.7%, rectum 41.6%), while codon 13 mutations were slightly more frequent in the right colon (right colon 40%, left colon 30.8%, rectum 29.2%, $P = 0.013$)

Identification of rare *KRAS* insertion mutations

In the pool of CRC cases, we identified two rare *KRAS* mutations which were defined as in-frame insertion mutations. The Insertion mutations in *KRAS* exon 2 of patient #286 and #833 were further validated by direct sequencing of the cloned PCR products (**Fig. 1**). In patient #286, an in-flame insertion of 3-nucleotide (GGA) between codons 10 and 11 was observed (c.30_31insGGA: p.G10_A11insG). This rare mutation, which suggested the insertion of a glycine residue between glycine (amino acid 10) and alanine (amino acid 11), was reported once in the patient with myeloid leukemia.[8] In patient #833, a tandem repeat sequences of codon 10 and 11 (GGA GCT) was in-flame inserted after codons 11 and introduced extra glycine and alanine residues between alanine (codon 11) and glycine (codon 12). This insertion mutation (c.33_34insGGAGCT:p.A11_G12insGA) has never been reported before. These two mutations are named $^{10}G^{11}$ and $^{11}GA^{12}$ respectively.

***KRAS* exon 2 insertions activate RAS signaling pathway and enhance NIH3T3 cells transformation**

To investigate whether the newly found $^{10}G^{11}$ and $^{11}GA^{12}$ *KRAS* mutation activate RAS activity, we constructed expression plasmids and transiently transfected into 293FT and NIH3T3 cells. As a control, expression plasmids carrying wild-type *KRAS* (*KRAS*-WT) and a well-known active *KRAS* mutant (*KRAS*-G12V) were used for comparison during the basic functional assay.

Compared with the cells transfected with WT expression plasmid, overexpression of $^{10}G^{11}$ and $^{11}GA^{12}$ *KRAS* mutants in cell lines resulted in elevated protein levels of both active RAS (Ras-GTP) and its downstream signaling molecule, phosphorylated extracellular signal-regulated kinase (p-ERK). The elevated levels of these two proteins are similar to the cells transfected with the *KRAS*-G12V mutant construct (**Fig. 2**). To further demonstrate the biological effect of $^{10}G^{11}$ and $^{11}GA^{12}$ *KRAS* mutants, NIH3T3 cells which stably transfected with empty vector, *KRAS*-WT, *KRAS*-G12V, $^{10}G^{11}$ or $^{11}GA^{12}$ mutant were prepared. Although NIH3T3 stable transfectants showed similar proliferation rate in MTT assay (data not shown), they have apparent differences

in anchorage-independent growth property. We demonstrated in soft-agar colony formation assay that only a few number of colonies of the cells transfected with either empty vector or *KRAS*-wild type expression vector were observed. In contrast, more colonies were counted in all three transfectants with mutant *KRAS* and the differences were statistically significant compared with cells transfected with *KRAS*-wild type (**Fig. 3**). Furthermore, the colony sizes of the mutant *KRAS* transfectants were, in general, bigger than that in *KRAS*-wild type transfectant. To assess the in vivo tumorigenicity of novel *KRAS* variants, NIH3T3 transfectants containing empty vector or different *KRAS* mutants were injected subcutaneously into the dorsal flank of Balb/c nude mice. Compared with *KRAS* wild type and empty vector controls, *KRAS* $^{10}G^{11}$ and $^{11}GA^{12}$ significantly enhanced in vivo tumor growth as showed in **Figure 4**. Collectively, these observations suggested that both newly identified *KRAS* mutants could activate the Raf-MEK-ERK pathway by elevating RAS-GTP level and contribute in vitro and in vivo cell transformation.

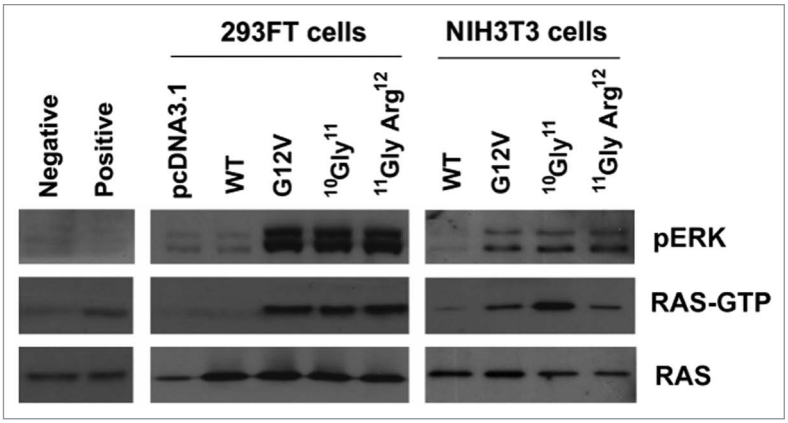

Figure 2. *KRAS* insertion mutants activated RAS signaling by enhancing cellular accumulation of active RAS (RAS-GTP) and activating p-ERK. NIH3T3 and 293FT cells were transfected with *KRAS* mutants, and RAS-GTP protein in the cell extract were immunoprecipitated with agarose beads containing Ras Binding Domain of Raf-1. Protein levels in both whole cell extracts (pan-RAS and pERK) and precipitated samples (RAS-GTP) were analyzed by western blot analysis as indicated. Representative results from 3 independent experiments were shown.

Discussion

In the current study, we report the *KRAS* mutation frequency in a large cohort of patients with colorectal cancer in Hong Kong. *KRAS* mutation is found in 44.5% (670 out of 1506) of colorectal cancers. The mutation rate is similar to *KRAS* studies previously reported.[13-20] **Table 4** summarized the *KRAS* mutation rates and the distribution of mutants in representative studies. Codon 12 is the most common *KRAS* mutation and the most frequently found mutation is G12D (35% of all mutations found). Our data demonstrate the predominance of *KRAS*-mutant carcinoma in right colon and in female patients. This is in keeping with some previous reports although other studies might not have demonstrated such relationship.[21,22] The preference of site of *KRAS* mutation might be correlate with the different molecular pathways involved in right and left side colon CRCs. The right and left side colon cancers have been considered as distinct tumor entities because of their epidemiological, clinicopathologic, and molecular biologic features. Right side colon cancer was found to be associated with female, older age, advanced stage, and poorly differentiated mucinous histology.[23-26] Higher rates of microsatellite instability and *KRAS* mutations were common molecular events found in right side colon cancer.[27,28] Whereas the left side tumor were more common to be chromosomal instable and harbor more *TP53* mutation.[27-30] The reason for the observed differences between left and right side colon adenocarcinoma remains unclear. It is likely to be multifactorial and complex including embryologic origin, and the effect of chemical and bacterial luminal microenvironments. Moreover, we have reported the predominant *KRAS* mutations in left colon are located in codon 12 and right colon in codon 13. This finding is different from a large population-based study which found significantly more codon-12 mutation cases in proximal (right colon) than distal (left colon) tumors (29.1% vs 20.5%; $P < 0.01$).[21] Another study also showed rectosigmoid tumor (left colon) had the highest frequency of codon 13 mutations.[31] There is no consistent trend, further study is necessary.

We report two rare in-frame insertion mutations in this study, c.30_31insGGA: p.G10_A11insG (duplication of codon 10) and c.33_34insGGAGCT:p.A11_G12insGA (duplication of codon 10–11). In-frame Insertion mutations in *KRAS* are rarely reported. Almost all reported *KRAS* in-frame insertions are tandem duplications. Three-nucleotide insertions resulting in codon 9, codon 10, and codon 12 duplications have been reported in colorectal cancer and leukemia.[8,32-34] A Netherland cohort study found a duplication of six nucleotides in a colorectal tumor, leading to two additional amino acids added in codon 9 of *KRAS*.[31] A 15-bp insertion in exon 3 that resulted in tandem duplication of codons 62–66 has been found in a case of primary lung adenocarcinoma.[35] Another study also reported the identical 15-bp in-frame insertion mutation in a colorectal carcinoma.[36]

Wild-type *KRAS* regulate cellular growth and differentiation by cycling between inactive GDP-bound form (Ras-GDP) and active GTP-bound form (Ras-GTP). Mutant *KRAS* is defective in intrinsic GTP hydrolysis. Therefore, it is accumulated in cells in active GTP-bound form, resulting in constitutive activation of downstream signaling through effector proteins. Both insertion mutations found in the current study ($^{10}G^{11}$ and $^{11}GA^{12}$) affect the phosphate-binding loop (codon 10–16) of *KRAS*. Our in vitro functional analyses have confirmed that similar to the *KRAS* mutant G12V, both rare mutants enhance the cellular accumulation of active RAS (Ras-GTP), and activate the Raf-MEK-ERK pathway. Using soft agar assays, we demonstrate the ability of both insertion variants in driving in vitro cell transformation.

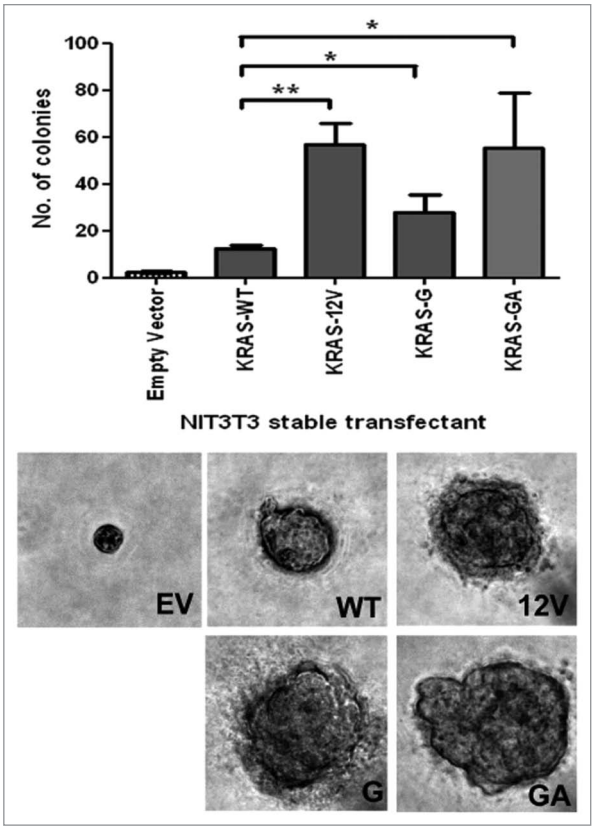

Figure 3. *KRAS* insertion mutants promoted anchorage-independent growth in soft agar. NIH3T3 cells stably transfected with pcDNA3.1 empty vector (EV), wild-type *KRAS* (WT), G12V *KRAS* mutant (G12V), ^{10}G^{11} and ^{11}GA^{12} mutants were cultured in soft agar for analysis. Representative microscopic pictures of colony from each transfectant were taken (Magnification, 400×). The number of colony in each transfectant was plot in the bar chart and the results shown were mean and standard deviation from three independent experiments. The *P* value of < 0.05 and < 0.001 were denoted as * and ** respectively.

We also show that both insertion mutants demonstrate enhanced tumorigenicity in nude mice. Our finding is concordant with previous in vivo analysis of *KRAS* ^{10}Gly^{11} mutation in acute leukemia which showed duplication of amino acid residue in codon 12 could lead to the activation of *KRAS*.[8] In addition, another RAS protein member, HRAS with an insertion mutation in codon 12 was reported to gain the ability in cell transformation.[8] These results suggest that both point mutation and insertion mutation within codon 12 and sites nearby could activate RAS protein through interrupting the GTP binding site of RAS family protein.

In summary, this study has provided a *KRAS* mutation database in colorectal cancer of local Chinese population and the correlation between *KRAS* status with gender and primary site in the colon. Furthermore, we report the identification and characterization of two rare *KRAS* insertion mutations. In vitro and in vivo functional studies confirm the oncogenic properties of these insertion mutations. *KRAS* mutations beyond the "hotspots" can be oncogenic by conveying selective growth advantage to the cells. These mutations might potentially contribute to primary resistance for anti-EGFR mAb targeted therapy. The clinical implication for these mutations requires further validation.

Materials and Methods

Patient sample

A total of 1506 consecutive colorectal adenocarcinoma specimens sent for *KRAS* mutational analysis in Prince of Wales Hospital, Hong Kong between 2008 and 2012 were included in this study. The study protocol was approved by the Joint CUHK-NTE Clinical Research Ethics Committee, Hong Kong.

Tumor DNA extraction

The location of tumor cells in the formalin-fixed, paraffin-embedded (FFPE) tissue were first marked on the standard H&E-stained histological slides. Subsequently, the corresponding tumor tissues on the unstained glass slide were microdissected manually for DNA extraction using QIAamp DNA tissue mini kit with standard procedure (Qiagen).

Sequencing analysis

Mutational hot spots including *KRAS* codons 12, 13, 61, and 146 were investigated by PCR-direct sequencing. PCR reactions were performed using primers listed in **Table 5**. Cycling sequencing reaction of the PCR fragments was performed with BigDye Terminator system (Applied Biosystems) using primers from both directions. The sequencing results were analyzed with the ABI PRISM® 3130XL Genetic Analyzer (Applied Biosystems). The data was collected and analyzed using Applied Biosystems sequencing analysis software.

Detection of the precise sequence of the rare mutation

PCR product corresponding to *KRAS* exon 1 was amplified from the patient genomic DNA and subsequently cloned using the TOPO-TA Cloning kit (Invitrogen). Ten colonies of each transformation were randomly selected for sequencing analysis.

Cell culture and transfection

Human embryonic kidney cells (293FT) and mouse embryonic fibroblast cells (NIH3T3) were obtained from Invitrogen and American Type Culture Collection (ATCC) respectively. Both cell lines were cultured in Dulbecco modified Eagle medium plus 10% FBS (Gibco, Invitrogen). Transfection of 293FT and NIH3T3 cells were performed using Lipofectamine™ LTX reagent (Invitrogen) following the manufacturer's protocol.

Site-direct mutagenesis and active RAS measurement

Full-length of *KRAS* cDNA was cut from pBabe K-Ras 12V vector (Addgene plasmid 12544)[37] and cloned into pcDNA3.1 (+) expression vector (Invitrogen) via *BamH1* and *Xba1* restriction sites. Corresponding *KRAS* mutations were introduced into the expression vector using QuickChange® II Site-Directed Mutagenesis Kit according to the manufacturer's recommendations (Stratagene). The desired mutations in each construct were finally confirmed by direct sequencing. The primer sequences for mutagenesis were listed in **Table 5**. Ras Activation Assay Kit (Millipore) was used to measure the level of active RAS (RAS-GTP) after transient transfection of corresponding plasmid into

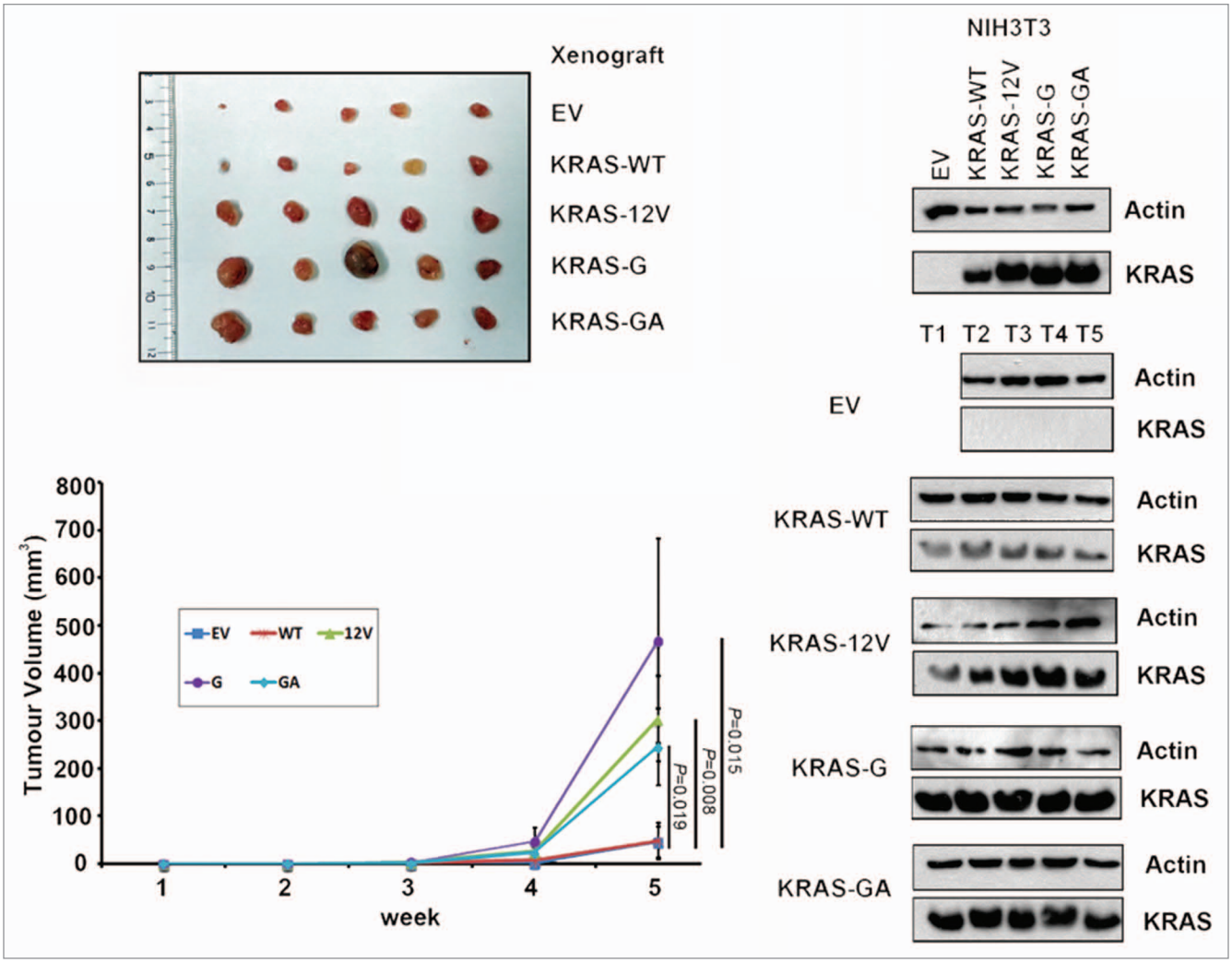

Figure 4. *KRAS* insertion mutants promoted in vivo growth of NIH3T3 cells. In vivo tumorgenic assay in nude mice showed that tumors formed in the sites implanted with NIH3T3 cells expressing *KRAS* mutants (G12V, ^{10}G^{11}, or ^{11}GA^{12}) were consistently larger than that implanted with wild-type *KRAS* (WT) and empty vector (EV) controls. By western blotting, the expression of KRAS protein in the NIH3T3 transfectants and tumors dissected from the xenografts (T1–T5) was detected.

the cell lines. In brief, 0.5 mg of cell extract was immunoprecipitated with agarose beads containing human Ras Binding Domain (RBD, residues 1–149) of Raf-1. After washing, the beads were mixed with protein loading buffer and 10% of the mixture was electrophoresed by 12% SDS-PAGE for western blot analysis as previously described.[38,39] The primary antibodies used were pan-RAS (RAS10, Millipore; 1:2000) and p-ERK1/2 (9102, Cell Signaling; 1:1000). HRP conjugated anti-mouse secondary antibody used was purchased from DAKO (1:20000 dilution).

Soft agar colony formation assay

NIH3T3 cells transfected with corresponding *KRAS* expression plasmids were selected in culture medium containing 400 μg/mL of G418 (Invitrogen) for one month before preparing colony formation assay. In the assay, culture medium containing 0.7% agarose was set as a bottom layer in 6-well dishes. A total of 3000 cells, which mixed with culture medium containing 0.35% agarose, were added over the bottom layer. After 25 d of incubation, colonies were stained with 0.005% crystal violet overnight and were counted under dissection microscope. Each experiment was performed in triplicate.

In vivo tumorigenicity

NIH3T3 transfectants (1×10^6 cells suspended in 0.1 mL phosphate-buffered saline), containing empty vector or different *KRAS* mutant, were injected subcutaneously into the dorsal flank of five 5-wk-old male Balb/c nude mice. The tumor volume was determined as previously described.[40] All experimental procedures were approved by the Animal Ethics Committee of the Chinese University of Hong Kong.

Statistical analysis

Statistical analysis of two times two contingency tables of categorical variables was performed using the Chi-square test or Fisher exact test, as appropriate. The *t* test was performed to compare continuous variables between two groups. All statistical analyses were performed by using statistical program SPSS

Table 4. Comparison of *KRAS* mutation distribution in reported series

Studies	Current study	COSMIC database	Rosty 2013[23]	Imamura 2012[41]	De Roock 2010[42]	Chang 2009[43]	Karapetis 2008[44]	Amado 2008[7]	Brink 2003[31]	Samowitz 2000[21]	Andreyev 1998[11]
n =	1506	17316	776	1261	747	228	394	427	737	1416	2214
Mutation rate %	44.5	34.9	28	35.8	36.3	36.4	41.6	43.1	36.8	31.8	37.7
Relative mutation distribution (%) by codon											
Codon 12	75.1	79.3	87	74.6	69.3	69.9	63.8	84.2	70	77.9	54
Codon 13	19.1	17.6	13	25.4	20.1	25.3	11.7	15.8	21.6	22.1	16.7
Codon 61	2.5	0.58			5.3	1.2					
Codon 146	2.7	0.19			5	2.4					
Relative mutation distribution (%) by nucleotide substitution											
G12D	37.6	35	161	35.2	27.4		35.7	38	26.1	31.1	30.6
G12V	20.0	21.5	95	20.8	19.8		28.1	21.7	24.4	21.4	23.4
G12C	6.7	8.3	44	9.6	7.3			7.6	5.9	9.5	
G12S	4.9	6.3	12	2.6	6.3			7.6	5.6	6.8	
G12A	4.3	6.7	20	4.4	6.9			8.2	5.6	3.5	
G12R	1.5	1.1	8	1.8	1.7			1.6	2.4	0.7	
G13D	18.8	17.4	110	24.1	20.1		11.7	15.8	20.2	20.8	16.7
G13C	0.3		3	0.7					0.3	0.4	
Q61H	1.3	0.3			2.3						
Q61L	0.7	0.2			1						
Q61R	0.3	0.1			1.3						
A146T	2.7	0.2			5						

Table 5. The sequences of oligonucleotides used in this study

PCR primers	Forward sequence	Reverse sequence
KRAS codon 12/13	GTATTAACCT TATGTGTGAC A	GTCCTGCACC AGTAATATGC
KRAS codon 61	TGCACTGTAA TAATCCAGAC TGTG	TGCACTGTAA TAATCCAGAC TGTG
KRAS codon 146	TCTGAAGATG TACCTATGGT CCTAGT	AAGAAGCAAT GCCCTCTCAA
Mutagenesis primers		
KRAS-WT	5′-GGTAGTTGGA GCTGGTGGCG TAGGCAAGA-3′	5′- TCTTGCCTAC GCCACCAGCT CCAACTACC-3′
KRAS-10G11	5′-GTGGTAGTTG GAGGAGCTGG TGGCGTAGGC AAG-3′	5′-CTTGCCTACG CCACCAGCTC CTCCAACTAC CAC-3′
KRAS-11GA12	5′-GGTAGTTGGA GCTGGAGCTG GTGGCGTAGG CAAG-3′	5′-CTTGCCTACG CCACCAGCTC CAGCTCCAAC TACC-3′

version 16.0. A two-tailed *P* value of <0.05 was regarded as statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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