Published in final edited form as:
Diagn Mol Pathol. 2010 September ; 19(3): 157–163. doi:10.1097/PDM.0b013e3181c93fd1.

NRAS Mutations Are Rare in Colorectal Cancer

Natsumi Irahara, PhD[*], **Yoshifumi Baba, MD, PhD**[*], **Katsuhiko Nosho, MD, PhD**[*], **Kaori Shima, DDS, PhD**[*], **Liying Yan, MS**[†], **Dora Dias-Santagata, PhD**[‡], **A. John Iafrate, MD, PhD**[‡], **Charles S. Fuchs, MD**[*,§], **Kevin M. Haigis, PhD**[‖], and **Shuji Ogino, MD, PhD**[*,¶]

[*] Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA

[†] EpigenDx, Worcester, MA

[‡] Department of Pathology, Massachusetts General Hospital and Harvard Medical School, Boston, MA

[§] Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

[‖] Molecular Pathology Unit and Center for Cancer Research, Massachusetts General Hospital, Charlestown, MA

[¶] Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

Abstract

Activating mutations in members of the *RAS* oncogene family (*KRAS*, *HRAS* and *NRAS*) have been found in a variety of human malignancies, suggesting a dominant role in carcinogenesis. In colon cancers, *KRAS* mutations are common and clearly contribute to malignant progression. The frequency of *NRAS* mutations and their relationship to clinical, pathologic, and molecular features remains uncertain. We developed and validated a Pyroseqencing assay to detect *NRAS* mutations at codons 12, 13 and 61. Utilizing a collection of 225 colorectal cancers from two prospective cohort studies, we examined the relationship between *NRAS* mutations, clinical outcome, and other molecular features, including mutation of *KRAS*, *BRAF*, and *PIK3CA*, microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP). Finally, we examined whether *NRAS* mutation was associated with patient survival or prognosis. *NRAS* mutations were detected in 5 (2.2%) of the 225 colorectal cancers and tended to occur in left-sided cancers arising in women, but did not appear to be associated with any of the molecular features that were examined.

Keywords

NRAS; colon cancer; clinical outcome; sequencing; signal transduction

Introduction

The *RAS* proto-oncogenes (*HRAS*, *KRAS* and *NRAS*) encode a family of GDP/GTP-regulated switches that convey extracellular signals to regulate the growth and survival properties of cells.[21] The four enzymes encoded by the three *RAS* genes are highly homologous to one

Correspondence to: Kevin M. Haigis, Ph.D. Molecular Pathology Unit Massachusetts General Hospital 149 13th St., 6.010 Charlestown, Massachusetts 02129, USA Phone: 617-643-0070 FAX: 617-726-5684 khaigis@partners.org.

NI, YB and KN contributed equally. CSF, KMH and SO are co-senior authors.

another, sharing a high degree of identity over the first 90% of the proteins. The extreme carboxy-termini of the proteins constitute the hypervariable region, which diverges radically in primary sequence and undergoes post-translational modification that confers important differences in trafficking and intracellular localization.

RAS family members are frequently found in their mutated, oncogenic forms in human tumors. Mutant RAS proteins are constitutively active, owing to reduced intrinsic GTPase activity and insensitivity to GTPase-activating proteins (GAPs). In total, activating mutations in the *RAS* genes occur in approximately 20% of all human cancers, mainly in codons 12, 13 or 61.[10, 46] Mutations in *KRAS* account for about 85% of all *RAS* mutations in human tumors, *NRAS* for about 15%, and *HRAS* for less than 1%.[12] Which particular *RAS* gene is mutated seems to be tumor specific; colonic, pancreatic and lung cancers have high frequencies of *KRAS* mutations. For example, we reported that *KRAS* gene mutations were present in 277 (36%) of 772 colorectal cancers analyzed.[24,] 36 *NRAS* mutations, by contrast, are common in myeloid leukemias and cutaneous melanomas.[4, 39] Nevertheless, the frequency of *NRAS* mutations in colorectal cancer remains unclear.

GTP-bound RAS transmits its signal through a variety of downstream "effector" pathways, for example the RAF→MEK→ERK and PI3K→AKT cascades. Activating *NRAS* mutations occur in up to 30% of cutaneous melanoma cases and *BRAF* mutations also occur at a high frequency in these cancers. Recently, it has been reported that mutations in *BRAF* or *NRAS* are associated with decreased survival in patients with metastatic melanoma.[16, 47] Interestingly, *BRAF* mutations are mutually exclusive with *NRAS* mutations in melanoma and with *KRAS* mutations in colorectal cancer.[1, 9, 38, 44, 45, 50, 52] By contrast, mutations in *PIK3CA* (which encodes the p110α catalytic subunit of PI3K) tend to co-exist with *KRAS* mutations in colorectal cancer.[22, 48] The patterns of mutational co-occurrence suggest that each RAS effector plays a different role in tumor development. In this respect, understanding the genetic context in which *NRAS* mutations arise is important.

In this study, we used Pyrosequencing to identify *NRAS* mutations at codons 12, 13 and 61, the mutational hotspots for all of the *RAS* genes, in a set of 225 colorectal cancers. We examined the relationship between *NRAS* mutations and other molecular, pathologic, and clinical features, including mutations in *KRAS*, *BRAF*, and *PIK3CA*, microsatellite instability (MSI), CpG island methylator phenotype (CIMP) status, and patient survival.

Materials and Methods

Tissue specimens

Experimental samples were identified by searching the databases of two large prospective cohort studies: the Nurses' Health Study (N = 121,700 women followed since 1976) and the Health Professionals Follow-up Study (N = 51,500 men followed since 1986).14 A subset of the cohort participants developed colorectal cancer during prospective follow-up. Previous studies on the Nurses' Health Study and Health Professionals Follow-up Study have described baseline characteristics of cohort participants and incident colorectal cancer cases and confirmed that our colorectal cancers were a good representative of a population-based sample.[7, 14] We collected paraffin-embedded tissue blocks from hospitals where cohort participants with colorectal cancers had undergone resections of primary tumors. On the basis of availability of tissue materials and assay results, a total of 225 colorectal cancers were included in this study. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue.6 Hematoxylin and eosin-stained tissue sections were examined by a pathologist (S.O.) unaware of clinical or other laboratory data.[29] The tumor grade was categorized as low (≥50% gland formation) vs. high (<50% gland formation). The presence and extent of extracellular mucin and signet ring cells

were recorded. Although many of the cases have been previously characterized for the status of CIMP, MSI, *KRAS*, *BRAF*, and p53,[29] we have not examined *NRAS* mutations in our specimens. Informed consent was obtained from all subjects. Tissue collection and analyses were approved by the Brigham and Women's Hospital and Harvard School of Public Health Institutional Review Boards.

Genomic DNA extraction and whole genome amplification

Genomic DNA was extracted from paraffin-embedded tumor tissue sections using QIAmp DNA Mini Kit (Qiagen, Valencia, CA).[28] Normal DNA was obtained from colonic tissue at resection margins. Whole genome amplification (WGA) of genomic DNA was performed by polymerase chain reaction (PCR) using random 15-mer primers.[11, 28] Previous studies showed that WGA did not significantly affect downstream genetic analysis.[3, 13]

Pyrosequencing for *NRAS*

The primers for PCR and Pyrosequencing were purchased from EpigenDx inc. (EpigenDx, Worcester, MA), and PCR was conducted according to the protocol recommended by the manufacturer. Each PCR mix contained 3 pmol of forward primer, 0.3 pmol of reverse primer, 2.7 pmol of Universal primer, 200 μmol each of dNTPs, 3.0 mmol $MgCl_2$, 1xPCR buffer (Qiagen, Valencia, CA), 0.75 U of HotStar Taq polymerase (Qiagen), and 1 μl of template WGA product in a total volume of 30 μl. PCR conditions were as follows: initial denaturing at 95°C for 15 minutes; 45 cycles of 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds; and final extension at 72°C for 5 minutes. The PCR products (each 10 μl) were sequenced using the Pyrosequencing PSQ96 HS System (Qiagen) according to the manufacturer's instructions. The nucleotide dispensation orders were: codon 12 - ACT GAC TAC GAC T, codon 13 - CAT GAC TAC TGA CTG, codon 61 - ACG TCT AGC AGT. To increase sensitivity, we designed a different Pyrosequencing primer for codon 13: 5′-GTGGTGGTTGGAGCAGGTG-3′. The use of three sequencing primers served as a quality control measure, because mutations in codon 13 are detected by at least two primers. To confirm the results, we repeated PCR-Pyrosequencing, with laboratory staff unaware of the previous results, using unique nucleotide dispensation orders and at a different facility (EpigenDx): codon 12 and 13 - TCG ATG CTA GTG TGC AGC G, codon 61 - CAT CGA TCA G.

Sanger sequencing analysis for *NRAS*

The mutational status of *NRAS* was confirmed by direct sequencing of PCR products generated using the following primer pairs: exon 2, 5′-ACGTTGGATGCAACAGGTTCTTGCTGGTGT-3′ (forward) and 5′-ACGTTGGATGgagagacaggatcaggtcagc-3′ (reverse); exon 3, 5′-ACGTTGGATGTGGTGAAACCTGTTTGTTGG-3′ (forward) and 5′-ACGTTGGATGcctttcagagaaaataatgctcct-3′ (reverse). PCR was performed in a volume of 20 μl, containing 1 unit of Taq polymerase (Invitrogen, Carlsbad, CA), 4 nmol of dNTPs (Invitrogen, Carlsbad, CA), 10 pmol of forward and reverse primers, 40 nmol of $MgCl_2$ and 40 ng of DNA. Thermocycling was performed at 95 °C for 8 min, followed by 45 cycles of 95 °C for 20 s, 58 °C for 30 s and 72°C for 1 min, and one last cycle of 72 °C for 3 min. The resulting PCR products were treated using 1 unit of shrimp alkaline phosphatase (USB, Cleveland, OH) and 5 units of exonuclease I (USB, Cleveland, OH) at 37°C for 20 minutes followed by 80°C for 15 minutes, and tested for the presence of mutations by bi-directional Sanger sequencing using the original PCR primers and the BigDye Terminator V1.1 Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer′s recommendations. Tumor and control human genomic DNA (Promega, Madison, WI) sequences were compared using the AB Sequencing Analysis Software v5.2 (Applied Biosystems).

PCR and Pyrosequencing for *KRAS*, *BRAF* and *PIK3CA*, and microsatellite analyses

PCR and Pyrosequencing targeted for *KRAS* codon 12 and 13,[28] *BRAF* codon 600,[30] and *PIK3CA* exons 9 and 20 were performed.24 Microsatellite instability (MSI) status was determined using D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487.[26] MSI-high was defined as the presence of instability in ≥30% of the markers, MSI-low as instability in 1-29% of the markers, and microsatellite stability (MSS) as the absence of instability. For 18q loss of heterozygosity (LOH) analysis using microsatellite markers (D18S55, D18S56, D18S67, and D18S487), LOH at each locus was defined as ≥40% reduction of one of two allele peaks in tumor DNA relative to normal DNA.31 Overall 18q LOH positivity was defined as the presence of any marker with LOH, and 18q LOH negativity as the presence of ≥2 informative markers and the absence of LOH.

Real-time PCR to measure CpG island methylation and Pyrosequencing to measure LINE-1 methylation

Bisulfite DNA treatment and real-time PCR (MethyLight) assays were validated and performed.[27] We quantified methylation at 8 CIMP-specific CpG islands [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*].[26, 29] CIMP-high was defined as ≥6/8 methylated promoters using the 8-marker CIMP panel, CIMP-low as 1/8-5/8 methylated promoters and CIMP-0 as 0/8 methylated promoters, according to the previously established criteria.[29] We also quantified methylation at 8 other loci (*CHFR*, *HIC1*, *IGFBP3*, *MGMT*, *MINT1*, *MINT31*, *p14*, and *WRN*).22, [36] The percentage of methylated reference (PMR) at a specific locus was calculated as previously described.[27] LINE-1 methylation level was measured by Pyrosequencing.32

Immunohistochemistry

We constructed tissue microarrays.6 Methods of immunohistochemistry were previously described as follows: cyclin D1,23 β-catenin,18 p21, p27, p53,33 fatty acid synthase (FASN) and COX-2.[25] All immunohistochemically-stained slides for each marker were interpreted by one of the investigators (p53, p21, p27, COX-2 and FASN by S.O.; cyclin D1 and β-catenin by K.N.) unaware of other data. A random sample of 108-402 tumors were re-examined by a second observer (p21, p27 and cyclin D1 by K.S.; p53, FASN by K.N.; β-catenin by S.O.; COX-2 by R. Dehari, Kanagawa Cancer Center, Japan) unaware of other data. The concordance between the two observers (all $p<0.0001$) was: 0.83 ($\kappa=0.64$, N=160) for cyclin D1; 0.87 ($\kappa=0.75$, N=118) for p53; 0.83 ($\kappa=0.62$, N=179) for p21; 0.94 ($\kappa=0.60$, N=114) for p27; 0.83 ($\kappa=0.65$, N=402) for β-catenin; 0.92 ($\kappa=0.62$, N=108) for COX-2; 0.93 ($\kappa=0.57$, N=246) for FASN, indicating good to substantial agreement.

Statistical analysis

Fisher's exact test was performed to assess associations between categorical data, using the SAS program (version 9.1; SAS Institute, Cary, NC). All P values were two-sided, and statistical significance was set at $P \leq 0.05$. A correction of multiple hypothesis testing was not attempted due to a limited power.

Results

NRAS mutation in colorectal cancer

We used Pyrosequencing to determine the frequency of *NRAS* codon 12, 13 and 61 mutations in sporadic colorectal cancers. The normal amino acid at codons 12 and 13 of the *NRAS* gene is glycine (GGT) and the normal amino acid at codon 61 is glutamine (CAA). In this study of 225 colorectal cancers, 5 cases (2.2%) harbored *NRAS* activating mutations (Figure 1). These mutations were confirmed by different nucleotide dispensation orders for Pyrosequencing and

by Sanger sequencing. The activating mutations found in the *NRAS* gene were c.34G>A (p.G12S), c.35G>A (p.G12D) and c.35G>T (p.G12V) in codon 12 and c.181C>A (p.Q61K) in codon 61. No codon 13 mutations were detected. These *NRAS* mutations have been described in other cancers (the Sanger Institute COSMIC database; www.sanger.ac.uk/genetics/CGP/cosmic/).

NRAS mutation and clinical, pathologic prognostic and molecular features

We examined genetic and epigenetic features as well as clinical outcome of 225 colorectal cancer cases according to *NRAS* mutation status (Table 1). A distribution of various molecular features detected in our colorectal cancers was essentially in agreement with the previous studies.[2, 19, 22, 34, 35, 37] Table 2 shows clinical, pathologic and molecular features of colorectal cancers with *NRAS* mutations. *NRAS* mutations were mutually exclusive with mutations in *BRAF*, *KRAS* and *PIK3CA*, although the sample size was limited. All of the cancers with *NRAS* mutations were located in the distal (left-side) colon and 4/5 arose in female patients. Nevertheless, due to the low frequency of *NRAS* mutation, none of those relations was statistically significant. There was no apparent association between the *NRAS* mutations and any of the other clinical or pathological features examined (Table 1). Likewise, there was no significant association between *NRAS* mutation and any of the other tumoral markers in Table 1, or to β-catenin, p53, p21, p27, cyclin D1, COX-2 and FASN (data not shown). In addition, no significant effect of *NRAS* mutation on patient survival was noted (data not shown).

Discussion

We conducted this study to examine the frequency of *NRAS* mutation in relation to various genetic and epigenetic alterations in colorectal cancer. The *RAS* pathway plays an important role in the development of various cancers,[17, 20, 43] and frequent activating mutations in the *KRAS* oncogene have been identified in colorectal cancer.[28] Nevertheless, there are only a few reports on *NRAS* mutations in colorectal cancer and none of these studies correlated *RAS* mutations with other molecular events. [40, 49] We developed a Pyrosequencing assay to detect *NRAS* mutations because this methodology has been shown to be applicable to paraffin-embedded tumors and is more sensitive than Sanger dideoxy sequencing in *KRAS* mutation analysis.[28] We found that *NRAS* mutation in colorectal cancer was rare (2.2%). This observation is consistent with publicly available cancer genome sequencing data from the Sanger Institute COSMIC database (www.sanger.ac.uk/genetics/CGP/cosmic/), which lists *NRAS* mutation frequency at approximately 3% in colon cancer.

Our resource of a population-based sample of colorectal cancer (relatively unbiased samples set compared with retrospective or single-hospital-based samples) derived from two prospective cohorts has enabled us to precisely estimate the frequency of specific molecular events (such as *KRAS, BRAF, PIK3CA* mutations, CIMP etc.) and to correlate mutations with clinical and pathological features of colon cancer. We detected *NRAS* mutations in only 5 of 225 colon caners using Pyrosequencing (Figure 1). Because of the low frequency, *NRAS* mutation was not significantly associated with any clinical or pathologic features or with patient survival. Nevertheless, there was a trend towards *NRAS* mutations in left-sided MSS tumors that arise in females.

Mutational activation of *RAS* via a point mutation at codon 12, 13 or 61 is well characterized as a marker for progression of normal or benign cells toward malignancy.[41, 42] Mutant RAS oncoproteins have decreased GTPase activity, essentially locking them into an activated state, and GTP-bound RAS transmits strong downstream signals that alter normal cellular functions.[5] Nevertheless, different cancers select for mutations in different *RAS* family members, suggesting that the family members exhibit cell type-specific expression patterns or functions. *NRAS* is, perhaps, best characterized in leukemia and melanoma, where mutations are relatively

common; *NRAS* mutations occur in 30% of melanomas and are mutually exclusive with mutations in *BRAF*, suggesting that these two events may be functionally equivalent. Unlike colon cancers, *KRAS* mutations are rare in melanomas.

Little is known regarding the impact of *NRAS* mutation in colorectal cancer. Some studies have shown that *NRAS* mutations seem to arise at a later stage in the development of malignancy, unlike *KRAS* mutations, which arise early.[8, 49] Recent studies utilizing mouse models have demonstrated clear phenotypic differences between mutant *KRAS* and *NRAS* in colon cancer.[15] Activated *KRAS* has a unique ability to promote tumor proliferation and to suppress differentiation, while activated *NRAS* suppressed apoptosis in a developing tumor. These data suggest that *KRAS* and *NRAS* mutations arise in response to unique selective pressures. *KRAS* mutations have recently been shown to arise under conditions of low glucose availability.[51] The data from animal studies suggest that *NRAS* mutations might arise under conditions of chronic apoptotic stress.

Although the results of our study do not reach statistical significance, the trends in the data may provide insight into *NRAS*-mutant colorectal cancers. For example, our data indicate that *NRAS* mutations are found in left-sided MSS cancers. The mutational pattern is similar to that of *KRAS*, but entirely distinct from *BRAF*, which is mutated predominantly in right-sided CIMP-high cancers.[22, 24] Thus, while *NRAS* and *BRAF* may play a similar role in melanoma progression, they appear to play distinct roles in colon cancer progression.

In conclusion, our cohort study shows that the frequency of *NRAS* activating mutations in colorectal cancers is low. Additional studies are needed to elucidate the mechanisms underlying the oncogenic properties of the *RAS* oncogenes in colorectal cancers.

Acknowledgments

Funding: This work was supported by the U.S. National Institute of Health (P01 CA87969 to S. Hankinson, P01 CA55075 to W. Willett, P50 CA127003 to C.S.F., K07 CA122826 to S.O., K01 CA118425 to K.M.H.); the Bennett Family Fund; the Entertainment Industry Foundation National Colorectal Cancer Research Alliance. K.N. was supported by a fellowship grant from the Japan Society for Promotion of Science. The content is solely the responsibility of the authors and does not necessarily represent the official views of NCI or NIH. Funding agencies did not have any role in the design of the study; the collection, analysis, or interpretation of the data; the decision to submit the manuscript for publication; or the writing of the manuscript.

References

1. Banerji U, Affolter A, Judson I, et al. BRAF and NRAS mutations in melanoma: potential relationships to clinical response to HSP90 inhibitors. Mol Cancer Ther 2008;7:737–739. [PubMed: 18375819]
2. Barault L, Veyrie N, Jooste V, et al. Mutations in the RAS-MAPK, PI(3)K (phosphatidylinositol-3-OH kinase) signaling network correlate with poor survival in a population-based series of colon cancers. Int J Cancer 2008;122:2255–2259. [PubMed: 18224685]
3. Boland CR, Thibodeau SN, Hamilton SR, et al. A National Cancer Institute Workshop on Microsatellite Instability for cancer detection and familial predisposition: development of international criteria for the determination of microsatellite instability in colorectal cancer. Cancer Res 1998;58:5248–5257. [PubMed: 9823339]
4. Bos JL. ras oncogenes in human cancer: a review. Cancer Res 1989;49:4682–4689. [PubMed: 2547513]
5. Campbell SL, Khosravi-Far R, Rossman KL, et al. Increasing complexity of Ras signaling. Oncogene 1998;17:1395–1413. [PubMed: 9779987]
6. Chan AT, Ogino S, Fuchs CS. Aspirin and the risk of colorectal cancer in relation to the expression of COX-2. N Engl J Med 2007;356:2131–2142. [PubMed: 17522398]
7. Colditz GA, Hankinson SE. The Nurses' Health Study: lifestyle and health among women. Nat Rev Cancer 2005;5:388–396. [PubMed: 15864280]

8. Demunter A, Stas M, Degreef H, et al. Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. J Invest Dermatol 2001;117:1483–1489. [PubMed: 11886512]
9. Dhomen N, Marais R. New insight into BRAF mutations in cancer. Curr Opin Genet Dev 2007;17:31–39. [PubMed: 17208430]
10. Diaz-Flores E, Shannon K. Targeting oncogenic Ras. Genes Dev 2007;21:1989–1992. [PubMed: 17699748]
11. Dietmaier W, Hartmann A, Wallinger S, et al. Multiple mutation analyses in single tumor cells with improved whole genome amplification. Am J Pathol 1999;154:83–95. [PubMed: 9916922]
12. Downward J. Targeting RAS signalling pathways in cancer therapy. Nat Rev Cancer 2003;3:11–22. [PubMed: 12509763]
13. Eads CA, Danenberg KD, Kawakami K, et al. MethyLight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 2000;28:E32. [PubMed: 10734209]
14. Giovannucci E, Liu Y, Rimm EB, et al. Prospective study of predictors of vitamin D status and cancer incidence and mortality in men. J Natl Cancer Inst 2006;98:451–459. [PubMed: 16595781]
15. Haigis KM, Kendall KR, Wang Y, et al. Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation and tumor progression in the colon. Nat Genet 2008;40:600–608. [PubMed: 18372904]
16. Houben R, Becker JC, Kappel A, et al. Constitutive activation of the Ras-Raf signaling pathway in metastatic melanoma is associated with poor prognosis. J Carcinog 2004;3:6. [PubMed: 15046639]
17. Jaiswal BS, Janakiraman V, Kljavin NM, et al. Combined targeting of BRAF and CRAF or BRAF and PI3K effector pathways is required for efficacy in NRAS mutant tumors. PLoS One 2009;4:e5717. [PubMed: 19492075]
18. Kawasaki T, Nosho K, Ohnishi M, et al. Correlation of beta-catenin localization with cyclooxygenase-2 expression and CpG island methylator phenotype (CIMP) in colorectal cancer. Neoplasia 2007;9:569–577. [PubMed: 17710160]
19. Kearney KE, Pretlow TG, Pretlow TP. Increased expression of fatty acid synthase in human aberrant crypt foci: possible target for colorectal cancer prevention. Int J Cancer 2009;125:249–252. [PubMed: 19358283]
20. Lea IA, Jackson MA, Dunnick JK. Genetic Pathways to Colorectal Cancer. Mutat Res. 2009
21. Malumbres M, Barbacid M. RAS oncogenes: the first 30 years. Nat Rev Cancer 2003;3:459–465. [PubMed: 12778136]
22. Nosho K, Irahara N, Shima K, et al. Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. PLoS ONE 2008;3:e3698. [PubMed: 19002263]
23. Nosho K, Kawasaki T, Chan AT, et al. Cyclin D1 is frequently overexpressed in microsatellite unstable colorectal cancer, independent of CpG island methylator phenotype. Histopathology 2008;53:588–598. [PubMed: 18983468]
24. Nosho K, Kawasaki T, Ohnishi M, et al. PIK3CA mutation in colorectal cancer: relationship with genetic and epigenetic alterations. Neoplasia 2008;10:534–541. [PubMed: 18516290]
25. Ogino S, Brahmandam M, Cantor M, et al. Distinct molecular features of colorectal carcinoma with signet ring cell component and colorectal carcinoma with mucinous component. Mod Pathol 2006;19:59–68. [PubMed: 16118624]
26. Ogino S, Cantor M, Kawasaki T, et al. CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 2006;55:1000–1006. [PubMed: 16407376]
27. Ogino S, Kawasaki T, Brahmandam M, et al. Precision and performance characteristics of bisulfite conversion and real-time PCR (MethyLight) for quantitative DNA methylation analysis. J Mol Diagn 2006;8:209–217. [PubMed: 16645207]
28. Ogino S, Kawasaki T, Brahmandam M, et al. Sensitive sequencing method for KRAS mutation detection by Pyrosequencing. J Mol Diagn 2005;7:413–421. [PubMed: 16049314]
29. Ogino S, Kawasaki T, Kirkner GJ, et al. Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 2007;9:305–314. [PubMed: 17591929]

30. Ogino S, Kawasaki T, Kirkner GJ, et al. CpG island methylator phenotype-low (CIMP-low) in colorectal cancer: possible associations with male sex and KRAS mutations. J Mol Diagn 2006;8:582–588. [PubMed: 17065427]
31. Ogino S, Kawasaki T, Kirkner GJ, et al. 18q loss of heterozygosity in microsatellite stable colorectal cancer is correlated with CpG island methylator phenotype-negative (CIMP-0) and inversely with CIMP-low and CIMP-high. BMC Cancer 2007;7:72. [PubMed: 17474983]
32. Ogino S, Kawasaki T, Nosho K, et al. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. Int J Cancer 2008;122:2767–2773. [PubMed: 18366060]
33. Ogino S, Meyerhardt JA, Cantor M, et al. Molecular alterations in tumors and response to combination chemotherapy with gefitinib for advanced colorectal cancer. Clin Cancer Res 2005;11:6650–6656. [PubMed: 16166444]
34. Ogino S, Nosho K, Irahara N, et al. A cohort study of cyclin d1 expression and prognosis in 602 colon cancer cases. Clin Cancer Res 2009;15:4431–4438. [PubMed: 19549773]
35. Ogino S, Nosho K, Kirkner GJ, et al. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. Gut 2009;58:90–96. [PubMed: 18832519]
36. Ogino S, Nosho K, Kirkner GJ, et al. PIK3CA mutation is associated with poor prognosis among patients with curatively resected colon cancer. J Clin Oncol 2009;27:1477–1484. [PubMed: 19237633]
37. Ogino S, Shima K, Nosho K, et al. A cohort study of p27 localization in colon cancer, body mass index, and patient survival. Cancer Epidemiol Biomarkers Prev 2009;18:1849–1858. [PubMed: 19505918]
38. Ollikainen M, Gylling A, Puputti M, et al. Patterns of PIK3CA alterations in familial colorectal and endometrial carcinoma. Int J Cancer 2007;121:915–920. [PubMed: 17471559]
39. Omholt K, Platz A, Kanter L, et al. NRAS and BRAF mutations arise early during melanoma pathogenesis and are preserved throughout tumor progression. Clin Cancer Res 2003;9:6483–6488. [PubMed: 14695152]
40. Oudejans JJ, Slebos RJ, Zoetmulder FA, et al. Differential activation of ras genes by point mutation in human colon cancer with metastases to either lung or liver. Int J Cancer 1991;49:875–879. [PubMed: 1959991]
41. Parikh C, Ren R. Mouse model for NRAS-induced leukemogenesis. Methods Enzymol 2008;439:15–24. [PubMed: 18374153]
42. Parikh C, Subrahmanyam R, Ren R. Oncogenic NRAS, KRAS, and HRAS exhibit different leukemogenic potentials in mice. Cancer Res 2007;67:7139–7146. [PubMed: 17671181]
43. Quinlan MP, Settleman J. Isoform-specific ras functions in development and cancer. Future Oncol 2009;5:105–116. [PubMed: 19243303]
44. Rossi DJ, Weissman IL. Pten, tumorigenesis, and stem cell self-renewal. Cell 2006;125:229–231. [PubMed: 16630811]
45. Samuels Y, Diaz LA Jr. Schmidt-Kittler O, et al. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. Cancer Cell 2005;7:561–573. [PubMed: 15950905]
46. Schubbert S, Shannon K, Bollag G. Hyperactive Ras in developmental disorders and cancer. Nat Rev Cancer 2007;7:295–308. [PubMed: 17384584]
47. Ugurel S, Thirumaran RK, Bloethner S, et al. B-RAF and N-RAS mutations are preserved during short time in vitro propagation and differentially impact prognosis. PLoS One 2007;2:e236. [PubMed: 17311103]
48. Velho S, Oliveira C, Ferreira A, et al. The prevalence of PIK3CA mutations in gastric and colon cancer. Eur J Cancer 2005;41:1649–1654. [PubMed: 15994075]
49. Vogelstein B, Fearon ER, Hamilton SR, et al. Genetic alterations during colorectal-tumor development. N Engl J Med 1988;319:525–532. [PubMed: 2841597]
50. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004;116:855–867. [PubMed: 15035987]
51. Yun J, Rago C, Cheong I, et al. Glucose Deprivation Contributes to the Development of KRAS Pathway Mutations in Tumor Cells. Science. 2009

52. Zhao L, Vogt PK. Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms. Proc Natl Acad Sci U S A 2008;105:2652–2657. [PubMed: 18268322]

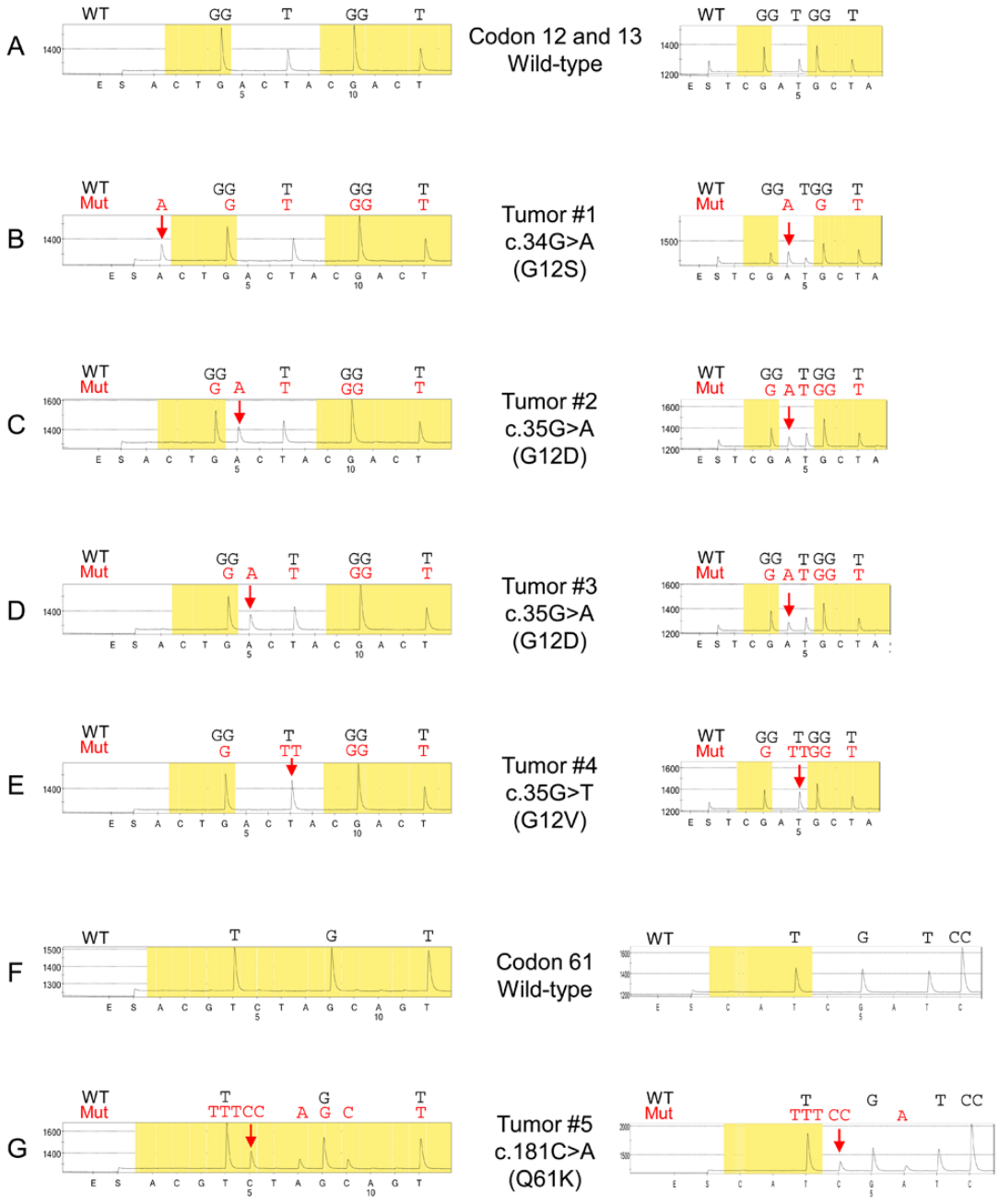

Figure 1.

NRAS Pyrosequencing assay. (A) Wild-type codon 12 and 13. (B) The c.34G>A (p.G12S) mutation (arrow). (C) (D) The c.35G>A (p.G12D) mutation (arrow). (E) The c.35G>T (p.G12V) mutation (arrow). (F) Wild-type codon 61. (G) The c.181C>A (p.Q61K) mutation (arrow) causes a shift in reading frame and results in new peaks at A and C (arrowhead), which serves as quality assurance. Mut, mutant; WT, wild-type.

Table 1

Characteristics of colorectal cancer patients according to *NRAS* mutation status.

Clinical, pathologic and molecular feature	Total No.	Wild-type *NRAS*	Mutant *NRAS*
Total No.	225	220	5
Sex			
Male	108 (48%)	107 (49%)	1 (20%)
Female	117 (51%)	113 (51%)	4 (80%)
Mean age ± SD	67.4 ± 8.7	67.4 ± 8.8	69.9 ± 3.6
Body mass index (BMI, kg/m^2)			
<30	172 (82%)	168 (82%)	4 (80%)
≥30	38 (18%)	37 (18%)	1 (20%)
Family history of colorectal cancer			
Absent	170 (76%)	166 (76%)	4 (80%)
Present	55 (24%)	54 (24%)	1 (20%)
Tumor location			
Proximal colon (cecum to transverse)	98 (46%)	98 (47%)	0
Distal colon (splenic flexure to sigmoid)	71 (33%)	67 (32%)	4 (80%)
Rectum	46 (21%)	45 (21%)	1 (20%)
AJCC stage			
I	59 (26%)	59 (26%)	0
II	53 (24%)	50 (24%)	3 (60%)
III	56 (25%)	55 (25%)	1 (20%)
IV	33 (15%)	32 (15%)	1 (20%)
Unknown	24 (10%)	24 (10%)	0
Tumor grade			
Low	187 (89%)	182 (88%)	5 (100%)
High	24 (11%)	24 (12%)	0
Mucinous component			
0%	105 (57%)	102 (57%)	3 (75%)
≥1%	47 (43%)	78 (43%)	1 (25%)
Signet ring cells			
0%	152 (92%)	148 (92%)	5 (100%)
≥1%	13 (7.9%)	13 (8.0%)	0
CIMP			
CIMP-low/0	174 (85%)	169 (85%)	5 (100%)
CIMP-high	30 (15%)	30 (15%)	0
MSI			
MSI-low/MSS	184 (83%)	179 (83%)	5 (100%)
MSI-high	37 (17%)	37 (17%)	0
18q loss of heterozygosity (LOH)			
(−)	65 (42%)	64 (43%)	1 (20%)
(+)	88 (58%)	84 (57%)	4 (80%)
Mean LINE-1 methylation (%) ± SD	61.5 ± 9.3	61.6 ± 9.3	58.6 ± 5.0

Clinical, pathologic and molecular feature	Total No.	Wild-type *NRAS*	Mutant *NRAS*
KRAS mutation			
(−)	132 (59%)	127 (58%)	5 (100%)
(+)	92 (41%)	92 (42%)	0
BRAF mutation			
(−)	189 (86%)	184 (86%)	5 (100%)
(+)	30 (14%)	30 (14%)	0
PIK3CA mutation			
(−)	184 (89%)	179 (89%)	5 (100%)
(+)	23 (11%)	23 (11%)	0

AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable; SD, standard deviation

Table 2

Characteristics of colorectal cancers with *NRAS* mutations

												PMR (degree of promotor methylation)[d]																				
Tumor ID	*NRAS*	Sex	Age	BMI(Kg/m^2)	Family history[a]	Tumor location[b]	AJCCstage	Tumor grade	Mucinous component	Signet ring cells	CIMP[c]	*CACNA1G*[e]	*CDKN2A(p16)*[e]	*CRABP1*[e]	*IGF2*[e]	*MLH1*[e]	*NEUROG1*[e]	*RUNX3*[e]	*SOCS1*[e]	*CHFR*	*HIC1*	*IGFBP3*	*MGMT*	MINT1	MINT31	p14	*WRN*	MSI	LINE1 methylation (%)	18q LOH	*KRAS*	*BRAF*	*PIK3CA*
1	G12S	F	71	<30	Absent	D	II	L	0%	0%	0	0	0	0	0	0	0	0	0	39	0	38	57	2	0	1.0	0	MSS	61.0	+	-	-	-
2	G12D	F	69	≥30	Present	R	II	L	0%	0%	L	0	171	73	0	0	0	0	0	0	0	22	0	2	0	0	7.9	MSS	59.3	-	-	-	-
3	G12D	F	68	<30	Absent	D	III	L	1–49%	0%	L	0	16	1.9	0	0	0	0	0	0	4.9	0	28	0.8	0.6	0	0	MSS	54.7	+	-	-	-
4	G12V	M	75	<30	Absent	D	II	L	0%	0%	0	0	0	0	0	0	0	0	0	0	1.0	0	0	3	0	0	0	MSS	65.1	+	-	-	-
5	Q61K	F	65	<30	Absent	D	IV	L	missing	0%	0	0	0	0	0	0	0	0	0	3.2	0	0	0	4	0	0	0	MSS	52.8	+	-	-	-

AJCC, American Joint Committee on Cancer; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; MSS, microsatellite stable; LOH loss of heterozygosity

[a] Family history is positive when any first-degree relative has been affected with colorectal cancer.

[b] D, distal colon (splenic flexure to sigmoid): R, rectum.

[c] CIMP status; L, CIMP-low; 0, CIMP-0.

[d] PMR (percentage of methylated reference; methylation index).

[e] 8 markers in the CpG island methylator phenotype (CIMP)-specific marker panel.