Clinicopathological characteristics and prognostic impact of colorectal cancers with *NRAS* mutations

TOSHIRO OGURA[1,4], MIHO KAKUTA[2], TOSHIMASA YATSUOKA[1], YOJI NISHIMURA[1], HIROHIKO SAKAMOTO[1], KENSEI YAMAGUCHI[3], MINORU TANABE[4], YOICHI TANAKA[1] and KIWAMU AKAGI[2]

Divisions of [1]Gastroenterological Surgery, [2]Molecular Diagnosis and Cancer Prevention, and [3]Gastroenterology, Saitama Cancer Center, Saitama 362-0806; [4]Department of Hepato-Biliary-Pancreatic Surgery, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

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Abstract. At present, molecular markers of colorectal cancer (CRC), including *KRAS*, *NRAS* and *BRAF* mutations, and the microsatellite status are evaluated for the development of personalized treatments. However, clinicopathological and molecular characteristics and the prognostic role of *NRAS* mutations remain unclear. In the present study, a total of 1,304 consecutive stage 0-IV CRC tumor samples were analyzed for *KRAS* (exon 2, 3 and 4), *NRAS* (exon 2 and 3) and *BRAF* (exon 15) mutations. Multivariate analysis was performed to assess the prognostic impact of *NRAS* mutations. *KRAS*, *NRAS* and *BRAF* mutations were identified in 553 (42.4%), 35 (2.7%), and 59 (4.5%) of 1,304 CRC cases, respectively. Tumors with *NRAS* mutations were more frequently located in the distal colorectum compared with those with *KRAS* or *BRAF* mutations. Multivariate analysis indicated that *KRAS* and *BRAF* mutations were found to be associated with poor prognosis [hazard ratio (HR)=1.44, 95% confidence interval (CI), 1.18-1.76 and HR=2.09; 95% CI, 1.33-3.28, respectively], whereas *NRAS* mutations were associated with a trend toward favorable prognosis (HR=0.53; 95% CI, 0.27-1.03). Characteristics and prognosis of CRC with *NRAS* mutations are different from those with *KRAS* or *BRAF* mutations.

Introduction

The epidermal growth factor receptor (EGFR) is one of the most important molecular targets for advanced colorectal cancer. Activation of this transmembrane receptor tyrosine kinase stimulates signaling pathways supporting cell proliferation, adhesion, migration, evasion of apoptosis, angiogenesis and survival (1-3). Oncogenic signaling pathways downstream of EGFR, including RAS/Raf/MAPK and PI3K/PTEN/Akt pathways, are important mechanisms of tumor progression.

Activating mutations in the *RAS* oncogene family are present in ~30% of all human cancers. *RAS* genes encode highly homologous proteins: *KRAS*, *NRAS* and *HRAS* (4). Mutations in the *KRAS* gene are frequency reported in various human neoplasms, including pancreatic cancer, biliary tract cancer and lung adenocarcinoma (4-6). Cancer types with a high rate of *NRAS* mutations include myeloid leukemia and cutaneous melanoma, whereas *HRAS* mutations are typical of bladder and cervical cancers (4,7,8). In colorectal cancer (CRC), rates of *KRAS*, *NRAS* and *HRAS* mutation are 30-42%, 2.2-5% and 0-0.8%, respectively (9-14). Relatively low rates of *NRAS* and *HRAS* mutations in CRC remain unexplained.

Anti-EGFR antibody therapy exhibits antitumor effects by inhibiting multiple EGFR signaling pathways, including RAS/RAF/MAPK and PI3K/PTEN/AKT pathways. Clinical trials have demonstrated that anti-EGFR monoclonal antibodies (i.e., cetuximab or panitumumab) are largely ineffective for metastatic CRC patients when tumors harbor mutations in the codon 12 or 13 of *KRAS* exon 2 (15-20). These mutations cause constitutive activation of the RAS/RAF/MAPK pathway, regardless of EGFR inhibition. Therefore, *KRAS* exon 2 mutations are recognized as predictive markers of anti-EGFR therapy resistance for metastatic CRC patients. Accordingly, these clinical trials routinely exclude CRC patients harboring *KRAS* exon 2 mutations.

Recent studies suggest that other activating mutations in *KRAS* or *NRAS*, in addition to *KRAS* exon 2, confer resistance to anti-EGFR therapy (9,11,13,21). Since *KRAS* and *NRAS* mutations tend to be mutually exclusive, they may be present in approximately half of metastatic CRC patients (9-14). Therefore, personalized cancer therapy should be tailored to the *KRAS* and *NRAS* mutation profile of each patient to improve treatment outcomes.

Previous studies have evaluated the clinicopathological features and prognostic influence of *KRAS* or *BRAF* mutations in colorectal cancer. The prognostic value of *KRAS* mutations in CRC remains controversial (22-24). In contrast, *BRAF* mutations are associated with proximal colon tumor location,

Correspondence to: Dr Kiwamu Akagi, Division of Molecular Diagnosis and Cancer Prevention, Saitama Cancer Center, 818 Komuro, Ina-machi, Kitaadati-gun, Saitama 362-0806, Japan
E-mail: akagi@cancer-c.pref.saitama.jp

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poor differentiation, mucinous component and microsatellite instability. Patients with *BRAF*-mutated tumors revealed lower survival rates compared with wild-type tumors, particularly those with *BRAF*-mutated and microsatellite-stable CRC (22,25-27). On the other hand, clinicopathological characteristics, molecular features, and the prognostic value of the *NRAS* mutation remain largely unknown (10,12). To date, analyses of *NRAS* mutations in colorectal cancer were performed as part of a subset analysis of clinical studies for treatment of metastatic CRC with anti-EGFR antibodies, and few studies have described *NRAS* mutations in the early stage of CRC. Irahara *et al* (12) associated *NRAS* mutations with left-sided cancers in females, but the data did not reach statistical significance since *NRAS* mutations were only detected in 5 (2.2%) of the 225 cases. Therefore, the prognostic role and clinical characteristics of *NRAS* mutations should be clarified using large tissue samples to guide future clinical studies on the predictive impact of the *NRAS* gene.

The present study used 1,304 consecutive samples of stage 0-IV CRC to investigate the impact of mutations in *NRAS* exon 2 and 3, in addition to *KRAS* and *BRAF*. We evaluated the relationship between *NRAS* mutations and other clinicopathological or molecular features, including *KRAS* and *BRAF* mutations, microsatellite instability (MSI) status and patient survival.

Materials and methods

Patients and tissue samples. The present study was conducted on 1,304 consecutive primary CRC patients at the Saitama Cancer Center from July 1999 to July 2008. Information on clinical data, including age at diagnosis, gender, tumor size, histological differentiation, tumor location, International Union against Cancer (UICC) stage and prognosis were collected from medical records. Tissue samples were surgically excised after obtaining informed consent from each patient. All tumor tissues were paired with normal colorectal tissues and immediately stored at -80˚C. The present study was approved by the Ethics Committee of the Saitama Cancer Center.

Mutation analysis of KRAS, BRAF and NRAS. Genomic DNA from each sample was extracted by standard SDS-proteinase K procedure, followed by ethanol precipitation. All tumor samples were tested for *KRAS* exon 2, 3 and 4; *BRAF* exon 15 (codon 600); *NRAS* exon 2 and 3; and MSI status.

KRAS mutations in exon 2 and 3 were detected by denaturing gradient gel electrophoresis (DGGE), and *BRAF* mutations in exon 15 by PCR-restriction fragment length polymorphism (RFLP), as previously described (28,29).

High resolution melting (HRM) analysis was used to identify mutations in *NRAS* exon 2 and 3 and in *KRAS* exon 4 using a Rotor-Gene Q (Qiagen, Hilden, Germany). Primer sets for *NRAS* were as follows: exon 2, 5'-GGTTTCCAACAGGT TCTTGC-3' (forward) and 5'-CACTGGGCCTCACCTCTA TG-3' (reverse); exon 3, 5'-CACACCCCCAGGATTCTTAC-3' (forward) and 5'-TGGCAAATACACAGAGGAAGC-3' (reverse). The primer set for *KRAS* exon 4 was as follows: 5'-GCCTTCTAGAACAGTAGACAC-3' (forward) and 5'-GA CATAACAGTTATGATTTTGCAGA-3' (reverse). The reaction mixture contained 7 μl of 2X LightCycler 480 High Resolution Melting Master Reaction Mix (Roche Diagnostics, Mannheim, Germany) with 0.21 μM of each forward and reverse primer, 3.2 mM $MgCl_2$, 20 ng purified genomic DNA, and water to a total volume of 14 μl. PCR cycling and melting conditions were as follows: initial denaturation at 95˚C for 5 min, followed by 40 cycles of 10 sec at 95˚C, 20 sec at 57˚C, and 10 sec at 72˚C. One heteroduplex cycle was performed at 95˚C for 1 min and 40°C for 1 min, followed by melting from 72˚C to 95˚C with 10 acquisitions per ˚C. HRM data were analyzed using the Rotor-Gene Q software ver.2.0.2.4.

The DNA sequence of *NRAS* exon 2 and 3 mutations was determined by HRM using primers particularly designed for HRM. Amplified products were labeled with GenomeLab™ DTCS Quick Start kit (Beckman Coulter Inc., Fullerton, CA, USA) according to the manufacturer's instructions and sequenced using the GenomeLab™ GeXP Genetic Analysis System (Beckman Coulter). Sequencing was performed in both directions, and sequence analysis was performed using the GenomeLab Genetic Analysis System v10.2 (Beckman Coulter).

Analysis of microsatellite status. The MSI status was determined using Bethesda markers: BAT25, BAT26, D5S346, D2S123 and D17S250. PCR and subsequent analyses were performed as previously described (30). CRC samples showing instability in two or more markers were defined as microsatellite instability-high (MSI-H), and the ones with none or one marker as microsatellite stable (MSS).

Statistical analysis. Possible associations between each mutation and clinicopathological parameters of CRC were assessed by the Chi-square or Fisher's exact test for categorical variables and Mann-Whitney U or Kruskal-Wallis test for continuous variables. Overall survival (OS) time was calculated from the date of surgery to the date of death by any cause or censored at the last follow-up visit. Cox proportional hazards analysis was used to estimate clinicopathological- and biomarker-specific survival hazard ratios (HRs) and 95% confidence intervals (CIs). A multivariable model stratification by UICC stage was performed. All P-values were calculated from two-sided test, and P-values <0.05 were considered statistically significant. All statistical analyses were performed with the SPSS Statistics v.20 (SPSS, Inc., Chicago, IL, USA).

Results

Patient characteristics. All 1,304 patients enrolled in the present study were diagnosed with either CRC stage 0 (n=48), stage I (n=248), stage II (n=407), stage III (n=384) or stage IV (n=217) (Table I). Three hundred and seventy-nine cancers were from the proximal colon (cecum to transverse colon), 544 from the distal colon (descending colon to sigmoid colon) and 381 from the rectum. The median follow-up period was 5.6 years (interquartile range, 4.1-7.8 years), during which there were 435 deaths (33%).

Frequency of KRAS, NRAS and BRAF mutations. All 1,304 CRC cases were examined for mutations in *KRAS* (exon 2, 3 and 4), *NRAS* (exon 2 and 3) and *BRAF* (exon 15), as well

Table I. Clinicopathological and molecular features of all of the CRC samples.

Features	Patients (n=1,304) n (%)
Gender	
Male	780 (59.8)
Female	524 (40.2)
Age ± SD (years)	63.8±10.4
Location	
Proximal	379 (29.1)
Distal	544 (41.7)
Rectum	381 (29.2)
Tumor size	
Mean ± SD (mm)	45.4±24.2
Histological features	
Well-differentiated	144 (11.0)
Moderately differentiated	1,078 (82.7)
Poorly differentiated	34 (2.6)
Others	48 (3.7)
Stage	
0	48 (3.7)
1	248 (19.0)
2	407 (31.3)
3	384 (29.4)
4	217 (16.6)
KRAS status	
Mutated-type	553 (42.4)
Wild-type	751 (57.6)
NRAS status	
Mutated-type	35 (2.7)
Wild-type	1,269 (97.3)
BRAF status	
Mutated-type	59 (4.5)
Wild-type	1,245 (95.5)
MSI status	
MSI-H	72 (5.5)
MSS	1,232 (94.5)

SD, standard deviation; MSI, microsatellite instability; MSI-H, microsatellite instability-high; MSS, microsatellite stable.

Table II. Frequency and type of *NRAS* mutation.

Nucleotide mutation	Amino acid change	Mutation frequency (n)	Coincidental *KRAS* mutation
Codon12			
c.35G>A	p.G12D	20.0% (7)	
c.34G>T	p.G12C	5.7% (2)	
c.35G>T	p.G12V	5.7% (2)	
Codon13			
c.37G>C	p.G13R	11.0% (4)	
c.38G>A	p.G13D	8.6% (3)	p.G57T
c.38G>T	p.G13V	2.9% (1)	
Codon61			
c.181C>A	p.Q61K	26% (9)	
c.182A>T	p.Q61L	5.7% (2)	
c.183A>C	p.Q61H	2.9% (1)	
c.183A>T	p.Q61H	2.9% (1)	
c.182A>G	p.Q61R	2.9% (1)	
Codon9			
c.26T>C	p.V9A	2.9% (1)	p.G12D
Codon68			
c.204A>T	p.R68S	2.9% (1)	p.G12V

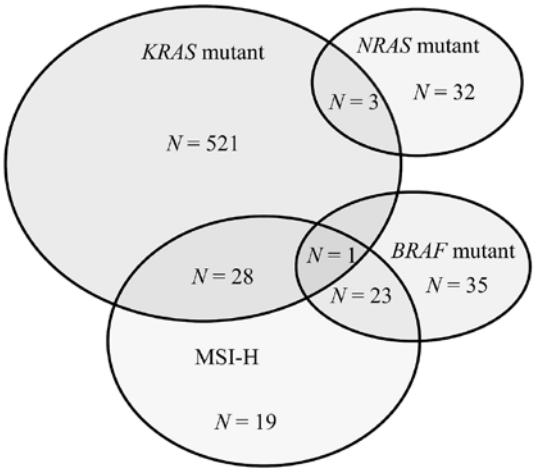

Figure 1. Associations between KRAS, NRAS and BRAF mutations and MSI. MSI-H, microsatellite instability-high.

as MSI status and clinicopathological factors (Table I). *KRAS* mutations were detected in 42.4% (n=553), *NRAS* in 2.7% (n=35) and *BRAF* in 4.5% (n=59) of patients. MSI-H was detected in 5.5% (n=72) of cases. Table II presents changes in the nucleotides and corresponding amino acids detected in *NRAS*, with p.G12D (codon 12), p.G13R (codon 13) and p.Q61K (codon 61) as the most frequently noted mutations.

Mapping associations between molecular markers revealed that 3 patients had both *KRAS* and *NRAS* mutations, whereas 1 patient had both *KRAS* and *BRAF* mutations (Fig. 1). *KRAS*/*NRAS* mutation combinations were as follows: p.G12D/p.V9A, pG12V/p.R68S and p.G57T/p.G13D. In contrast, *NRAS* and *BRAF* mutations were mutually exclusive. Regarding the MRI status, 28 patients with *KRAS* mutations also had MSI-H tumors compared with 23 patients with *BRAF* mutations. None of the patients with *NRAS* mutations had MSI-H tumors.

BRAF mutations were significantly more frequent in MSI-H than in MSS tumors (P<0.001), whereas no significant association was observed between MSI status and *KRAS* or *NRAS* mutations.

Table III. Mutation rates of *KRAS* and *NRAS* genes for each exon.

Gene	Patients with mutations, n (%)
KRAS	
Exon 2	495 (38.0)
Exon 3	26 (2.0)
Exon 4	32 (2.5)
NRAS	
Exon 2	20 (1.5)
Exon 3	15 (1.2)

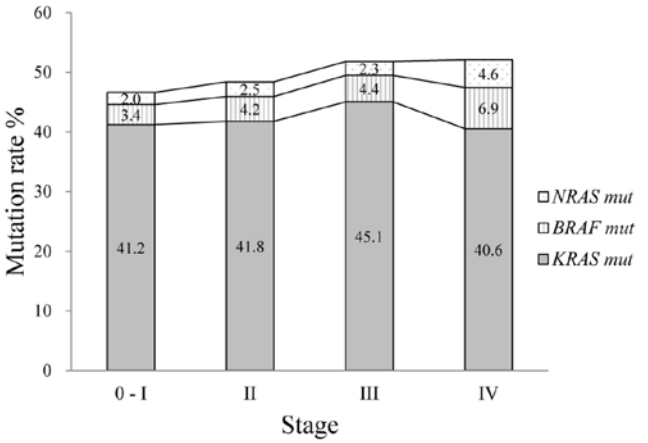

Figure 2. Mutation rates of the *KRAS*, *NRAS* and *BRAF* genes according to UICC staging.

Frequency of KRAS and NRAS mutations in each exon. In the *KRAS* gene, most mutations were located in exon 2, with 495 of 1,304 cases (38.0%), whereas exon 3 or 4 mutations were detected in 26 (2.0%) and 32 (2.5%) cases, respectively (Table III). In the *NRAS* gene, 20 (1.5%) mutations were identified in exon 2 and 15 (1.2%) mutations in exon 3.

Impact of KRAS, NRAS and BRAF mutation status on clinicopathological and molecular characteristics of the colorectal cancer patients. CRC patients were categorized into three groups on the basis of *KRAS*, *NRAS* and *BRAF* mutations, and they were compared in terms of gender, age, colorectal tumor location, tumor maximum size, histological differentiation, mucinous component, depth of tumor invasion, UICC stage, extramural venous invasion and MSI status (Table IV). *BRAF*-mutated tumors were more frequently associated with mucinous component tumors (*KRAS*, P=0.003; *NRAS*, P=0.002), poorly differentiated tumors (*KRAS*, P<0.001; *NRAS*, P=0.013), female gender (*KRAS*, P=0.022) and MSI-H (*KRAS* and *NRAS*, P<0.001). *NRAS*-mutated tumors were more frequently located in the distal colorectum compared with *KRAS*- or *BRAF*-mutated tumors (P=0.015 and P<0.001, respectively). Compared with triple wild-type tumors (*KRAS*, *NRAS* and *BRAF* wild-type), *KRAS*- and *BRAF*-mutated tumors were more commonly noted in the proximal colon (P<0.001 and P<0.001, respectively), whereas no significant difference was observed between *NRAS*-mutated tumors and triple wild-type tumors (P=0.201).

Mutation rates of *KRAS*, *NRAS* and *BRAF* for each UICC stage are presented in Fig. 2. *KRAS* mutations were detected at similar frequencies in stage 0-I to IV. *NRAS* mutations tended to occur more frequently in stage IV cancers than in stage 0-III cancers compared with *KRAS* mutations (P=0.061).

Impact of KRAS, NRAS and BRAF mutations on CRC patient survival. Univariate analysis was conducted in regards to age, gender, tumor location, stage, histological subtype, mucinous component, extramural venous invasion, MSI status, *KRAS*, *NRAS* and *BRAF* mutations (Table V). Patients with *KRAS* and *BRAF* mutations had significantly worse survival compared with wild-type cases [HR=1.25; 95% confidence interval (CI) 1.03-1.52; P=0.027 and HR=1.73; 95% CI, 1.15-2.60; P=0.009, respectively]. Four other variables were significantly associated with poor prognosis, namely age ≥65 years (HR=1.39; 95% CI, 1.15-1.69; P=0.001), UICC stage (stage II: HR=2.33; stage III: HR=3.58; stage IV: HR=14.14; P<0.001 respectively), histological subtype (HR=1.82; 95% CI, 1.31-2.52; P<0.001), and extramural venous invasion (HR=3.28; 95% CI, 2.50-4.30; P<0.001). The only predictor of good prognosis was female gender (HR=0.73; 95% CI, 0.60-0.89; P=0.002). In multivariable analysis, *KRAS* and *BRAF* mutations were associated with significantly higher mortality rates when stratified according to UICC staging (HR=1.44; 95% CI, 1.18-1.79; P<0.001 and HR=2.09; 95% CI, 1.36-3.28; P=0.001, respectively). Notably, *NRAS*-mutated tumors demonstrated a trend towards favorable prognosis (HR=0.53; 95% CI, 0.27-1.03; P=0.059).

Discussion

The present study investigated clinicopathological and prognostic features of *KRAS*, *NRAS* and *BRAF* mutations in tumors from 1,304 consecutive CRC patients. An important finding was that patients undergoing CRC tumor resection at all stages were targeted by all these mutations. In addition, to the best of our knowledge, this is the first large study to present statistically significant comparisons between these three categories of *RAS/RAF* mutations in CRC patients.

NRAS mutations were observed in 35 (2.7%) of the 1,304 patients, of which 20 (1.5%) patients revealed a mutation in exon 2 and others (1.2%) in exon 3. These data are consistent with previous studies that reported *NRAS* mutations in 2.2-5.0% of CRC tumors, with approximately equal frequency in exon 2 and 3 (9,10,12,29). Moreover, we showed that *NRAS* mutations are detected in early stages of CRC and tend to occur more frequently in stage IV cancers than in stage 0-III cancers. Therefore, *NRAS* mutations appear to be acquired at early and advanced stages of CRC (31). Nonetheless, the tendency of higher *NRAS* mutation rates in stage IV CRC should be ascertained in a larger scale study. The frequency of *KRAS* mutations was also compatible with previous studies, but the frequency of *BRAF* mutations (4.5%) declined below 7.4-10.1% of the values previously reported (10,13,14). On the other hand, Yokota *et al* (32) reported an incidence of 4.7% for *BRAF* mutations (15/319 patients) in Japanese CRC patients. Such agreement with our Japanese study suggests that racial

Table IV. Clinicopathological characteristics according to the *KRAS, NRAS* and *BRAF* mutation status.

Characteristics	Triple wild-type n (%)	*KRAS* mt n (%)	*NRAS* mt n (%)	*BRAF* mt n (%)	P-value		
					KRAS vs. *NRAS*	*KRAS* vs. *BRAF*	*NRAS* vs. *BRAF*
Patient	661				0.222	0.022	0.633
Male	431 (65.2)	309 (55.9)	16 (45.7)	24 (40.7)			
Female	230 (34.8)	244 (44.1)	19 (54.3)	35 (59.3)			
Age ± SD (years)	63.3±10.3	64.2±10.4	65.5±9.5	64.2±11.5	0.716	0.64	0.997
Location					0.015	<0.001	<0.001
Proximal	142 (21.4)	189 (34.2)	4 (11.4)	46 (77.9)			
Distal	311 (47.1)	209 (37.8)	16 (45.7)	9 (15.3)			
Rectum	208 (31.5)	155 (28.0)	15 (42.9)	4 (6.8)			
Tumor size					0.456	0.417	0.928
Mean ± SD (mm)	44.1±24.3	46.1±22.7	48.0±23.0	52.6±33.9			
Histologic feature					0.916	<0.001	0.013
Well-differentiated	58 (8.8)	78 (14.1)	6 (17.1)	3 (5.1)			
Moderately differentiated	573 (86.6)	437 (79.0)	29 (82.9)	41 (69.4)			
Poorly differentiated	16 (2.4)	11 (2.0)	0 (0.0)	8 (13.6)			
Mucinous	11 (1.7)	25 (4.5)	0 (0.0)	7 (11.9)			
Others	3 (0.5)	2 (0.4)	0 (0.0)	0 (0.0)			
Mucinous component					0.068	0.003	0.002
+	34 (5.1)	98 (17.7)	2 (5.7)	20 (33.9)			
-	627 (94.9)	455 (82.3)	33 (94.3)	39 (66.1)			
Depth of tumor invasion					0.63	0.483	0.838
Tis	15 (2.3)	33 (6.0)	0 (0.0)	0 (0.0)			
T1	62 (9.4)	45 (8.1)	4 (11.4)	4 (6.8)			
T2	110 (16.6)	68 (12.3)	3 (8.6)	7 (11.9)			
T3	404 (61.1)	352 (63.7)	24 (68.6)	39 (66.0)			
T4	70 (10.6)	55 (9.9)	4 (11.4)	9 (15.3)			
UICC stage					0.353	0.151	0.75
0	15 (2.3)	33 (6.0)	0 (0.0)	0 (0.0)			
1	143 (21.6)	89 (16.1)	6 (17.1)	10 (16.9)			
2	210 (31.8)	170 (30.7)	10 (28.6)	17 (28.8)			
3	188 (28.4)	173 (31.3)	9 (25.7)	17 (28.8)			
4	105 (15.9)	88 (15.9)	10 (28.6)	15 (25.5)			
Extramural venous invasion					0.231	0.105	0.689
+	471 (71.3)	365 (66.0)	24 (68.6)	46 (78.0)			
-	190 (28.7)	188 (34.0)	11 (31.4)	13 (22.0)			
MSI-H					0.171	<0.001	<0.001
+	20 (3.0)	29 (5.2)	0 (0.0)	24 (40.7)			
-	641 (97.0)	524 (94.8)	35 (100)	35 (59.3)			

MSI-H, microsatellite instability-high.

or environmental factors may affect the frequency of *BRAF* mutations. The strong overlap between *BRAF* mutation and MSI-H status that we detected suggests that the low frequency in the *BRAF* mutation is affected by the low MSI-H frequency reported among Asians compared with Westerners (33).

In the present study, 3 cases of 1,304 had mutations in both *KRAS* and *NRAS*, which is inconsistent with previous reports of mutual exclusivity. Notably, all three tumors presented rare mutations (*NRAS* p.V9A, *NRAS* p.R68S and *KRAS* p.G57T), whereas these tumors had common mutations (*KRAS* p.G12D, *KRAS* p.G12V and *NRAS* p.G13D). The oncogenic activity of these minor mutations is unknown, except for *KRAS* p.G57T (28). Therefore, *KRAS* and *NRAS* mutation detection only in major mutation lesion may have missed these rare mutations.

Table V. Univariate and multivariate analyses of the covariates associated with overall survival.

Covariates	Univariate HR (95% CI)	P-value	Multivariate HR(95% CI)	P-value
Age ≥65 years	1.39 (1.15-1.69)	0.001	1.53 (1.27-1.86)	<0.001
Female	0.73 (0.60-0.89)	0.002	0.69 (0.56-0.84)	<0.001
Tumor location (proximal vs. distal colorectum)	0.88 (0.72-1.08)	0.22	1.01 (0.81-1.25)	0.96
KRAS-mutant	1.25 (1.03-1.52)	0.027	1.44 (1.18-1.76)	<0.001
NRAS-mutant	0.83 (0.43-1.61)	0.57	0.53 (0.27-1.03)	0.059
BRAF-mutant	1.73 (1.15-2.60)	0.009	2.09 (1.33-3.28)	0.001
MSS (vs. MSI-high)	1.59 (0.98-2.58)	0.062	1.56 (0.92-2.64)	0.10
UICC stage				
II	2.33 (1.58-3.44)	<0.001	-[a]	
III	3.58 (2.46-5.22)	<0.001	-[a]	
IV (vs. Stage 0 and I)	14.14 (9.68-20.7)	<0.001	-[a]	
Histological subtype (vs. well and mod)	1.82 (1.31-2.52)	<0.001	1.59 (1.09-2.32)	0.016
Mucinous component	1.23 (0.93-1.62)	0.14	0.81 (0.59-1.11)	0.2
Extramural venous invasion	3.28 (2.50-4.30)	<0.001	1.78 (1.31-2.37)	<0.001

[a]UICC Stage was a stratifying variable in the multivariate analysis. HR, hazard ratio.

Colorectal tumors with *NRAS* mutations were found more frequently in the distal colon and rectum compared with tumors with *KRAS* or *BRAF* mutations, while the distribution of *BRAF*-mutated tumors was consistent with previous studies reporting that *BRAF*-mutated tumors are primarily located in the proximal colon (22,32,34). It was proposed that cellular transformation and mutations occur more frequently in the proximal colon due to close contact of epithelial cells with stimulating bowel content (34). However, this theory does not explain the higher frequency of *NRAS*-mutated tumors in the distal colon and rectum compared with those with *KRAS* or *BRAF* mutations. Elucidating factors responsible for distinct locations of *KRAS*- and *NRAS*-mutated tumors may be crucial to our understanding of *NRAS* mutations in CRC patients.

The prognosis of advanced CRC patients carrying *NRAS* mutations has been reported in the form of subset analysis of clinical studies using anti-EGFR drugs such as cetuximab and panitumumab (9,13,14). Although there is currently no consistent view regarding the efficacy of anti-EGFR drugs for CRC patients with *NRAS* mutations, several reports consider this form of therapy inappropriate for such CRC patients. However, the fundamental malignant potential of the *NRAS* mutation should be considered to determine the efficacy of anti-EGFR drugs. To the best of our knowledge, this is the first study to compare patient survival in *NRAS*-mutated and triple wild-type CRCs. Numerous studies report an association between *BRAF* mutations and poor clinical outcome (15,22,23,27,32,35). Although the prognostic value of the *KRAS* mutation is still controversial, several studies suggest a poor prognosis (24,35,36). Since *NRAS* is a *RAS* family member, we expected *NRAS*-mutated CRC patients to have a poor prognosis compared with those without *RAS* mutations. However, multivariate analysis showed a tendency toward a better prognosis for *NRAS*-mutated CRC patients. Therefore, the present study suggests that CRC cases with *NRAS* mutations exhibit different characteristics than CRC cases with *KRAS* mutations.

Recent studies report that common *KRAS* exon 2 mutations targeting the RAS/RAF/MAPK pathway are currently used to determine patient eligibility for anti-EGFR monoclonal antibody therapy (19,37,38). Minor mutations (*KRAS* exon 3 and 4, *NRAS*, *BRAF*) are expected to become the next predictive biomarkers. In fact, a recent clinical trial on anti-EGFR monoclonal antibodies excluded patients with the KRAS exon 2 mutation and those with these less frequent mutations (39). In the present study, the incidence of *KRAS* exon 2 mutations was 38%, whereas the combined mutation rate for *KRAS* exon 3 and 4, *NRAS* and *BRAF* was 49.3%. For stage IV CRC patients subjected to anti-EGFR monoclonal antibody therapy, the total mutation rate increased from 37.3 to 51.6%. If the validity of excluding CRC patients with these minor *RAS* mutations from anti-EGFR monoclonal antibody therapy is verified by future trials, more than 50% of CRC patients would greatly benefit from personalized medicine for enhanced efficacy and a better prognosis.

In conclusion, the present study demonstrated that *KRAS*- and *NRAS*-mutated CRC tumors exhibit distinct characteristics and distributions along the colorectum. Future molecular biology studies should address the significance of these differences between *NRAS*- and *KRAS*-mutated CRC and confirm possible positive prognoses associated with *NRAS* mutations.

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