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ORIGINAL ARTICLE

G12V and G12C mutations in the gene *KRAS* are associated with a poorer prognosis in primary colorectal cancer

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Abstract

Purpose The increased incidence of colorectal cancer (CRC) has necessitated the development of novel prognostic and predictive factors from which new diagnostic tests could evolve. Evidence suggests the *KRAS* gene represents such a factor; its mutations are considered to be early indicators of CRC progression. This study assessed the prognostic impact of specific known *KRAS* codon 12/13 mutations on survival in patients with CRC.

Methods Formalin-fixed paraffin-embedded tissue blocks or sections from primary were obtained from patients registered between 2014 and 2016 for genomic DNA extraction. KRAS gene was analyzed by direct sequencing or Luminex assay. The primary endpoint was the frequency of KRAS gene mutations and the secondary endpoints were differences in KRAS mutation rates by various stratification factors. Univariate and multivariate analyses were performed to investigate relationships between KRAS mutation rates and patient background factors.

Results Sequencing of 200 CRC primary tumor samples demonstrated 74 (37.5%) with *KRAS* mutations in codons 12 (77%; 57/74) and 13 (23%; 17/74), all of which were TNM stages I–III. Tumors with *KRAS* mutations were more frequently located in the right side of the colon. Multivariate analysis indicated that G12V or G12C mutations were associated with poor prognosis [hazard ratio (HR) = 3.77, 95% confidence interval (CI), 1.54–8.39 and HR = 6.57; 95% CI, 1.90–17.7, respectively] in terms of recurrence-free survival.

Conclusion *KRAS* codon 12G-to-V or G-to-C mutations are independent prognostic factors in patients with stage I–III CRC.

Keywords Colorectal · Cancer · *KRAS* · Prognosis

Introduction

The mitogen-activated protein kinase signaling pathway incorporates a variety of transduction cascades; of these, the Ras-Raf-Mek-extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is often dysregulated in human colorectal cancer (CRC) [1]. This pathway regulates multiple critical cellular functions including proliferation, growth, and senescence [1].

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Activating somatic mutations in the *KRAS* and mutations that activate regulators and effectors of Ras proteins are common in tumor development and cancer. A recent clinical study indicated that in the Japanese population up to 37.6% of colorectal adenocarcinomas include *KRAS* mutations; these were mainly point mutations in codons 12 and 13 [2]. Other report has been reported that *KRAS* mutations occur in up to 50% of colorectal adenocarcinoma; approximately 90% of the activating mutations in the *KRAS* are scored at codon 12 and codon 13 in exon 1, while only 5% are located at codon 61 in exon 2 [3]. Mutations of codons 12, 13, or 61 of N-ras, H-ras, or K-ras activate the oncogenic properties of Ras proteins and it has been proposed that they do so by inhibiting GTPase activity [4]. *KRAS* mutations, remaining longer in the GTP-bound state, result in a more persistent growth-promoting signal. The mutant K-Ras protein has reduced GTPase activity that results in increased cell proliferation potentially related to early tumorigenesis. In vitro studies have suggested that specific KRAS point mutations in codon 12 may confer increased oncogenic

Table 1 TNM stage

TNM stage *n* (%)	KRAS WT *n* (%)	Codon 12 *n* (%)	Codon 13 *n* (%)
Stage I 47 (23.5)	34 (71.7)	11 (24)	2 (4.3)
Stages II–III 153 (76.5)	92 (60.1)	46 (30)	15 (9.8)
P value	0.130	0.256	0.232

potential through the inhibition of apoptosis, loss of contact inhibition, and increased contact-independent growth, but the clinical relevance of these findings has yet to be clarified [5–8].

However, *KRAS* mutations were determined to be predictors of the efficacy of anti-epithelial growth factor receptor (EGFR) antitumor therapy [9]. The analyses of *KRAS* mutations in CRC were performed as part of a subset analysis of clinical studies for treatment of metastatic CRC with anti-EGFR antibodies. However, there are minimal data regarding KRAS mutations in the early stage of CRC, and both their overall prognostic value and their relationship with clinicopathological characteristics remain unclear.

This study was conducted to determine whether codon 12 *KRAS* mutations are prognostic for CRC or its clinicopathologic characteristics. We sequenced *KRAS* in primary CRC tumor samples and identified those samples with codon 12 or 13 mutations, and then determined the relationships, if any, between specific somatic mutations and various clinicopathologic factors, including recurrence-free survival.

Material and methods

Patient selection

A total of 200 individuals comprising part of a cohort of consecutive patients with CRC treated via curative resection at the Teikyo University Hospital, Japan, from 2014 through 2016 were included. Only patients identified as having stage I–III CRC according to the 8th edition of the American Joint Committee on Cancer (AJCC) staging system [10] were included in the study.

The exclusion criteria were (1) patient received adjuvant chemotherapy, (2) history of familial adenomatous polyposis or Lynch syndrome, and (3) multiple primary malignancies.

Table 2 Frequency of KRAS mutations

Somatic mutation	*N* (%)
G12A	2 (2.7)
G12D	28 (37.8)
G12C	7 (9.4)
G12S	3 (4.1)
G12V	17 (23)
G13D	16 (21.6)
G13C	1 (1.4)

Standard demographic and clinicopathologic data were collected on each patient, including sex, age, tumor characteristics, date of last follow-up, date and type of recurrence, and date of death; other recorded characteristics included AJCC tumor (T) and necrosis (N) stages, tumor site (right vs. left), and nodal status. Tissue samples were surgically excised only 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 conducted in accord with the Declarations of Helsinki and was approved by the Ethics Committee of the Teikyo University.

Follow-up

Surgical resection was defined as radical when there was no evidence of distant metastasis and the tumor clearance was both macroscopically and histologically complete. The patients were followed up every 3 months for the first 3 years, every 6 months for the next 2 years, and once annually thereafter. Every follow-up included a physical examination and testing for serum carcinoembryonic antigen (CEA) and ca19–9 (carbohydrate antigen 19–9). At the 1-year postoperative follow-up, all patients also underwent full colonoscopies, and then once every 3–5 years if no polyps were identified. Chest and abdominal computed tomography scans were generally obtained every 6 months. Recurrence was defined as emergence of clinical, radiological, and/or pathological diagnosis of tumors locally or distantly from the original position.

KRAS mutation analysis

KRAS testing was performed centrally at Hoken Kagaku Laboratories (Kanagawa, Japan) using the resected or biopsied primary CRC samples collected for this study. All samples were histologically reviewed by pathologists to select appropriate areas for the *KRAS* mutation assay. The selected area on the unstained slide (10-μm-thick) was scraped manually and the sample deparaffinized. DNA was isolated using a QIAamp DNA FFPE Tissue Kit (Qiagen, Manchester, UK) and quantified on a Nano Drop c2000 (Thermo Fisher Scientific, Waltham, MA, USA). An assay kit (KRAS RGQ PCR kit; Qiagen) utilizing the Scorpions and Amplification Refractory Mutation system to detect wild-type (control) and specific mutant forms (GLY12ALA, GLY12ASP, GLY12ARG, GLY12CYS, GLY12SER, GLY12VAL, GLY13ASP) of *KRAS* codons 12 and 13 was used according

Table 3 Association of clinicopathological features with KRAS mutational status

Clinicopathological feature	$n = 126$ (%)	Codon 12 $n = 57$ (%)	Codon 13 $n = 17$ (%)	P value
Age (median, range)	66, 28–90	69, 34–93	70, 50–83	0.08
Gender				
Male	84 (66.7)	29 (50.9)	11 (64.7)	0.07
Female	42 (33.3)	28 (49.1)	6 (35.3)	
T stage				
T1 or T2	42 (66.7)	13 (22.8)	3 (17.6)	0.07
T3 or T4	84 (33.3)	44 (77.2)	14 (82.4)	
N stage				
N0	79 (62.7)	33 (57.9)	9 (52.9)	0.40
N1 or N2	47 (37.3)	24 (42.1)	8 (47.1)	
Histology				
Well or moderate	116 (7.25)	53 (93.0)	16 (94.1)	0.76
Others	10 (92.8)	4 (7.0)	1 (5.9)	
Tumor location				
Right side	34 (27)	26 (45.6)	6 (35.3)	0.02
Left side	92 (73)	31 (54.4)	11 (64.7)	

to the manufacturer's instructions. Real-time PCR was performed on a Rotor-Gene Q MDx 5plex HRM platform (Qiagen). The cycling conditions were 15 min at 95 °C, followed by 40 cycles at 95 °C for 30 s and 60 °C for 1 min. Fluorescence was measured at 60 °C. Data on each mutation were analyzed with the associated Therascreen KRAS program ver.1.0.4 (Qiagen).

Statistical analysis

Comparisons between groups were made with the chi-squared test or Fisher's exact test for proportions, and the Mann–Whitney *U* test for continuous variables. Recurrence-free survival (RFS) was calculated from the date of surgery to that recurrence using the Kaplan–Meier method. Cox regression analysis was used to identify factors significantly associated with RFS. Factors found to be statistically significant in the log-rank test were entered into the stepwise Cox regression model to produce the final model of independent prognostic factors. $P \leq 0.05$ was considered statistically significant. JMP 14 software (SAS Institute Inc., Cary, NC, USA) was used to perform all statistical analyses.

Results

Patient characteristics

All 200 patients enrolled in the present study were diagnosed with either CRC stage I 23.5% ($n = 47$) or stages II–III 765.5% ($n = 153$) (Table 1). The mutation rate of codon 12

Table 4 Univariate and multivariate analysis of relapse-free survival stratified by clinicopathological features

	Univariate	P value	Multivariate	P value
Age > 66	1.15 (0.62–2.18)	0.655		
Male	0.985 (0.51–1.84)	0.962		
Female				
T stage		0.0003		0.008
T1/T2	1 (Reference)		1 (Reference)	
T3/T4	4.81 (1.93–16.1)		5.00 (1.77–21.0)	
N stage		0.0052		0.052
N0	1 (Reference)		1 (Reference)	
N1, N2	2.412 (1.23–4.61)		1.88 (1.01–3.63)	
Well, moderate	1 (Reference)	0.386		
Poor, Muc, others	1.63 (0.48–4.06)			
Tumor site		0.702		
Right side	1 (Reference)			
Left side	1.13 (0.57–2.16)			
Wild type	1 (Reference)	0.022	1 (Reference)	0.031
All codon 12 mutants	2.27 (1.20–4.28)		2.05 (1.08–3.85)	
All codon 13 mutants	0.713 (0.11–2.44)		0.58 (0.09–1.99)	

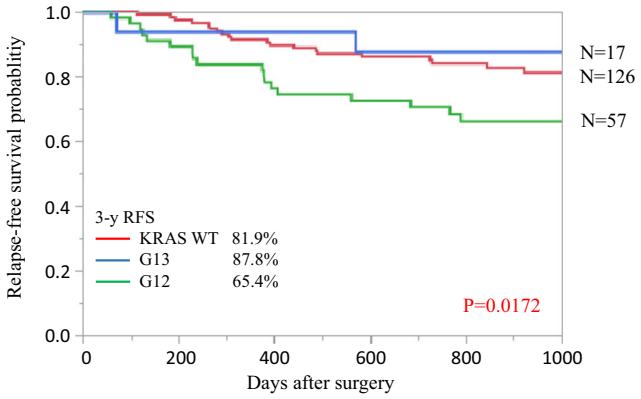

Fig. 1 Kaplan–Meier curves. Relapse-free survival for patients with colorectal cancer stratified by codon mutation

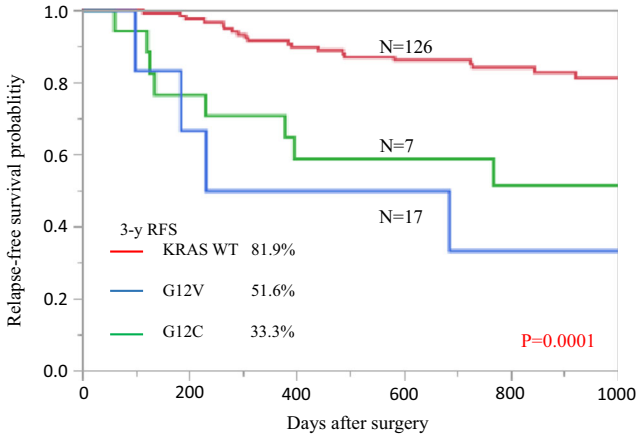

Fig. 2 Kaplan–Meier curves. Relapse-free survival for patients with colorectal cancer stratified by codon 12 point mutation

in stage I CRC was 24%; the mutation rate of codon 13 was 4.3%. In stage II–III CRC was 30% ($P = 0.26$) and 15% ($P = 0.23$). Compared with early-stage cancer, advanced cancer tended to have a lower percentage of wild-type *KRAS* ($P = 0.13$).

Mutation characteristics of KRAS gene

As Table 2 shows, in the 200 tumor samples, a total of 74 *KRAS* mutations (37%; 74/200) were detected, such that 28.5% (57/200) of the samples included codon 12 mutations and 8.5% (17/200) included codon 13 mutations. The p.G12D (49.2%; 28/57) and p.G12V (29.8%; 17/57) point mutations were the most common codon 12 mutations; p.G13D was most common (94.1%; 16/17) in codon 13. A significant correlation was found between mutant *KRAS* and tumor location (right side of body; $P = 0.02$), but no other tested characteristics (Table 3).

Relapse-free survival

Thirty-three patients (16.5%; 33/200) developed a recurrence after a median 850-day-postoperative follow-up period. As Table 4 shows, univariate analysis identified T stage, N stage, and *KRAS* mutation status as predictive of RFS. In multivariate analysis and while controlling for other factors, *KRAS* status remained statistically significant (HR 2.05; 95% CI, 1.08–3.85, $P = 0.031$). In N stage, P value was 0.052. N stage did not remain statistically, but this may be due to the small number of cases.

The median length of RFS for the entire cohort was 770 days, and 77.1% of the overall group reached the 3-year RFS milestone. A significantly smaller proportion of the patients with mutant *KRAS* than those with wild-type *KRAS* reached the 3-year RFS milestone (69.7% vs. 82.1%, respectively; $P = 0.01$). Three-year RFS among patients with mutations in codon 12 was 65.4% compared with 87.8% for patients with codon 13 mutations ($P = 0.45$). Among the six most common *KRAS* mutations assayed, none was associated with shorter RFS relative to the wild-type patient group (Fig. 1).

Univariate analysis of the five most common *KRAS* mutations demonstrated that the G12V and G12C mutations were most strongly associated with a greater likelihood of long-term recurrence (Table 5). Both G12V ($n = 17$; HR, 3.77; 95% CI, 1.54–8.39; $P = 0.002$) and G12C ($n = 7$; HR, 6.57; 95% CI, 1.90–17.7; $P < 0.001$) mutations were significantly associated with an increased risk for long-term recurrence versus the wild-type form (Fig. 2).

Table 5 Univariate analysis of relapse-free survival according to codon 12 KRAS mutation

Mutation	Univariate hazard ratio	P value
Wild type	1 (Reference)	
G12A	1.47 (0.16–6.03)	0.414
G12D	1.12 (0.37–2.83)	0.810
G12C	6.57 (1.90–17.7)	0.0007
G12S	3.36 (0.53–11.7)	0.105
G12V	3.77 (1.54–8.394)	0.002

Patients with codon 12 mutation

When comparing between codon 12 mutations, G12V or G12C mutations were associated with worse RFS than G12A, G12D, or G12S mutations, such that the former were considered high risk and the latter low-risk mutations. In patients with codon 12 mutation, univariate analysis identified T stage and N stage and KRAS G12 mutation status as predictive of RFS (Table 6). On multivariate analysis controlling for other factors, KRAS status remained statistically significant (HR 4.97; 95% CI, 1.50–14.6), $P = 0.001$). Median relapse-free survival for patients with high-risk mutations was

Table 6 Univariate and multivariate analysis of relapse-free survival stratified by clinico-pathological features (patients with codon 12 mutation)

	Univariate	*P* value	Multivariate	*P* value
Age > 66	1.78 (0.70–5.10)	0.227		
Male	0.77 (0.31–1.93)	0.580		
Female				
T stage		0.013		0.001
T1/T2	1 (Reference)		1 (Reference)	
T3/T4	6.71 (1.39–12.1)		7.92 (1.50–14.6)	
N stage		0.043		0.273
N0	1 (Reference)		1 (Reference)	
N1, N2	2.58 (1.03–6.95)		1.69 (0.66–4.67)	
Well, moderate	1 (Reference)	0.589		
Poor, Muc, others	1.53 (0.48–4.06)			
Tumor site		0.617		
Right side	1 (Reference)			
Left side	1.26 (0.51–3.26)			
KRAS		0.004		0.001
Low risk (G12A + D + S)	1 (Reference)		1 (Reference)	
High risk (G12C + V)	4.05 (1.54–12.6)		4.97 (1.50–14.6)	

20.1 months, and was significantly lower than patients with low-risk mutations (28.3 months; $P = 0.004$, Fig. 3).

Discussion

The present study investigated clinicopathologic and prognostic features of *KRAS* mutations in primary tumors from 200 consecutive patients with stage I–III CRC. Our analyses showed a significant proportion of the patients (37.5%) had tumors bearing a *KRAS* mutation in codons 12 or 13, and the majority (57/74) were in codon 12. Notably, the only relationship between mutation state and clinicopathologic features was a significant bias of mutant forms towards right-side tumors. *KRAS* mutation state was associated with worse RFS as a result of the G12V and G12C point mutations, whereas the other codon 12 and 13 mutations were not significantly different from each other or wild-type *KRAS*, and thus considered low risk.

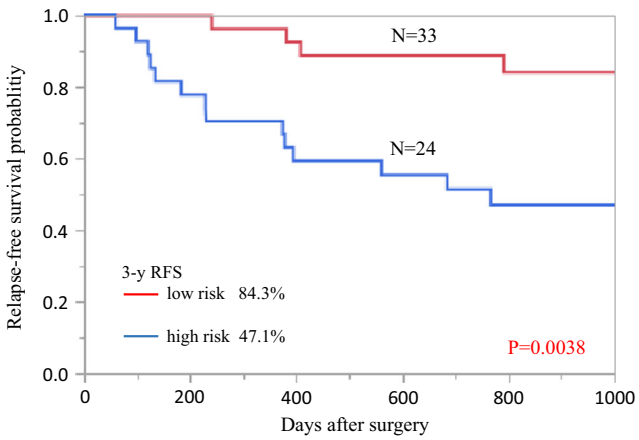

Fig. 3 Kaplan–Meier curves. Relapse-free survival for patients with colorectal cancer stratified by codon 12 mutation risk factor

The *KRAS* mutation rate of Japanese CRC patients was found to be 37.6% in a previous study [2]. The findings herein verify that; however, gender, age, the site of the primary lesion, and the year that the sample was prepared were found to be independent risk factors for *KRAS* mutations in the prior work [2]. *KRAS* mutations were identified in 37% of patients included for survival analysis. Other reports indicate similar rates in stage II and III CRC [11, 12] for both G12C (8.9 vs. 10.0%) and G12V (24.4 vs. 21.1%) mutations [13].

Within our population, mutations in *KRAS* codon 12 were independently associated with a worse RFS versus wild-type *KRAS*. By contrast, mutations in codon 13 were not. We found that codon 12 mutations increased the risk of recurrence roughly 100% over the wild-type form, especially in the cases of the G12V and G12C mutations (hazard ratios of 3.77 and 6.57, respectively). These findings are consistent with those in other reports [13].

The effects of specific codon 12 mutations on clinical outcomes have also been demonstrated in a large multicenter study of 2721 patients with metastatic CRC [14]. Notably, only the G12V point mutation was a predictive factor of worse overall survival among patients with advanced CRC, suggesting such a mutation induces more aggressive tumor behavior [14, 15].

Studies analyzing the behavior of G12V *KRAS* mutants noted that GTPase activity of the G12V form is 25% of that of the G12D mutant form and just 10% of that of the wild type [6, 16, 17]. The G12V mutation also has reduced affinity for binding GTPase-activating proteins, further reducing GTPase function [18]. This appears to alter the threshold at which apoptosis is induced [7], and potentially to increase the cell's

transforming ability and thus generate a more aggressive biologic phenotype [19]. In addition, G12V and G12D mutations appear to also significantly inhibit GTP binding by the protein [6, 16–20].

In conclusion, the present study demonstrated that CRC tumors with *KRAS* mutations have a distinct phenotype and, in the case of G12V or G12C point mutations, are independent factors predicting a poorer prognosis in patients with stage I–III CRC. *KRAS* mutations should be considered in predicting the clinical outcome of patients and in individualizing their therapies.

This is an interesting finding that should be further examined with greater amount of research in hope that it may constitute an additional prognostic factor for colorectal cancer.

Compliance with ethical standards

The present study was conducted in accord with the Declarations of Helsinki and was approved by the Ethics Committee of the Teikyo University.

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