The prognostic value of *KRAS* mutations in patients with colorectal cancer

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Abstract. Our aim was to evaluate the *KRAS* genotypes of Japanese colorectal cancer (CRC) patients and to assess the effect of these genotypes on clinical outcome. A total of 99 patients with stage I-IV CRC who underwent resection were prospectively studied for *KRAS* mutations by direct sequencing. *KRAS* mutations were found in 37 (37.4%) of 99 patients. Of these, 11.1% were the *KRAS* p.G13D mutation and the remaining 26.2% were other *KRAS* mutations. The cumulative 5-year survival rates for patients with wild-type *KRAS*, *KRAS* 12 and *KRAS* p.G13D mutations were 81.4, 61.4 and 42.0%, respectively (P=0.0397). The *KRAS* genotype had no effect on stage IV patient prognosis without anti-epithelial growth factor receptor (EGFR) antibody therapy. However, in stage I-III patients significant or trends in prognostic factors for disease-free survival (DFS) were pathological T stage, lymphatic vessel involvement and *KRAS* p.G13D. Multivariate analysis identified T4 pathological stage (P=0.0076) and the *KRAS* p.G13D mutation (P=0.0499) as the most significant independent prognostic factors associated with DFS. In Japanese CRC patients *KRAS* p.G13D had prognostic impact on DFS in stage I-III disease, while the prognosis of stage IV patients without anti-EGFR antibody therapy was unaffected by *KRAS* status.

Introduction

Colorectal cancer (CRC) is one of the major causes of cancer-related death. In order to improve the prognosis of CRC patients, the role of chemotherapy has been considerably expanded over the past decades. The administration of molecular targeted therapy, including bevacizumab and anti-epithelial growth factor receptor (EGFR) antibodies, has further prolonged the survival times of CRC patients (1-3). The *KRAS* mutation in CRC is now a well-established useful marker for predicting tumor responsiveness, especially involving the use of the anti-EGFR antibodies cetuximab and panitumumab. Many studies have reported that 30-40% of CRC patients have *KRAS* mutations and that they failed to respond to anti-EGFR antibody therapy (3-10). Most of the mutations are found in codons 12 and 13. However, recent reports have suggested that CRC tumors with the codon 13 mutation might show a different response to treatment than tumors with other *KRAS* mutations (11,12). In fact, some reports have suggested that cetuximab but not panitumumab has shown therapeutic efficacy regarding CRC with codon 13 (11-13).

The *KRAS* mutations are monoallelic and appear during carcinogenesis, mostly between the stages of early and intermediate adenoma, maintaining a constant incidence in late adenomas and carcinomas. Therefore, many studies have been conducted to assess the prognostic significance of *KRAS* mutations in CRC (14-25). Since the 1990's, it has been suggested that alteration of codons 12 or 13, both of which are important for protein functionality, might have a stronger prognostic value (26-31). However, the prognostic value of *KRAS* mutations in CRC remains controversial, especially in the case of codons 12 or 13, although *KRAS* mutations have been associated with a poor response to modern anti-EGFR antibody therapy in relation to metastatic CRC. In addition, recent attention has been drawn to the fact that *KRAS* mutations could be a useful marker for predicting tumor responsiveness rather than as a prognostic factor.

More recently, the Japan Study Group of *KRAS* Mutation in CRC has reported on the clinicopathological features of *KRAS* mutations in a large-scale Japanese study population (32). They clarified the clinicopathological features of *KRAS* mutations, rather than their prognostic significance, in Japanese patients with CRC. Only one study has reported the prognostic value of *KRAS* and *BRAF*, which is a downstream *KRAS* molecule, in Japanese patients with advanced and recurrent CRC (33). The study concluded that the *KRAS* codon 13 mutation showed a trend towards poor patient survival, and that *BRAF* mutations and not *KRAS* mutations are a powerful prognostic factor in advanced and recurrent CRC (33). Despite

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these studies, the prognostic impact of *KRAS* mutations in Japanese patients remains unknown in those patients who had recurrence or curative surgery for advanced CRC. The aim of the current study was to evaluate the *KRAS* genotypes of Japanese patients with Union for International Cancer Control (UICC) stage I-IV CRC who did not have anti-EGFR antibody therapy, and to assess the relationship of these genotypes with clinical outcome.

Patients and methods

Patients and sample collection. A total of 99 patients (56 males and 43 females; mean age, 66.9 years; age range, 42-91 years) who underwent CRC resection at the Mie University Hospital between 2002 and 2004, and from whom resected cancer tissue could be collected with informed consent for use as samples, were enrolled in this study. All tumors were histologically confirmed to be adenocarcinoma of the colon and rectum. Exclusion criteria included a history of previous neoplasm, patients from families with familial adenomatous polyposis or hereditary non-polyposis CRC, and patients who had undergone chemotherapy or radiotherapy prior to surgery. In this study, there were no patients who received anti-EGFR antibody therapy, because it had not been approved in Japan. To assess the precise prognostic value of *KRAS* status, we also excluded patients with stage IV disease who had received curative resection for synchronous metastasis.

All patients were classified according to the UICC stage classifications using resected specimens. There were 20 patients with stage I disease, 30 patients with stage II disease and 25 patients with stage III disease. Twenty-four patients with distant metastases were classified as having stage IV disease. Drug approval in Japan takes considerably more time than in the Western world. Molecular targeted agents including bevacizumab, cetuximab and panitumumab were approved for use in 2007, 2008 and 2010, respectively. Then, all stage IV patients underwent 5-FU with irinotecan or oxaliplatin based chemotherapy. None of the patients received molecular targeting agents including anti-EGFR antibodies and bevacizumab. Adjuvant chemotherapy using 5-FU based chemotherapy was introduced for all stage III and some of the stage II patients. Clinicopathological data, such as age, gender, tumor size and location were retrieved from patient records. The specimens used for *KRAS* genotyping were paraffin-embedded tissues. At the time of analysis, the median follow-up period for all patients was 34 months. For the *KRAS* genotyping, appropriate approvals were obtained from the institutional review board.

DNA extraction from formalin-fixed, paraffin-embedded (FFPE) tumor tissue sections. Tumor cell-rich area in a hematoxylin and eosin-stained section was marked under a microscope, and tissue was scratched from the area of another deparaffinized unstained section. DNA for pieces of the scratched tissue sample was isolated using the QIAamp FFPE Tissue kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol.

The concentration and purity of the extracted DNA was determined by spectrophotometry (NanoDrop® ND-1000). The extracted DNA was stocked at -20˚C until use.

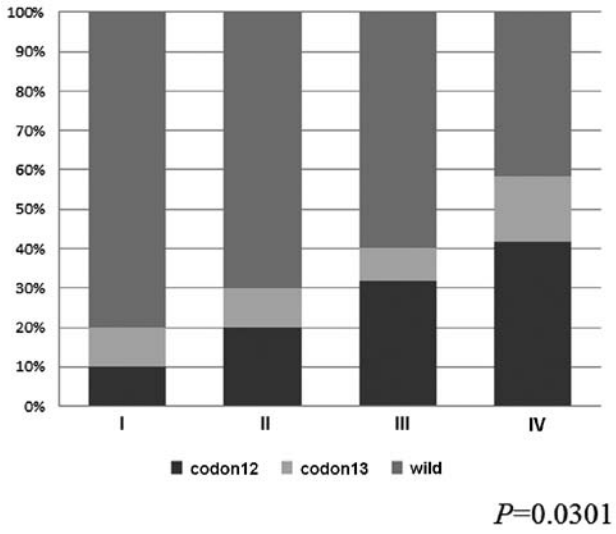

Figure 1. The frequency of *KRAS* mutation according to colorectal cancer stage classification.

PCR amplification and direct sequencing. To detect point mutation at *KRAS* codons 12 and 13, we used direct sequencing as previously described by Karapetis *et al* (4). Exons 2 of the *KRAS* gene was amplified by PCR. The PCR products were visualized using agarose gel electrophoresis with ethidium bromide staining. The PCR DNA fragment was extracted from agarose gel, and directly sequenced using an ABI 3130xl Genetic Analyzer (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions.

Statistical analysis. We used JMP version 7 (SAS Institute Inc., Cary, NC, USA) to perform statistical analyses. Contingency tables were analyzed using Fisher's exact test or the χ^2 test with Yate's correction. Associations between continuous variables and categorical variables were evaluated using the Mann-Whitney U test. Survival curves were constructed according to the Kaplan-Meier method, and differences were analyzed using the log-rank test. A P-value of <0.05 was considered as significant. Variables found to be significant at this level were considered eligible for Cox proportional hazard regression.

Results

Frequency of KRAS gene mutation in Japanese CRC patients. *KRAS* mutations were found in 37 (37.4%) of the 99 patients. The most prevalent mutations were 10 (27.0%) patients with GGT→GAT (G12D) and eight (21.6%) with GGT→GTT (G12V) within codon 12, and 11 (29.7%) patients with GGC→GAC (G13D) within codon 13. In addition, there were three (8.1%) patients with GGT→TGT (G12c), three (8.1%) with GGT→AGT (G12S) and two (5.4%) with GGT→GAT (G12D) mutations. Of these, 11% were the *KRAS* p.G13D mutation and the remaining 26.2% were other *KRAS* mutations. The frequency of *KRAS* mutation according to UICC stage was 20% in stage I, 30% in stage II, 40% in stage III and 58% in stage IV. The frequency of *KRAS* mutations was positively correlated with stage classification mutations (P=0.0301), which resulted in a trend

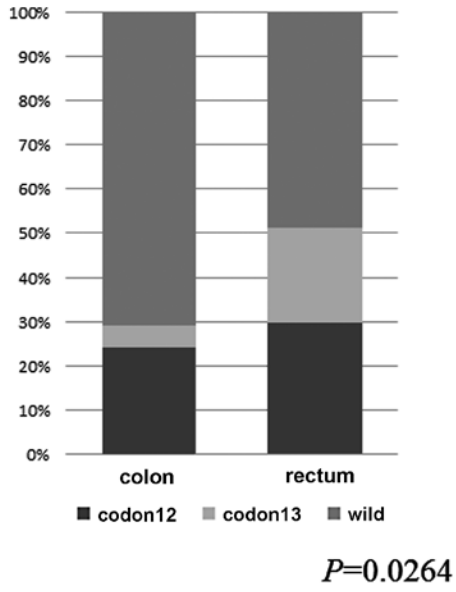

Figure 2. The frequency of *KRAS* mutation according to tumor location.

towards a high frequency of *KRAS* 12 mutations in advanced stage disease (Fig. 1); while the *KRAS* p.G13D mutation occurred at a constant rate in all disease stages. Furthermore, the *KRAS* p.G13D mutation was found more frequently in the rectum than in the colon (21.6 vs. 4.8%; P=0.0102), whereas *KRAS* 12 mutations were found at comparable levels in the rectum and colon (29.7 vs. 24.2%; P=0.5448) (Fig. 2).

Association of KRAS mutations with clinicopathological features. When all patients were divided into three groups according to their *KRAS* status (wild-type vs. *KRAS* 12 mutations vs. *KRAS* p.G13D), a significant association emerged between *KRAS* mutations and lymph node metastases (P=0.0076), and distant metastases (P=0.0289) (Table I). There were no significant differences in age, gender, primary site, tumor size, pathological type, tumor invasion, vessel and lymphatic invasion.

Prognostic analyses. Fig. 3 shows the cumulative overall survival (OS) curves for all patients subdivided according to

Table I. Relationship of *KRAS* status with clinicopathological features.

	Wild-type (n=62) (%)	*KRAS* 12 (n=26) (%)	*KRAS* p.G13D (n=11) (%)	P-value
Age (years)	67.9	65	65.9	ns
Gender (male/female)	40/22	9/16	6/5	ns
Colon/rectum	44/18	15/11	3/8	ns
Tumor size (mm)	40.4	50.2	41.4	ns
Pathology				
Well	15	6	2	
Moderately	41	17	8	ns
Poorly	3	1	0	
Mucinous	3	2	1	
pT				
T1	11	1	0	
T2	10	5	2	ns
T3	29	13	5	
T4	12	7	4	
pN				
N0	41 (66)	10 (38)	5 (46)	
N1	12 (19)	11 (42)	3 (27)	0.0076
N2	8 (13)	1 (5)	3 (27)	
N3	1 (2)	4 (15)	0	
Lymphatic invasion				
0-1/2-3	24/38	8/18	3/8	ns
Venous invasion				
0-1/2-3	50/12	19/7	9/2	ns
Distant metastasis				
Yes/no	9/62 (15)	10/26 (38)	4/11 (36)	0.0289

N0, no evidence of lymph node metastasis; N1, metastasis in 1-3 lymph nodes; N2, metastasis in 4 or more intermediate lymph nodes; N3, metastasis in main or lateral lymph nodes. ly0, no invasion; ly1, minimal invasion; ly2, moderate invasion; ly3, sever invasion. v0, no invasion; v1, minimal invasion; v2, moderate invasion; v3, severe invasion; ns, not statistically significant.

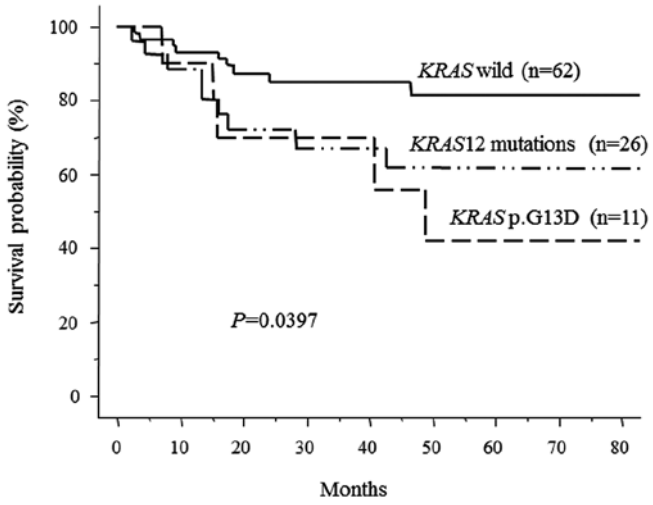

Figure 3. The cumulative overall survival curves of all patients according to *KRAS* status.

KRAS status. The cumulative 5-year survival rate of *KRAS* wild-type and *KRAS* 12 mutations, and the *KRAS* p.G13D mutation was 81.4, 61.4 and 42.0%, respectively (P=0.0397). On the basis of Cox univariate proportional hazards analysis, tumor size (>5 vs. <5 cm) [hazard ratio (HR), 2.674; 95% confidence interval (CI), 1.155-6.188; and P=0.0216], pathological T stage (T4 vs. others) (HR, 15.761; 95% CI, 6.122-40.577; and P<0.0001), lymphatic vessel involvement (HR, 5.600; 95% CI, 1.312-23.895; and P=0.020), vessel involvement (HR, 2.848; 95% CI, 1.232-6.582; and P=0.0144), distant metastasis (HR, 14.910; 95% CI, 5.802-38.314; and P<0.0001), *KRAS* mutation (mutation vs. wild-type) (HR, 2.884; 95% CI, 1.247-6.668; and P=0.0133) and *KRAS* p.G13D mutation (p.G13D vs. wild-type) (HR, 3.462; 95% CI, 1.158-10.351; and P=0.0262) were significant prognostic factors for OS. However, no *KRAS* mutations were identified as independent risk factors for predicting poor prognosis using multivariate analysis.

Next, we evaluated the prognostic value of *KRAS* mutations according to CRC stage classification. In this study, all stage IV patients underwent therapeutic chemotherapy using irinotecan (CPT-11) and/or oxaliplatin (L-OHP) based chemotherapy without any molecular agents including cetuximab or panitumumab. There were no differences in the median survival times (MST) of patients between *KRAS* and wild-type mutations (16.2 vs. 17.8 months). Regardless of *KRAS* mutant status, *KRAS* genotype had no effect upon the OS of stage IV patients without anti-EGFR antibody therapy (*KRAS* p.G13D, MST 15.7 months; *KRAS* 12 mutations, MST 17.8 months; wild-type, MST 16.2 months).

To further investigate the prognostic value of *KRAS* mutation, we evaluated the correlation between *KRAS* mutations and DFS in patients with stage I-III disease. Significant prognostic factors or a trend in prognostic factors for DFS were pathological T stage (T4 vs. others; P=0.0015), lymphatic vessel involvement (P=0.0645) and *KRAS* p.G13D (p.G13D vs. wild-type; P=0.0895). Furthermore, multivariate analysis identified pathological T4 stage (HR, 6.993; 95% CI, 1.678-29.145; and P=0.0076) and the *KRAS* p.G13D mutation (HR, 5.934; 95% CI, 1.001-35.174; and P=0.0499) as the most significant independent prognostic factors associated with DFS (Table II).

Discussion

KRAS is a proto-oncogene encoding a small 21 kD guanosine triphosphate (GTP)/guanosine diphosphate (GDP) binding protein, and is responsible for the regulation of cellular response to many extracellular stimuli (34). The major signal transduction pathways, including the RAS-RAF mitogen activated protein kinase (MAPK), lead to the expression of proteins involved in cell proliferation, differentiation and survival (35,36).

Despite the many studies that have investigated the prognostic value of *KRAS* mutations in CRC treatment (14-25), uncertainty remains. Large cohort studies such as the collaborative RASCAL study have indicated that *KRAS* mutations increased the risk of recurrence and death (37). A second RASCAL II study involving 3,498 CRC patients found that of the 12 possible *KRAS* mutations on codons 12 and 13, only the substitution of glycine to valine on codon 12 had a significant impact on failure free survival and OS. Although the various *KRAS* mutations did not have prognostic value in relation to patients with metastasis, the mutation had greater impact in Dukes' C than in Dukes' B cancers (25). These studies did not identify the *KRAS* 13 mutation as being prognostic. However, a recent report by De Roock *et al* (11) suggested that tumors with the *KRAS* p.G13D mutation may have a worse prognosis, and that the use of cetuximab was associated with longer OS and progression-free survival (PFS) among patients with tumors containing the *KRAS* p.G13D mutation than with tumors containing other *KRAS* mutations.

In order to evaluate the precise prognostic value of *KRAS* status for CRC patients, we need to exclude several biases including the use of anti-EGFR antibody therapy for stage IV disease and curability of the patients. Therefore, our exclusion criteria were patients with stage IV disease who had received

Table II. Multivariate analysis of factors associated with disease-free survival.

Variables	HR	95% CI	P-value
Pathological T (T4 vs. others)	6.993	1.678-29.145	0.0076
Lymphatic vessel involvement (ly 2-3 vs.ly 0-1)	4.807	0.583-36.639	0.1447
KRAS mutations			
KRAS 12 mutations vs. wild-type	3.288	0.691-15.640	0.1346
KRAS p.G13D vs. wild-type	5.934	1.001-35.174	0.0499

curative metastasectomy, and who had undergone chemotherapy with anti-EGFR antibody therapy. Furthermore, we evaluated the prognosis of patients with stage I-III and IV disease separately. Our study found that the DFS of stage I-III patients with *KRAS* p.G13D mutated tumors showed a worse trend than was the case for *KRAS* wild-type mutated tumors, and that the *KRAS* p.G13D mutation was the most significant independent prognostic factor associated with DFS. However, there were no significant correlations between *KRAS* mutations including the *KRAS* p.G13D mutation and OS in Japanese CRC patients, with respect to stage IV patients who underwent therapeutic chemotherapy without anti-EGFR antibodies. These results suggest that the *KRAS* p.13D mutation may be a possible prognostic factor for cancer recurrence in Japanese CRC patients via enhanced tumor growth by mechanisms such as cell proliferation. It is still not clear whether none or only one of the two codons is important in the prognosis of CRC patients. According to Guerrero *et al* (38), *in vitro* data suggested that *KRAS* 13 mutated tumors had a higher level of apoptosis and were less aggressive as compared with *KRAS* 12 mutated tumors. This observation contradicts our prognostic findings that suggested that the *KRAS* p.G13D mutation was an independent prognostic factor associated with DFS; although it was in agreement with our finding of a positive correlation between *KRAS* 12 mutations and advanced disease stage classification.

In 2011, the Japan Study Group of *KRAS* Mutation in CRC reported the results from a large observation study with information on 5,887 CRC patients (32). The frequency of *KRAS* mutations and the *KRAS* p.G13D mutation in this study was 37.6 and 7.3%, respectively, which was similar to our results (37.4 and 11.1%, respectively). The study suggested that the clinicopathological features of tumors with the *KRAS* p.G13D mutation were not similar to those of the *KRAS* wild-type mutation and other mutations. Tumors with the *KRAS* p.G13D mutation might show a different response to treatment than tumors with other *KRAS* mutations. Thus, *KRAS* p.G13D and other *KRAS* mutations may have differing usefulness as prognostic indicators in Japanese CRC patients because of their differing biological effects. The major limitations of our study were the small number of patients enrolled, and the fact that it was retrospective.

In conclusion, we found that in Japanese CRC patients the *KRAS* p.G13D mutation had prognostic impact in relation to DFS in stage I-III disease, while the prognosis of stage IV patients without anti-EGFR antibody therapy was unaffected by *KRAS* status. Elucidation of the exact effects of *KRAS* 12 mutations and the *KRAS* p.G13D mutation on survival in Japanese CRC patients will require further study.

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