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Clinicopathologic and prognostic associations of *KRAS* and *BRAF* mutations in small intestinal adenocarcinoma

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Activating *KRAS* and/or *BRAF* mutations have been identified as predictors of resistance to anti-epidermal growth factor receptor (EGFR) chemotherapy in colorectal cancer. But the status of *KRAS* and *BRAF* mutations and their clinicopathologic and prognostic significance has not been extensively evaluated in small intestinal adenocarcinomas. In this work, the *KRAS* and *BRAF* genes in 190 surgically resected small intestinal adenocarcinoma cases were sequenced and their association with various clinicopathologic variables, including survival of the patients, was analyzed. *KRAS* or *BRAF* mutations were observed in 63 (33%) cases. Sixty-one cases had *KRAS* mutations and 2 had *BRAF* mutations and the two types of mutation were mutually exclusive. The majority of *KRAS* mutations were G>A transition (43/61 cases, 71%) or p.G12D (31/61 cases, 51%). The patients with mutant *KRAS* tended to have higher pT classifications ($P=0.034$) and more frequent pancreatic invasion ($P=0.020$) than those with wild-type *KRAS*. Multivariate logistic regression analysis showed that certain mutated *KRAS* subtypes (G>A transitions and G12D mutations) were significantly correlated with higher pT classification ($P=0.015$ and 0.004, respectively) than wild-type *KRAS* and other *KRAS* mutations. The patients with *KRAS* or *BRAF* mutation had a tendency to shorter overall survival than those with wild-type *KRAS* and *BRAF* ($P=0.148$), but subgroup analysis demonstrated the patients with *KRAS* mutations showed worse survival (median, 46.0 months; $P=0.046$) than those with wild-type *KRAS* (85.4 months) in lower pT classification (pT1–pT3) group. In summary, *KRAS* and, infrequently, *BRAF* mutations are observed in a subset of small intestinal adenocarcinomas, and are associated with higher pT classification and more frequent pancreatic invasion. *KRAS* mutation is a poor prognostic predictor in patients with lower pT classification tumors. Anti-EGFR targeted therapy could be applied to about two-thirds of small intestinal adenocarcinoma patients, namely those with wild-type *KRAS* and *BRAF* if they have metastatic disease, similar to colorectal cancer patients.
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Although the small intestine accounts for 90% of the mucosal surface area of the gastrointestinal tract, small intestinal adenocarcinoma is rare and accounts for only approximately 2% of gastrointestinal malignancies.[1] Epidemiologic observations have shown an increasing incidence of small intestinal adenocarcinoma in recent years, with an estimated 9160 new cases in 2014 in the United States.[2]

Small intestinal adenocarcinoma has a poor prognosis at all stages, which appears to be intermediate

between that of colon and gastric cancer.[3] Lymph node metastasis and distal location (jejunum and ileum) are the most important independent prognostic factors.[4] As a result of the nonspecific nature of presenting clinical manifestations and the lack of effective tools for exploring the small intestine, small intestinal adenocarcinomas are usually diagnosed at an advanced stage,[5] at which chemotherapy can only prolong overall survival. The cytotoxic agents used are generally the same as those used to treat patients with advanced colorectal cancers, and include oral or intravenous fluoropyrimidines, platinum and irinotecan.[5] Unfortunately, there is no standard treatment and the place of new targeted therapies has still to be defined.[1] Moreover, the number of tumor samples collected remains limited because of the low incidence of the disease, which explains the lack of information regarding somatic genetic alterations that occur during small intestinal carcinogenesis.

The *RAS-RAF* mitogen-activated protein kinase cascade has a crucial role in tumor cell proliferation. The *KRAS* oncogene is mutated in approximately 30–40% of colorectal cancers, mainly at codon 12 or 13. *KRAS* mutations are predictive of a lack of efficacy of anti-epidermal growth factor receptor (EGFR) monoclonal antibodies, such as panitumumab and cetuximab, in patients with metastatic colorectal cancer.[6] Recently, several case reports have described the effect of anti-EGFR therapy in patients with wild-type *KRAS* small intestinal adenocarcinomas.[7,8] Moreover, a number of studies of combined treatments, such as CAPOX (capecitabine and oxaliplatin) with bevacizumab, CAPOX with irinotecan, CAPOX with panitumumab, GEMOX (gemcitabine and oxaliplatin) with erlotinib and Nab-paclitaxel, are exploring the effects of anti-EGFR agents in small intestinal adenocarcinoma.[9] *BRAF* mutations, which always occur in the absence of *KRAS* mutations, have also been associated with resistance to anti-EGFR treatment in colorectal cancers.

KRAS and *BRAF* are two of the most frequently studied oncogenes, but they have been little studied in small intestinal adenocarcinoma.[1,3,10–20] In addition, because most of these few analyses were performed on only small numbers of small intestinal adenocarcinoma patients, they need to be validated.

In this study, we analyze the mutational status of *KRAS* and *BRAF* in small intestinal adenocarcinomas and consider the utility of targeted therapy in the light of their frequency. In addition, we evaluate their clinicopathologic and prognostic significance.

Materials and methods

Study Population

This study was approved by the Institutional Review Board of Incheon St. Mary's Hospital (OC14OIMI0133). A total cohort of 197 surgically resected primary small intestinal adenocarcinoma cases was collected from the surgical pathology archives of 22 South Korean institutions by the Korean Small Intestinal Cancer Study Group, as previously reported.[21] Carcinomas extending from the surrounding gastrointestinal tract organs, such as the stomach, ampulla of Vater, pancreas, cecum or appendix, into the small bowel were excluded from the analysis. Clinical and pathologic data collected as part of a previous study were used again in this study.[21]

Histologic types and tumor grading were classified according to the WHO classification.[22] Briefly, tumor grading was classified as low-grade (well (>95% with gland formation) and moderately differentiated (50–95% with gland formation) adenocarcinomas) and high-grade (poorly differentiated (1–49% with gland formation) and undifferentiated (no gland formation)) carcinomas.[22]

Molecular Analysis

Ten sections with 10 μm in thickness from formalin-fixed paraffin-embedded tissue blocks were used and manually dissected to extract genomic DNA. Genomic DNA was extracted with a QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Mutations in codons 12 and 13 of *KRAS* exon 1 and codon 600 of *BRAF* exon 15 were identified by cycle sequencing. The *KRAS* and *BRAF* genes were PCR amplified with primers for *KRAS* (F: 5′-TGACATGTTCTAATATAGTCAC-3′, R: 5′-AC AAGATTTACCTCTATTGTT-3′) and primers for *BRAF* (F: 5′-TCATAATGCTTGCTCTGATAGGA-3′, R: 5′-GGCCAAAAATTTAATCAGTGGA-3′). In general, PCR reactions were run in a total volume of 25 μl with 0.3 μM of each primer using AmpliTaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Samples were subjected to initial denaturation at 95 °C for 15 min, 40–45 cycles at 95 °C for 50 s, annealing for 50 s and elongation at 72 °C for 1 min, followed by final elongation at 72 °C for 7 min. PCR products were column-purified using a QIAquick PCR Purification Kit (Qiagen) or enzymatically treated with ExoSAP-IT (USB, Cleveland, OH, USA). The sequencing primers were identical to the PCR primers, and all samples were sequenced in both directions using a BigDye Terminator Cycle Sequencing Kit, version 1.1 (Applied Biosystems). The sequencing reactions were analyzed on an ABI Prism 3100 Genetic Analyzer with Sequencing Analysis software, version 3.7 (Applied Biosystems).

Statistical Analysis

Statistical analyses were performed using SPSS software (version 17.0; SPSS, Chicago, IL, USA). Student's *t*-test, the χ^2 test and Fisher's exact test were used to examine associations between categorical variables. Multivariate relationships were estimated by fitting logistic regression models. Overall patient survival was defined as the time from surgical

Table 1 *KRAS* mutation status among small intestinal adenocarcinomas

			Tumor location (%)		
KRAS mutation	*Amino acid*	n	*Proximal (duodenum)*	*Distal (jejunum, ileum)*	P-*value*
Transition of base		61	38 (62)	23 (38)	0.038[a]
G>A		43	26 (60)	17 (40)	
G>C		5	1 (20)	4 (80)	
G>T		13	11 (85)	2 (15)	
Mutation sequence					0.042[a]
Codon 12		50	31 (62)	19 (38)	
GGT>GCT	G12A (alanine)	4	0	4 (100)	
GGT>GTT	G12V (valine)	6	6 (100)	0	
GGT>CGT	G12R (arginine)	1	1 (100)	0	
GGT>GAT	G12D (aspartate)	31	18 (58)	13 (42)	
GGT>TGT	G12C (cysteine)	7	5 (71)	2 (29)	
GGT>AGT	G12S (serine)	1	1 (100)	0	
Codon 13		11	7 (64)	4 (36)	
GGC>GAC	G13D (aspartate)	11	7 (64)	4 (36)	

[a]Statistically significant ($P < 0.05$).

resection to death or last follow-up examination. Survival rates were calculated by the Kaplan–Meier method. Associations between survival rates and various clinicopathologic factors were assessed using the log-rank test. We also investigated the significance of any prognostic factors using Cox proportional hazards modeling. *P*-values < 0.05 were considered to denote statistical significance.

Results

Patient Characteristics

Of the total of 197 patients, 190 with interpretable *KRAS* or *BRAF* sequencing results were included in our study. The mutation status of *KRAS* and *BRAF* was evaluable in 190 and 178 patients, respectively. We could not perform complete sequencing on the other samples because of a lack DNA or degradation of the DNA.

Patient ages ranged between 23 and 86 years (mean, 59.0 years; s.d., 14.0 years). The male to female ratio was 1.7. The median follow-up period after surgical resection was 28.6 months (range, 0–168.4 months). Predisposing conditions were observed in 22 cases (12%), including 16 cases of sporadic adenoma, 3 of Peutz–Jeghers syndrome, 2 of Meckel's diverticulum and 1 of Crohn's disease. Forty patients were classified as 'suspected Lynch syndrome' based on revised Bethesda guideline. There were no patients with familial adenomatous polyposis, Gardner syndrome, gluten-sensitive enteropathy or intestinal duplication. Tumors were located in the duodenum in 105 cases (55%), the jejunum in 56 cases (30%) and the ileum in 29 cases (15%).

KRAS and *BRAF* Mutations

KRAS mutations were observed in 32% (61/190 cases) of the small intestinal adenocarcinoma patients, whereas *BRAF* mutation was detected in only 1% (2/178 cases). The *KRAS* and *BRAF* mutations were mutually exclusive.

The *KRAS* mutations were mostly in codon 12 (Table 1). Fifty patients (50/61 cases, 82%) had *KRAS* mutations in codon 12, and 11 patients (11/61 cases, 18%) had *KRAS* mutations in codon 13. Based on the mutation sequence of *KRAS*, the most frequent *KRAS* mutation subtype—expressed in the recommended genetic nomenclature[23]—was p.G12D (31/61 cases, 51%), followed by p.G13D (11/61, 18%), p.G12C (7/61, 11%), p.G12V (6/61, 10%), p.G12A (4/61, 6%), p.G12R (1/61, 2%) and p.G12S (1/61, 2%). There was a significant difference in the mutation sequences of *KRAS* according to tumor location ($P = 0.042$): p.G12D, p.G12C and p.G13D were more common in the proximal (duodenal) adenocarcinomas. p.G12A was detected only in distal adenocarcinomas, whereas p.G12V, p.G12R and p.G12S were found only in proximal adenocarcinomas. The most common base substitution in the *KRAS* mutations was a G to A transition (G>A; 43/61 cases, 71%), followed by G>T (13/61, 21%) and G>C (5/61, 8%). An association between the type of base substitution and tumor location was also observed ($P = 0.038$): G>A and G>T substitutions were common in the proximal small intestine, whereas G>C was common in the distal small intestine.

The Association Between *KRAS* or *BRAF* Mutations and Clinicopathologic Factors

The associations between *KRAS* mutation and clinicopathologic factors are summarized in Table 2. The patients with mutant *KRAS* tended to have higher T classifications ($P = 0.034$) and more frequent pancreatic invasion ($P = 0.020$) than those with wild-type *KRAS*. However, high-grade carcinomas were more common among adenocarcinomas

with wild-type *KRAS* ($P=0.036$). There was no association between *KRAS* mutation and other clinicopathologic factors, including age and gender, growth pattern, tumor location, histologic type, nodal metastasis, other loop invasion, retroperitoneal seeding, and perineural and lymphovascular invasion.

Similarly, patients with mutant *KRAS* or *BRAF* had higher T classification ($P=0.040$) and more frequent pancreatic invasion ($P=0.017$), whereas high-grade carcinomas were more frequent in adenocarcinomas with wild-type *KRAS* and *BRAF* ($P=0.024$).

Association Between *KRAS* Mutation and Clinicopathologic Factors According to Tumor Location

We analyzed in detail the relationship between pathologic factors and *KRAS* mutation according to tumor location (Table 3). Proximal adenocarcinoma patients with *KRAS* mutations tended to have tumors with high T classifications ($P=0.022$) and more frequent pancreatic invasion ($P=0.045$), whereas distal cases with *KRAS* mutations had lower-grade tumors ($P=0.028$).

We next evaluated the relationship between pathologic factors and *KRAS* mutation subtypes according to tumor location (Tables 4 and 5). In the proximal adenocarcinomas, *KRAS* G>A and G12D mutations were closely associated with late T classification ($P=0.015$ and 0.009, respectively) and pancreatic invasion ($P=0.011$ and 0.003, respectively). By contrast, neither *KRAS* G>A nor G12D mutations were associated with any pathologic factors among the distal adenocarcinomas.

Association Between Subtypes of *KRAS* Mutations and Clinicopathologic Factors

We assessed the correlation of *KRAS* mutational subtype with clinicopathologic variables (Tables 6 and 7). In a univariate association analysis stratified by *KRAS* mutation subtype, *KRAS* G>A mutation was significantly more frequent in tumors with a higher T classification ($P=0.015$) and pancreatic invasion ($P=0.017$) than in *KRAS* wild-type tumors and those with mutations other than G>A. The *KRAS* G>A mutation was not related to tumor location or differentiation status. In a multivariate logistic regression analysis including location, differentiation, T stage and pancreatic invasion, the *KRAS* G>A mutation remained significantly more frequent in late T stage tumors (odds ratio = 1.588, 95% CI: 1.085–2.324; $P=0.017$).

In a univariate analysis stratified by *KRAS* mutation subtype, the *KRAS* G12D mutation was more closely related to higher T classification ($P=0.004$) and pancreatic invasion ($P=0.016$) than the *KRAS* wild-type and mutations other than G12D. In a

Table 2 Correlation between clinicopathological factors and *KRAS* mutation status in small intestinal adenocarcinomas

	KRAS status (%)		
Clinicopathologic factor	*Wild*	*Mutated*	P-*value*
No. of patients	129	61	
Size (mean ± s.d., cm)	4.4 ± 2.6[a]	4.5 ± 2.2	0.868
Age			
≤50	38 (29)	15 (25)	0.485
>50	91 (71)	46 (75)	
Sex			
Male	83 (64)	36 (59)	0.479
Female	46 (36)	25 (41)	
Growth pattern[a]			
Polypoid	24 (20)	11 (18)	0.885
Nodular	8 (6)	3 (5)	
Infiltrative	90 (74)	46 (77)	
Location			
Proximal (duodenum)	67 (52)	38 (62)	0.180
Distal (jejunum, ileum)	62 (48)	23 (38)	
Histologic subtype			
Tubular	113 (88)	59 (96)	0.288
Mucinous	8 (6)	1 (2)	
Signet ring cell	4 (3)	0	
Undifferentiated	4 (3)	1 (2)	
Differentiation			
Low	92 (71)	52 (85)	0.036[b]
High	37 (29)	9 (15)	
pT classification[c]			
pT1–pT3	61 (48)	18 (31)	0.034[b]
pT4	67 (52)	40 (69)	
pN classification[a]			
N0	55 (48)	29 (50)	0.787
N1	60 (52)	29 (50)	
Pancreas invasion			
No	90 (70)	32 (52)	0.020[b]
Yes	39 (30)	29 (48)	
Other loop invasion			
No	125 (97)	60 (98)	1.000
Yes	4 (3)	1 (2)	
Retroperitoneal seeding			
No	121 (94)	57 (93)	1.000
Yes	8 (6)	4 (7)	
Perineural invasion			
No	88 (68)	42 (69)	0.930
Yes	41 (32)	19 (31)	
Lymphovascular invasion			
No	61 (47)	30 (49)	0.807
Yes	68 (53)	31 (51)	

Abbreviation: s.d., standard deviation.
[a]Calculated using only patients with adequate data.
[b]Statistically significant ($P<0.05$).
[c]Excluding patients with pTis.

Table 3 Correlation between clinicopathologic features and *KRAS* mutation status according to tumor location

	Proximal (duodenum) (%)		*Distal (jejunum, ileum) (%)*	
	Wild	*Mutated*	*Wild*	*Mutated*
Size (mean ± s.d., cm)	4.2 ± 2.5	4.6 ± 2.3	4.6 ± 2.8[a]	4.2 ± 2.1
P-value	0.394		0.512	
Age				
≤50	20 (30)	9 (24)	18 (29)	6 (26)
>50	47 (70)	29 (76)	44 (71)	17 (74)
P-value	0.497		0.789	
Sex				
Male	43 (64)	23 (61)	40 (65)	13 (57)
Female	24 (36)	15 (39)	22 (35)	10 (43)
P-value	0.710		0.499	
Growth pattern[a]				
Polypoid	10 (17)	8 (22)	14 (23)	3 (13)
Nodular	4 (6)	2 (5)	4 (6)	1 (4)
Infiltrative	46 (77)	27 (73)	44 (71)	19 (83)
P-value	0.867		0.620	
Histologic subtype				
Tubular	59 (88)	37 (97)	54 (87)	22 (96)
Mucinous	4 (6)	1 (3)	4 (6)	0
Signet ring cell	3 (4)	0	1 (2)	0
Undifferentiated	1 (2)	0	3 (5)	1 (4)
P-value	0.489		0.866	
Differentiation				
Low	50 (75)	31 (82)	42 (68)	21 (91)
High	17 (25)	7 (18)	20 (32)	2 (9)
P-value	0.415		0.028[b]	
pT classification[c]				
pT1–pT3	26 (39)	6 (17)	35 (56)	12 (52)
pT4	40 (61)	29 (83)	27 (44)	11 (48)
P-value	0.022[b]		0.725	
pN classification[a]				
N0	31 (48)	18 (49)	24 (48)	11 (52)
N1	34 (52)	19 (51)	26 (52)	10 (48)
P-value	0.926		0.736	
Pancreas invasion				
No	29 (43)	9 (24)	61 (98)	23 (100)
Yes	38 (57)	29 (76)	1 (2)	0
P-value	0.045[b]		1.000	
Other loop invasion				
No	67 (100)	37 (97)	58 (94)	23 (100)
Yes	0	1 (3)	4 (6)	0
P-value	0.362		0.570	
Retroperitoneal seeding				
No	66 (99)	38 (100)	55 (89)	19 (83)
Yes	1 (1)	0	7 (11)	4 (17)
P-value	1.000		0.479	
Perineural invasion				
No	47 (70)	26 (68)	41 (66)	16 (70)
Yes	20 (30)	12 (32)	21 (34)	7 (30)
P-value	0.853		0.765	
Lymphovascular invasion				
No	33 (49)	20 (53)	28 (45)	10 (43)
Yes	34 (51)	18 (47)	34 (55)	13 (57)
P-value	0.739		0.890	

Abbreviation: s.d., standard deviation.
[a]Calculated using only patients with sufficient available data.
[b]Statistically significant ($P < 0.05$).
[c]Excluding patients with pTis.

Table 4 Correlation between clinicopathologic features and the presence of *KRAS* G>A mutations according to tumor location

	Proximal (duodenum) (%)		*Distal (jejunum, ileum) (%)*	
	Wild-type and other mutation	*KRAS G>A mutation*	*Wild-type and other mutation*	*KRAS G>A mutation*
Size (mean ± s.d., cm)	4.3 ± 2.5	4.5 ± 2.2	4.6 ± 2.7[a]	4.1 ± 2.3
P-value	0.740		0.525	
Age				
≤50	23 (29)	6 (23)	20 (29)	4 (24)
>50	56 (71)	20 (77)	48 (71)	13 (76)
P-value	0.550		0.768	
Sex				
Male	52 (66)	14 (54)	43 (63)	10 (59)
Female	27 (34)	12 (46)	25 (37)	7 (41)
P-value	0.273		0.737	
Growth pattern[a]				
Polypoid	13 (18)	5 (19)	15 (22)	2 (12)
Nodular	5 (7)	1 (4)	4 (6)	1 (6)
Infiltrative	53 (75)	20 (77)	49 (72)	14 (82)
P-value	1.000		0.789	
Histologic subtype				
Tubular	71 (90)	25 (96)	59 (87)	17 (100)
Mucinous	4 (5)	1 (4)	4 (6)	0
Signet ring cell	3 (4)	0	1 (1)	0
Undifferentiated	1 (1)	0	4 (6)	0
P-value	0.869		0.586	
Differentiation				
Low	61 (77)	20 (77)	47 (69)	16 (94)
High	18 (23)	6 (23)	21 (31)	1 (6)
P-value	0.975		0.059	
pT classification[b]				
pT1–pT3	29 (38)	3 (12)	39 (57)	8 (47)
pT4	47 (62)	22 (88)	29 (43)	9 (53)
P-value	0.015[c]		0.445	
pN classification[a]				
N0	35 (46)	14 (54)	27 (48)	8 (53)
N1	41 (54)	12 (46)	29 (52)	7 (47)
P-value	0.492		0.725	
Pancreas invasion				
No	34 (43)	4 (15)	67 (99)	17 (100)
Yes	45 (57)	22 (85)	1 (1)	0
P-value	0.011[c]		1.000	
Other loop invasion				
No	78 (99)	26 (100)	64 (94)	17 (100)
Yes	1 (1)	0	4 (6)	0
P-value	1.000		0.579	
Retroperitoneal seeding				
No	78 (99)	26 (100)	61 (90)	13 (76)
Yes	1 (1)	0	7 (10)	4 (24)
P-value	1.000		0.219	
Perineural invasion				
No	55 (70)	18 (69)	46 (68)	11 (65)
Yes	24 (30)	8 (31)	22 (32)	6 (35)
P-value	0.970		0.817	
Lymphovascular invasion				
No	41 (52)	12 (46)	30 (44)	8 (47)
Yes	38 (48)	14 (54)	38 (56)	9 (53)
P-value	0.611		0.827	

Abbreviation: s.d., standard deviation.
[a]Calculated using only patients with sufficient available data.
[b]Excluding patients with pTis.
[c]Statistically significant ($P < 0.05$).

Table 5 Correlation between clinicopathologic features and the presence of *KRAS* G12D mutations according to tumor location

	Proximal (duodenum) (%)		*Distal (jejunum, ileum) (%)*	
	Wild-type and other mutation	*KRAS G12D mutation*	*Wild-type and other mutation*	*KRAS G12D mutation*
Size (mean ± SD, cm)	4.3 ± 2.5	4.5 ± 2.3	4.6 ± 2.7[a]	3.9 ± 1.9
P-value	0.792		0.407	
Age				
≤50	24 (28)	5 (28)	21 (29)	3 (23)
>50	63 (72)	13 (72)	51 (71)	10 (77)
P-value	1.000		0.751	
Sex				
Male	56 (64)	10 (56)	45 (63)	8 (62)
Female	31 (36)	8 (44)	27 (37)	5 (38)
P-value	0.481		1.000	
Growth pattern[a]				
Polypoid	16 (20)	2 (11)	16 (22)	1 (8)
Nodular	5 (6)	1 (6)	5 (7)	0
Infiltrative	58 (74)	15 (83)	51 (71)	12 (92)
P-value	0.798		0.333	
Histologic subtype				
Tubular	79 (91)	17 (94)	63 (87)	13 (100)
Mucinous	4 (5)	1 (6)	4 (6)	0
Signet ring cell	3 (3)	0	1 (1)	0
Undifferentiated	1 (1)	0	4 (6)	0
P-value	1.000		1.000	
Differentiation				
Low	68 (78)	13 (72)	51 (71)	12 (92)
High	19 (22)	5 (28)	21 (29)	1 (8)
P-value	0.553		0.169	
pT classification[b]				
pT1–pT3	31 (37)	1 (6)	42 (58)	5 (38)
pT4	52 (63)	17 (94)	30 (42)	8 (62)
P-value	0.009[c]		0.185	
pN classification[a]				
N0	40 (48)	9 (50)	28 (47)	7 (64)
N1	44 (52)	9 (50)	32 (53)	4 (36)
P-value	0.854		0.301	
Pancreas invasion				
No	37 (43)	1 (6)	71 (99)	13 (100)
Yes	50 (57)	17 (94)	1 (1)	0
P-value	0.003[c]		1.000	
Other loop invasion				
No	86 (99)	18 (100)	68 (94)	13 (100)
Yes	1 (1)	0	4 (6)	0
P-value	1.000		1.000	
Retroperitoneal seeding				
No	86 (99)	18 (100)	65 (90)	9 (69)
Yes	1 (1)	0	7 (10)	4 (31)
P-value	1.000		0.060	
Perineural invasion				
No	63 (72)	10 (56)	48 (67)	9 (69)
Yes	24 (28)	8 (44)	24 (33)	4 (31)
P-value	0.157		1.000	
Lymphovascular invasion				
No	45 (52)	8 (44)	31 (43)	7 (54)
Yes	42 (48)	10 (56)	41 (57)	6 (46)
P-value	0.574		0.471	

Abbreviation: s.d., standard deviation.
[a]Calculated using only patients with sufficient available data.
[b]Excluding patients with pTis.
[c]Statistically significant ($P < 0.05$).

Table 6 Correlation between clinicopathologic features and the presence of *KRAS* G>A mutations

	Frequency analysis			*Multivariate logistic regression analysis*		
	Wild-type and other mutations (%)	*KRAS G>A mutation (%)*	P-value	*OR*	*95% CI*	P-value
Location						
Proximal	79 (54)	26 (60)	0.435	1.313	0.700–2.463	0.396
Distal	68 (46)	17 (40)				
Differentiation						
Low	108 (73)	36 (84)	0.167	0.747	0.474–1.177	0.209
High	39 (27)	7 (16)				
pT classification[a]						
pT1–pT3	68 (47)	11 (26)	0.015[b]	1.588	1.085–2.324	0.017[b]
pT4	76 (53)	31 (74)				
Pancreas invasion						
No	101 (69)	21 (49)	0.017[b]	2.507	0.553–11.369	0.234
Yes	46 (31)	22 (51)				

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Excluding patients with pTis.
[b]Statistically significant ($P < 0.05$).

Table 7 Correlation between clinicopathologic features and the presence of *KRAS* G12D mutations

	Frequency analysis			*Multivariate logistic regression analysis*		
	Wild-type and other mutations (%)	*KRAS G12D mutation (%)*	P-*value*	*OR*	*95% CI*	P-*value*
Location						
Proximal	87 (55)	18 (58)	0.732	0.625	0.269–1.453	0.275
Distal	72 (45)	13 (42)				
Differentiation						
Low	119 (75)	25 (81)	0.490	1.181	0.720–1.936	0.510
High	40 (25)	6 (19)				
pT classification[a]						
pT1–pT3	73 (47)	6 (19)	0.004[b]	0.519	0.324–0.833	0.007[b]
pT4	82 (53)	25 (81)				
Pancreas invasion						
No	108 (68)	14 (45)	0.016[b]	0.330	0.050–2.175	0.249
Yes	51 (32)	17 (55)				

Abbreviations: CI, confidence interval; OR, odds ratio.
[a]Excluding patients with pTis.
[b]Statistically significant ($P < 0.05$).

Figure 1 Survival of patients with small intestinal adenocarcinomas based on *KRAS* or *BRAF* mutational status. (**a**) Small intestinal adenocarcinoma patients with *KRAS* mutations tended to have relatively shorter overall survival outcomes (median, 21.5 months) than those with wild-type *KRAS* (38.5 months), but this effect does not reach statistical significance ($P = 0.116$, log-rank test). (**b**) Small intestinal adenocarcinoma patients having either *KRAS* or *BRAF* mutation (median, 22.6 months) also tended to have shorter survival times than those with wild-type *KRAS* and *BRAF* (38.5 months; $P = 0.148$). There were no significant differences in the survival time distributions of (**c**) patients with *KRAS* codon 12 mutations (median, 21.0 months) and those with codon 13 mutations (21.5 months; $P = 0.805$), (**d**) patients with *KRAS* G>A mutations (median, 21.0 months) and those with *KRAS* wild-type or mutations other than G>A (36.5 months; $P = 0.398$), and (**e**) patients with *KRAS* G12D (median, 17.3 months) and *KRAS* wild-type patients and those with mutations other than G12D (30.7 months; $P = 0.507$).

multivariate logistic regression analysis including location, differentiation, T classification and pancreatic invasion, *KRAS* G12D mutation remained significantly more frequent in adenocarcinomas with advanced T classification (odds ratio = 0.519, 95% CI: 0.324–0.833; $P = 0.007$).

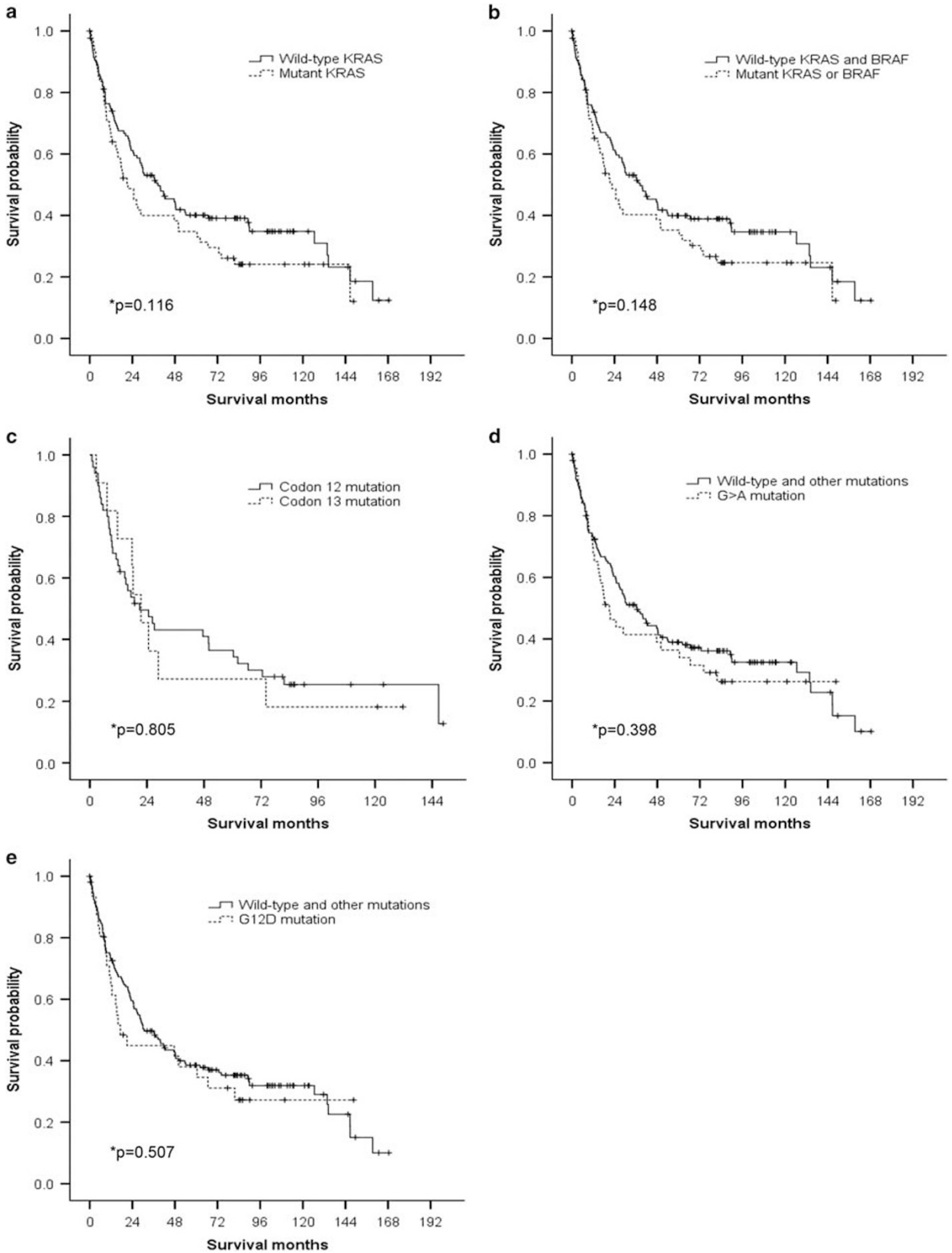

Figure 1 For caption see page 409.

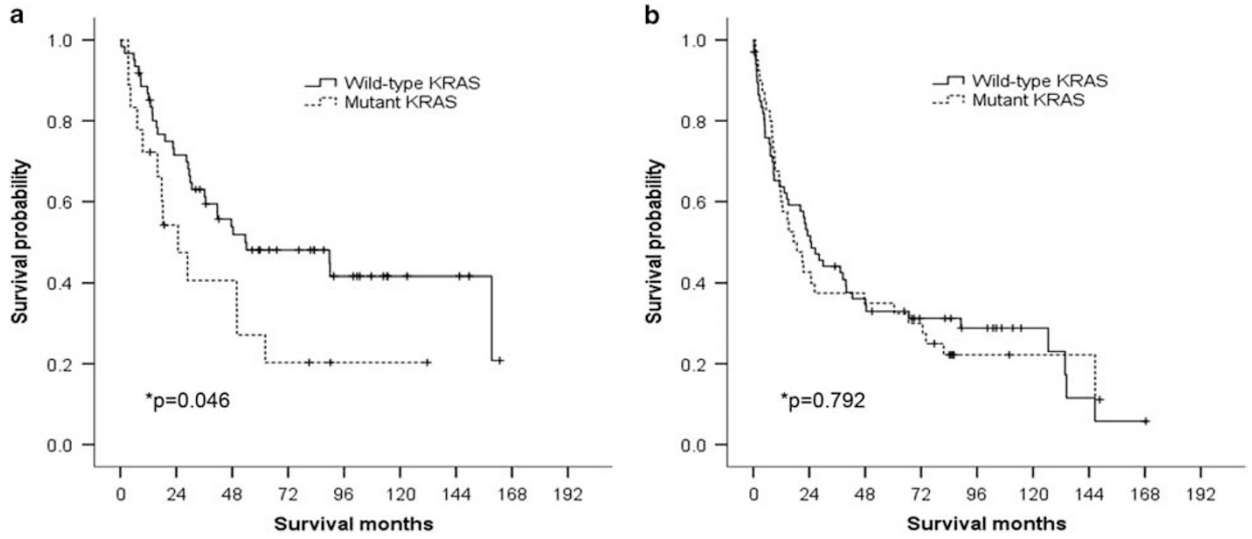

Figure 2 Survival of small intestinal adenocarcinomas patients with *KRAS* mutation based on T classification. (**a**) In lower pT classification (pT1-pT3) subgroup, the survival time for patients with *KRAS* mutation (median, 46.0 months) was significantly shorter than those with wild-type *KRAS* (85.4 months; $P=0.046$, log-rank test). (**b**) In higher pT classification subgroup (pT4), there was no significant difference in survival time distribution between the patients with *KRAS* mutations (median, 50.1 months) and those with wild-type *KRAS* (54.8 months; $P=0.792$).

Survival Analysis

Small intestinal adenocarcinoma patients with *KRAS* mutations (median survival time, 21.5 months) had shorter overall survival outcomes than those with wild-type *KRAS,* but the difference did not reach statistical significance (38.5 months; $P=0.116$, log-rank test; Figure 1a). The survival times of the two *BRAF* mutation carriers were also relatively short ($P=0.675$). There were no significant differences in survival time distribution between the patients with *KRAS* or *BRAF* mutations (median, 22.6 months) and those with wild-type *KRAS* and *BRAF* (38.5 months; $P=0.148$; Figure 1b).

When overall survival times were compared based on mutational status, the median survival time of the patients with codon 12 *KRAS* mutations was not significantly different (21.0 months) from those with codon 13 mutations (21.5 months; $P=0.805$; Figure 1c). Small intestinal adenocarcinoma patients with *KRAS* G>A mutations were associated with shorter survival time (median, 21.0 months) than those with *KRAS* wild-type and those with other than G>A mutations (36.5 months), but this effect did not reach statistical significance ($P=0.398$; Figure 1d). There were no significant differences in survival time distributions between carriers with *KRAS* G12D (median, 17.3 months) and *KRAS* wild-type patients and those with mutations other than G12D (30.7 months; $P=0.507$; Figure 1e).

In terms of tumor location, no relation between *KRAS* mutation and overall survival was seen in proximal versus distal adenocarcinomas. In addition, *KRAS* mutation subtype was also not related to the patient survival in either proximal or distal adenocarcinomas.

Table 8 Multivariate analysis of the small intestinal adenocarcinoma patients with lower T classifications (pT1–pT3)

		95% CI		
	Relative risk	*Lower*	*Upper*	P-*value*
KRAS mutation	2.817	1.360	5.833	0.005[a]
Age ($\leq$ 50 *vs* >50)	1.835	0.897	3.756	0.097
Sex	1.789	0.905	3.535	0.094
Location	1.134	0.804	1.600	0.473
Differentiation	1.062	0.714	1.580	0.768
pN classification	1.793	0.768	4.185	0.177
Retroperitoneal seeding	0.950	0.106	8.540	0.963
Perineural invasion	1.266	0.547	2.927	0.582
Lymphovascular invasion	4.142	2.044	8.394	<0.0001[a]

Abbreviation: CI, confidence interval.
[a]Statistically significant ($P<0.05$).

When survival of small intestinal adenocarcinoma patients was compared depending on T classifications, significant survival differences were observed. In lower T classification (pT1–pT3) group, the survival time for patients with *KRAS* mutation (median, 46.0 months) was significantly shorter than those with wild-type *KRAS* (85.4 months; $P=0.046$; Figure 2a). However, in higher T classification (pT4) group, there was no significant difference in survival time distribution between the patients with *KRAS* mutations (median, 50.1 months) and those with wild-type *KRAS* (54.8 months; $P=0.792$; Figure 2b).

After grouping according to tumor location, we also compared the survival of the patients. In the proximal adenocarcinomas, *KRAS* mutation was not

Table 9 Previous studies of *KRAS* and/or *BRAF* mutations in small intestinal adenocarcinoma

			KRAS mutation		*BRAF mutation*	
Reference	*Country*	*Tumors analyzed* (n)	*Mutation site or codons*	n *(%)*	*Mutation site or codons*	n *(%)*
Laforest *et al*[1]	France, Germany	83: D (39), J (28), I (16)	NS	NS (43)	NS	NS (6)
Aparicio *et al*[3]	France	63: D (32), J (18), I (13)	12, 13, 61, 146	21/49 (43)	V600E	1/40 (3)
Fu *et al*[10]	USA	78: all D	12, 13	27/78 (35)		
Warth *et al*[11]	Germany	37: D (15), J (7), I (7), J/I (8)	NS	11/36 (31)	V600E, etc.[a]	5/36 (14)[a]
Blaker *et al*[12]	Germany	21: D (3), J (2), I (6), J/I (10)	Exon 1	12/21 (57)	Exons 11, 15	1/21 (5)
Mitomi *et al*[13]	Japan	7: J (5), I (2)	Exon 1	5/7 (71)		
Nishiyama *et al*[14]	Japan	35: D (12), J/I (23)	12	2/22 (9)		
Muneyuki *et al*[15]	Japan	20: D (10), J (6), I (4)	12, 13, 61	5/20 (25)		
Achille *et al*[16]	Italy	12: all D	12	5/12 (42)		
Rashid *et al*[17]	USA	23: D (6), D/J (2), J (8), I (7)	12, 13, 61	9/23 (39)		
Younes *et al*[18]	USA	28: D (12), J (11), I (5)	12, 13, 61	4/28 (14)		
Arai *et al*[19]	Japan	15: D (2), J (12), I (1)	12, 13, 61	8/15 (53)		
Sutter *et al*[20]	USA	8: D (6), I (2)	12	5/6 (83)		

Abbreviations: D, duodenum; J, jejunum; I, ileum; NS, not stated.
[a]V600E ($n=4$) and a 3-bp deletion (c1869–1871; $n=1$).

related to the patient survival in either lower or higher pT classification. However, in the distal adenocarcinomas, the patients with *KRAS* mutations (median, 33.0 months) had significantly shorter overall survival outcomes than those with wild-type *KRAS* (78.7 months; $P=0.026$) in a lower pT classification group. On the contrary, in higher pT classification group of distal adenocarcinomas, the patients with *KRAS* mutation (median, 30.1 months) had slightly longer survival time than those with wild-type *KRAS* (22.1 months), but it was not statistically significant ($P=0.428$).

Prognostic Significance of *KRAS* Mutation in Lower pT Classification Tumors

The independent prognostic significance of *KRAS* mutation and other clinicopathologic factors in small intestinal adenocarcinomas of lower pT classification, which were considered significant by univariate analyses, was further evaluated using the Cox proportional hazards modeling (Table 8). According to this multivariate analysis, *KRAS* mutation ($P=0.005$) and the presence of lymphovascular invasion ($P<0.0001$) are poor independent prognostic predictors of overall survival in small intestinal adenocarcinoma patients with lower pT classification.

Discussion

We have sequenced the *KRAS* and *BRAF* genes of a large number of patients ($n=190$) in this study and found *KRAS* mutations in 32% (61 cases). Although a few previous studies of small intestinal adenocarcinomas have examined *KRAS* and/or *BRAF* mutations, which are well-known oncogenes in colorectal cancer, most of these analyses were performed on small numbers of patients (Table 9).[1,3,10–20] The frequency of *KRAS* mutations among small intestinal adenocarcinomas ranged from 9 to 83% in the previous studies. Previous studies from Japan with homogeneous ethnic groups showed a wide range of frequencies of *KRAS* mutation (9–71%),[13–15,19] and other studies with heterogeneous ethnic groups, such as studies from USA, also reported various frequencies of *KRAS* mutation (14–83%).[10,17,18,20] Various *KRAS* mutation frequencies in the previous studies may be related with different detection methods rather than diverse ethnic background. Several different techniques for detecting *KRAS* and/or *BRAF* mutation were used, including restriction fragment length polymorphism, allele-specific oligonucleotide hybridization, PCR-single-strand conformation polymorphism, allele discrimination assay and Sanger sequencing.[1,3,10–20] In addition to different detection techniques, differences in sample size may contribute to the wide range of *KRAS* mutation frequencies observed. The overall prevalence of *KRAS* mutations in the previous studies was 38% (150/400). In those studies with >35 *KRAS* mutations tested,[3,10,11] the prevalence of *KRAS* mutation was reported to be between 31 and 43%. This is similar to the prevalence of *KRAS* mutations in this study. In addition, the prevalence of *KRAS* mutations among small intestinal adenocarcinomas in the previous studies was similar to that among colorectal cancers.[3,6]

On the other hand, *BRAF* mutations occur at a lower rate (0–13%) than *KRAS* mutations in colorectal cancers.[23] *BRAF* mutations are also rarely encountered among small intestinal adenocarcinomas, where the prevalence of *BRAF* mutation ranges from 3 to 14%.[1,3,11,12] In this study, *BRAF* mutations were observed in only 1% of the small intestinal adenocarcinomas. In accord with previous reports for colorectal cancers[23] and small intestinal adenocarcinomas,[1,3,12] *KRAS* and *BRAF* mutations

were mutually exclusive ways in our observation as the same with results of the previous studies.[3,12] Although one study reported concurrent *KRAS* and *BRAF* mutation in squamous cell carcinoma of the lung,[24] to the best our knowledge, there is no report of concurrent *KRAS* and *BRAF* mutation in small intestinal adenocarcinomas. Further studies with larger number of cases are needed for identifying the existence of concurrent *KRAS* and *BRAF* mutations in small intestinal adenocarcinomas. In summary, the molecular alterations in small intestinal adenocarcinomas are close to those in colorectal cancers, with a high frequency of *KRAS* mutations and infrequent *BRAF* mutations.

Several retrospective studies have indicated that chemotherapy prolongs overall survival in patients with advanced small intestinal adenocarcinomas, but there is no standard frontline regimen owing to a lack of randomized trials.[5,25] Patients with advanced small intestinal adenocarcinomas are often treated with the same chemotherapy regimens as patients with advanced colorectal cancers or gastric cancers, especially 5-fluorouracil (FU) or 5-FU-based schedules.[9] The combination of 5-FU and a platinum-based agent has been considered more effective than other regimens such as oxaliplatin-based chemotherapy.[25,26] The main challenge for the near future is to identify molecular markers involved in small bowel carcinogenesis that predict chemosensitivity, and thus to improve survival.[5] Overman *et al*[27] observed that a high proportion of small intestinal adenocarcinomas express both EGFR and vascular endothelial growth factor (VEGF), suggesting that these patients may benefit from therapeutic strategies targeting EGFR and VEGF. Targeted therapies such as monoclonal antibodies against VEGF or EGFR combined with chemotherapy have already exhibited significant efficacy in metastatic colorectal cancers.[5] Given the similar prevalence of *KRAS* mutations in small intestinal adenocarcinomas and colorectal cancers, we think that targeted therapy with anti-EGFR monoclonal antibody would be particularly appropriate for small intestinal adenocarcinoma patients with wild-type *KRAS* and *BRAF* as it is for colorectal cancer patients.

Different genetic mutations may be responsible for different biological effects. In the literature on colorectal cancer, codon 12 mutations of the *KRAS* are associated with a mucinous phenotype.[23] By contrast, codon 13 mutations tend to be nonmucinous and are characterized as more aggressive with a greater metastatic potential.[23] In this study, we did not find any difference in clinicopathologic findings in terms of mucinous/nonmucinous histologic subtype with respect to codon 12/13 mutations of *KRAS* among the small intestinal adenocarcinomas.

Patients with *KRAS* and/or *BRAF* mutations had shorter survival than those with wild-type genes, but the effect was not statistically significant. There has been considerable controversy regarding the prognostic significance of *KRAS/BRAF* mutations.[3,10] Many studies have investigated the prognostic value of *KRAS/BRAF* mutations on retrospectively collected cohorts of colorectal cancers patients, with conflicting results.[23,28–32] Initially, *KRAS* was found to be an important prognostic indicator, but this finding was later restricted to G12V mutations.[31] Colorectal cancer patients with G>A and G>C mutations tended to have a worse prognosis than those with G>T mutations.[30] In addition, mutation of G>T but not G>A or G>C increased the risk of recurrence and death of colorectal cancer patients.[32] To the best of our knowledge, there have been only two previously reported studies of small intestinal adenocarcinomas concerning the prognostic significance of *KRAS* mutations in Western populations,[3,10] and no reports about the prognostic significance of *BRAF* mutations. Aparicio *et al*[3] reported that mutated *KRAS* status in small intestinal adenocarcinoma was an independent predictor of longer overall survival, but this was meaningful only in stage IV patients. Whereas about a third of study population (20/63, 32%) was stage IV patients in the study by Aparicio *et al*, no case of stage IV was present in our study. Fu *et al*[10] proposed that *KRAS* G>A mutation was significantly correlated with late disease stage and poor tumor differentiation, and carriers had an increased risk of distant metastasis and relapse, as well as significantly shorter overall survival. However, in their study, only duodenal adenocarcinomas were included. In this study, *KRAS* G>A mutation as well as G12D mutation was closely related to advanced T classification, as in the study of Fu *et al*.

In addition, we found that mutated *KRAS* status was an independent predictor of poor survival in small intestinal adenocarcinoma, particularly in patients of lower pT classification. In the literature about colorectal cancers, *KRAS* mutations have been detected in the earliest neoplastic lesions found in colonic mucosa, and appear to exert a strong influence on the growth of polyps and early cancers. Nash *et al*[33] reported that the patients with mutant *KRAS* and microsatellite stability had significantly worse survival than other groups among the early-stage colorectal cancer (stages I and II). To the best of our knowledge, this study is the first report to identify the prognostic significance of *KRAS* mutations in lower pT classification of small intestinal adenocarcinomas.

Codons 61 and 146 mutation are additional hotspots for *KRAS* mutations, and data from a small number of studies of colorectal cancers suggest that mutation at these sites predicts resistance to anti-EGFR therapy.[34] Despite their growing clinical relevance, the clinicopathological and molecular features of colorectal cancers with *KRAS* codon 61 or 146 mutations remain largely unknown. A few studies have encountered mutations of codons 61 and 146 of *KRAS* in small intestinal adenocarcinomas.[3,15,17–19] Arai *et al*[19] described only one case of a codon 61 *KRAS* mutation among eight cases of mutant *KRAS* genes in small intestinal adenocarcinomas. Aparicio

et al[3] only documented *KRAS* codon 61 ($n=2$) and codon 146 ($n=1$) mutations in a total of 21 cases of mutated *KRAS*. As we only examined codons 12 and 13 *KRAS* mutations, we do not have information regarding the frequency of *KRAS* mutations of codons 61 and 146. Therefore, further studies are needed to define the predictive value of these mutations in small intestinal adenocarcinomas.

In conclusion, our data from 190 small intestinal adenocarcinoma patients demonstrate that *KRAS* and, infrequently, *BRAF* mutations are observed in a subset of small intestinal adenocarcinomas. *KRAS* mutations are associated with higher pT classification and pancreas invasion. Small intestinal adenocarcinoma patients with either *KRAS* or *BRAF* mutation have a tendency toward shorter survival than those with wild-type *KRAS*. Mutation of *KRAS* oncogene is a worse prognostic predictor in small intestinal adenocarcinomas of lower pT classification. Our observations suggest that targeted therapies such as anti-EGFR chemotherapy could be beneficial in the two-thirds of small intestinal adenocarcinoma patients with wild-type *KRAS* and *BRAF* if they have metastatic disease.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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