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Original Article

Not all mutations of *KRAS* predict poor prognosis in patients with colorectal cancer

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Abstract: The mutation of Kirsten rat sarcoma viral oncogene homolog (*KRAS*) has been reported to be prognostically important in patients with colorectal cancer (CRC). In this study, we investigated whether all *KRAS* mutations predict poor prognosis in patients with CRC. Our analysis of characteristics of *KRAS* mutations revealed the mutation rate for codon 12 was 72.7%, of which G12D was the highest (47.5%) followed by G12V (30.6%), and the mutation rate for codon 13 was 22.0%, of which all were G13D. In support of the concept that prognostic value of the *KRAS* codon-12 mutations is different from the codon-13 mutations, results from our Cox proportional hazard model studies showed that codon-12 mutations correlated with worse overall survival (OS; HR = 2.846, 95% CI: 1.967-4.118, P < 0.001) and progression free survival (PFS; HR = 2.011, 95% CI: 1.450-2.789, P < 0.001). No prognostic significance was revealed for codon-13 mutations. On further analysis, we found that mortality risk was significantly increased with G12D and G12V (G12D: HR = 2.802, 95% CI: 1.793-4.381, P < 0.001; G12V: HR = 2.802, 95% CI: 1.793-4.381, P < 0.001), as was the risk of disease progression (G12D: HR = 2.079, 95% CI: 1.396-3.099, P < 0.001; G12V: HR = 2.408, 95% CI: 1.517-3.822, P < 0.001). To conclude, our results support the concept that codon-12 mutations were predictive for a poor prognosis in Chinese patients with CRC. Specifically, G12D and G12V were independent prognostic factors for worse OS and PFS.

Keywords: Colorectal cancer, *KRAS* mutations, overall survival, prognosis, progression free survival

Introduction

KRAS is an important downstream molecular switch of cell-surface growth signal receptors such as epidermal growth factor receptor (EGFR), which is closely related to cell proliferation [1, 2]. Several studies have shown that *KRAS* is involved in the regulation of different signaling pathways in the development of colorectal cancer (CRC), such as RAS/RAF/MAPK and RAS/PI3K/AKT [3]. The abnormal activation of *KRAS* is one of the primary reasons for the uncontrolled proliferation of tumor cells. The protein encoded by *KRAS* has GTPase activity that may affect cell proliferation, differentiation, and apoptosis by participating in the cellular signal transfer process [4]. However, the impact of *KRAS* mutations on the prognosis of patients with CRC remains controversial and the results from previous studies failed to reach a consensus [5-10]. Even patients with the same *KRAS* mutations who receive the same surgical treatment may experience different postoperative survival times. This indicates that the different codons of *KRAS,* and even different site mutations, may have diverse effects on tumor biological behavior [11-13].

Very few studies have focused on the characteristics of *KRAS* mutation subtypes in Chinese patients with CRC. In the current study, we detected seven common mutations in codons 12 and 13 of *KRAS* exon 2 in 1164 specimens from Chinese CRC patients and investigated the prognostic value of distinct codon-specific *KRAS* mutations and their association with clinicopathologic characteristics for evidence of potential clinical application in CRC.

Materials and methods

Study population

Based on the database of Fujian Provincial Hospital (Fuzhou, China), a total of 1164

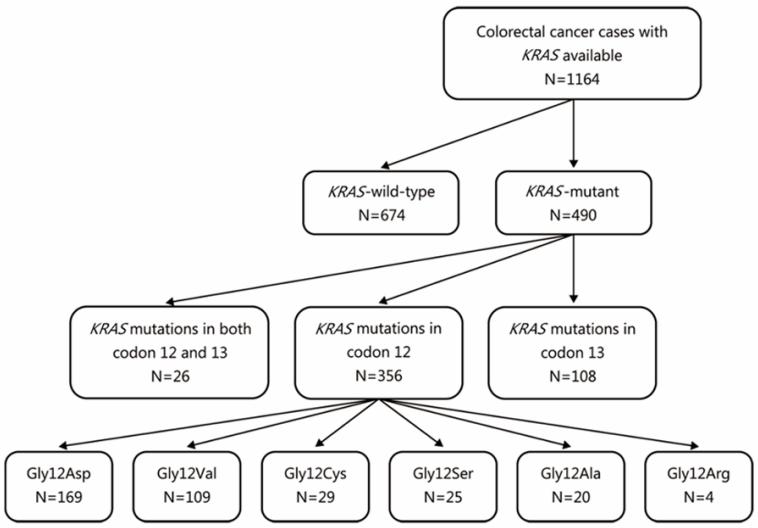

Figure 1. Flow chart of the current study. Cases with *KRAS* mutations in both codons 12 and 13 (N = 26) were excluded from the study to allow for the assessment of the effectiveness of *KRAS* codon 12 mutations as effective prognostic factors for patients with colorectal cancer, independent of *KRAS* codon 13 mutations.

patients with histologically confirmed CRC between June 2012 and September 2015 were identified, among which 26 cases had multiple mutation sites and were excluded from the analysis (**Figure 1**). Standard demographic and clinicopathologic data were collected on each patient, including gender, age, disease status, tumor characteristics, perioperative status, date of last follow-up, date of disease progression, and date of death. The cohort included 657 males and 481 females with an age range of 20-89 years (mean 62). Patients reported to have tumors with *BRAF* mutations, a history of other tumors in addition to CRC, severe heart or cerebrovascular disease, and those who received neoadjuvant therapy or anti-epidermal growth factor receptor agents in the perioperative period were excluded from the study. All patients with CRC had undergone surgery, including 1015 cases of primary radical resection and 123 cases of palliative surgery. Characteristics of the primary tumor, including tumor site, American Joint Committee on cancer T stage, nodal status, and metastasis status (8^{th}) were recorded. The number, size, pathological type, and histological type of the tumors were determined from excised specimens. The largest lesion was used as the index lesion in the case of patients with multiple tumors. Data on *KRAS* mutational status, serum carcinoembryonic antigen (CEA) levels, serum carbohydrate antigen 19-9 (CA19-9) levels were also recorded. Patients with *KRAS* mutations were classified according to the specific *KRAS* mutation (G12D, G12V, G12C, G12S, G12A, G12R, and G13D). The Fujian Provincial Hospital institutional review board approved the study. Patient informed consent specific to this study was not required given its retrospective nature. Data from the clinical follow-up of the patients included progression free survival (PFS) and overall survival (OS) at last follow-up. Progression of disease was defined as the presence of a biopsy-confirmed tumor post-surgery with pathology showing colorectal adenocarcinoma cells or lesions considered suspicious in follow-up computed tomography imaging and elevated serum CEA levels.

DNA preparation and quantitative polymerase chain reaction (PCR)

Three 5-μm thick paraffin-embedded tissue sections were cut and placed in 1.5-mL EP tubes. After deparaffination with xylene, the genomic DNA was extracted using a DNA isolation kit (AmoyDx; Amoy Diagnostics Co., Ltd., Xiamen, China). A Nanodrop 2000 spectrophotometer was used to determine the concentration of DNA and the A_{260}/A_{280} of the DNA samples were between 1.8 and 2.1. An AmoyDx® *KRAS* Mutation Detection Kit (Amoy Diagnostics Co., Ltd.) was used according to the manufacturer's instructions to determine the *KRAS* status of each DNA sample. A Scorpions amplification refractory mutation system (Amoy Diagnostics Co., Ltd.) was used according to the kit instructions to detect the seven *KRAS* mutation sites in the codons 12 and 13 of exon 2 for the paraffin-embedded tissue. The real-time quantitative PCR amplification profile included one cycle of 42°C for 5 min and 95°C for 5 min; followed by 10 cycles of 95°C for 25 sec, 64°C for 20 sec, and 72°C for 20 sec; followed by 30 cycles of 93°C for 20 sec, 60°C for 35 sec and 72°C for 20 sec. The fluorescein amidite (FAM)

Table 1. Characteristics of specific mutations in *KRAS*

Mutation type	Mutation site	Base change	Frequency
Transitions			
1st position	G12S	GGT→AGT (Ser)	5.1% (25/490)
2nd position	G12D	GGT→GAT (Asp)	34.5% (169/490)
	G13D	GGC→GAC (Asp)	22.0% (108/490)
Transversions			
1st position	G12C	GGT→TGT (Cys)	5.9% (29/490)
	G12R	GGT→CGT (Arg)	0.8% (4/490)
2nd position	G12V	GGT→GTT (Val)	22.3% (109/490)
	G12A	GGT→GCT (Ala)	4.1% (20/490)
	Composite		5.3% (26/490)

and hexachloro-fluorescein (HEX) dye signals were measured at the 60°C step during the final 30 cycles of amplification. PCR analysis was performed in the Molecular Diagnostics Laboratory of Fujian Provincial Hospital.

Statistical analysis

Data were analyzed using the Chi-squared (χ^2) test or Fisher's exact test to compare proportions. The Kaplan-Meier method was performed for survival analysis and log rank test was used to compare the survival distributions. Cox's proportional hazards regression model was used to identify the impact of factors on OS and PFS. Hazard ratio (HR) was calculated at 95% confidence interval (CI). $P < 0.05$ was considered statistically significant. All statistical analysis was performed using SPSS statistical software (version 22.0; IBM Corp., Armonk, NY, USA).

Results

Characteristics of KRAS mutations in colorectal cancer

The mutation rate of *KRAS* in CRC of the patient population in the current study was 42.1% (490/1164). Among the patients evaluated with *KRAS* mutations, codon 12 mutations were detected in 356 patients (72.7%, 356/490) and codon 13 mutations were detected in 108 patients (22.0%, 108/490). The six common mutation sites of codon 12 were G12D 47.5% (169/356), G12V 30.6% (109/356), G12C 8.2% (29/356), G12S 7.0% (25/356), G12A 5.6% (20/356), and G12R 1.1% (4/356). The codon 13 mutations were all G13D. The mutated form was dominated by single point mutations, which accounted for 94.7% (464/490) of the mutants and the composite site mutations accounted for only 5.3% (26/490). *KRAS* mutations in codons 12 and 13 were all missense mutations, but the amino acid base of each single-site mutation varied. We determined that G12S, G12D, and G13D were transitions (G > A) that accounted for 61.6% (302/490) of the changes; while G12C, G12R, G12V, and G12A were transversions (142 G > T, 20 G > C) that accounted for other 33.1% (162/490) changes (**Table 1**).

Relationship between KRAS mutations and clinicopathological characteristics of colorectal cancer

The mutation rate of *KRAS* was different between genders, primary tumor sites, tumor histology types, and preoperative serum tumor marker levels (CEA and CA19-9). *KRAS* mutations were significantly more common in female CRC patients than that in male patients (44.5% vs 38.1%, $P < 0.05$). Compared with that of the left-sided colon, the right colon presented with a significantly greater number of *KRAS* mutations (47.2% vs 35.5%, $P < 0.05$). The mutation rate of *KRAS* in CRC patients with elevated serum CA19-9 levels preoperatively was higher than that in normal patients (48.9% vs 36.7%, $P < 0.05$). Additionally, *KRAS* mutations were also more frequently observed in patients with preoperatively elevated serum CEA levels compared to that in normal patients (46.6% vs 35.0%, $P < 0.05$).

The mutation rate of *KRAS* was different in diverse pathologic types of CRC. Mucinous adenocarcinoma demonstrated the highest rate (52.9%) followed by tubular adenocarcinoma (40.3%) with other types of CRC having the lowest rate (22.2%). The differences among the groups were statistically significant ($P < 0.05$). However, no other significant associations were identified for the remaining clinicopathologic characteristics (**Table 2**). Specifically, the mutation rate for codon 12 in rectal cancer (81.7%) was higher than that in right colon cancer

Table 2. Relationship between *KRAS* mutation and clinicopathologic characteristics of colorectal cancer

Characteristics	*KRAS* status		χ^2	*P*	*KRAS* mutant status		χ^2	*P*
	Wild-type	Mutations			Codon 12	Codon 13		
Total	674	464			356	108		
Gender			4.768	0.029			0.494	0.482
Male	407	250			195	55		
Female	267	214			161	53		
Age (y)			0.004	0.953				
≥ 60	405	278			...	...		
< 60	269	186			...	...		
Size of tumor			0.049	0.825				
≥ 5 cm	260	182			...	...		
< 5 cm	414	282			...	...		
Tumor site			8.082	0.018			6.425	0.040
Right colon	134	120			86	34		
Left colon	207	114			82	32		
Rectum	333	230			188	42		
Pathological type			3.729	0.155				
Protrude	227	182			...	...		
Ulcerative	422	265			...	...		
Infiltrative	25	17			...	...		
Histological type			12.806	0.002			0.468	0.791
Tubular adenocarcinoma	590	399			304	95		
Mucinous adenocarcinoma	49	55			44	11		
Others	35	10			8	2		
pT stage			0.338	0.561				
pT1-2	99	74			...	...		
pT3-4	575	390			...	...		
pN stage			0.109	0.741				
pN0	355	249			...	...		
pN1-2	319	215			...	...		
pM stage			0.375	0.540				
pM0	598	417			...	...		
pM1	76	47			...	...		
Disease stage			0.390	0.823				
I + II	335	232			...	...		
III	263	185			...	...		
IV	76	47					0.585	0.444
CEA (ng/ml)			15.674	< 0.001	150	50		
< 5	371	200			206	58		
≥ 5	303	264						
CA19-9 (U/ml)			15.494	< 0.001			1.175	0.278
< 27	482	280			210	70		
≥ 27	192	184			146	38		

CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9.

(71.7%) and left colon cancer (71.9%), whereas the mutation rate of codon 13 in right colon cancer (28.3%) and left colon cancer (28.1%) was higher than that in rectal cancer (18.3%).

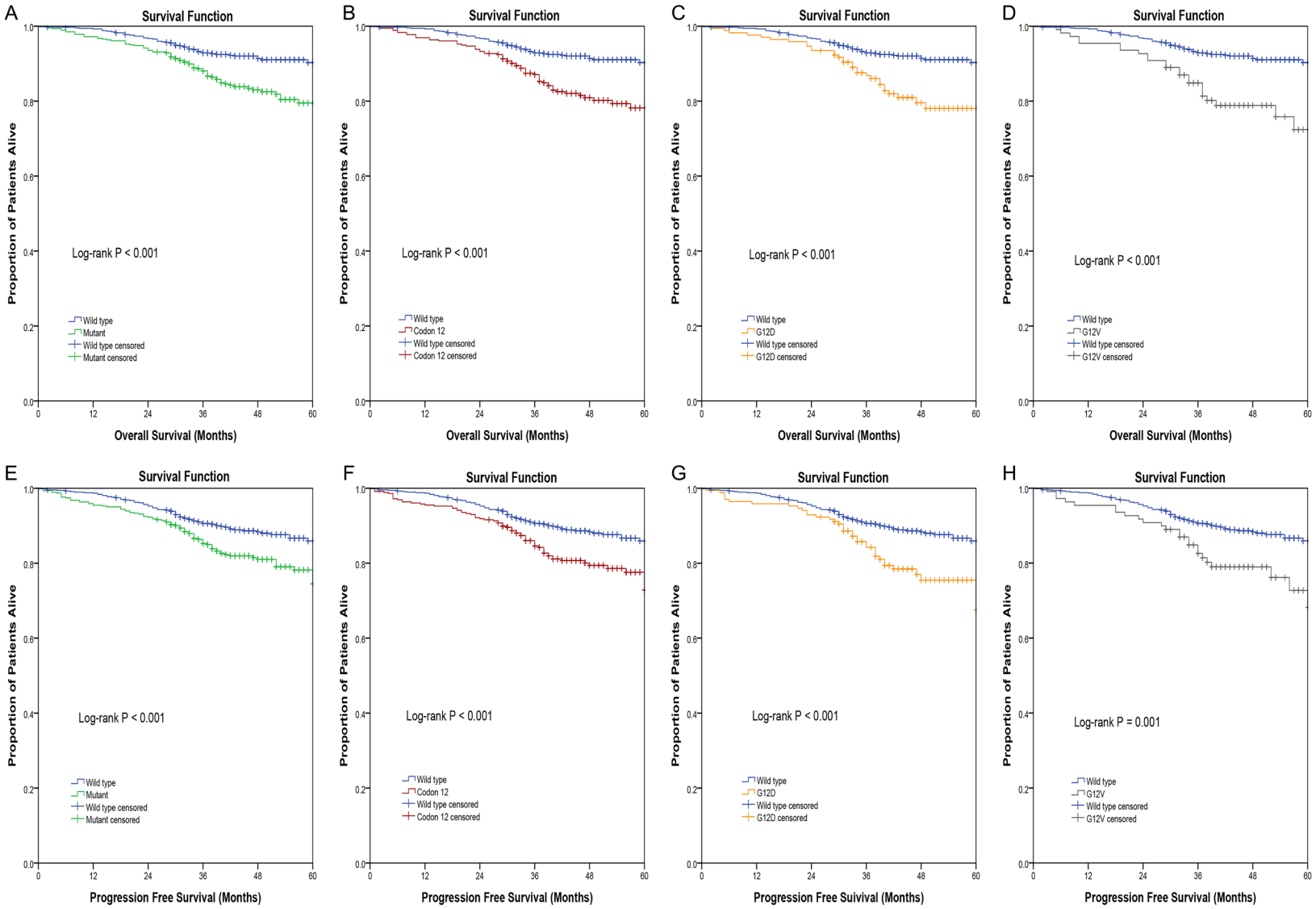

Figure 2. Kaplan-Meier curves of colorectal cancer patients according to *KRAS* mutation status. Overall survival in months according to (A) *KRAS* mutation status, (B) *KRAS* codon 12 mutation status, (C) *KRAS* G12D mutation status, (D) *KRAS* G12V mutation status. Progression free survival in months according to (E) *KRAS* mutation status, (F) *KRAS* codon 12 mutation status, (G) *KRAS* G12D mutation status, (H) *KRAS* G12V mutation status. Log rank test was used to compare the survival distributions. $P < 0.05$ was considered significant.

The mutation rate for codon 12 was not significantly different between right colon cancer and left colon cancer (P > 0.05), as was codon 13 mutation (**Table 2**).

Overall survival

As a whole, the 5-year OS among patients with a mutated *KRAS* was 79.5%, compared with that of 90.3% for patients with a wild-type *KRAS* (P < 0.001). Patients with *KRAS* mutations had worse OS compared with that of patients with wild-type *KRAS*. Of note, the 5-year OS of patients with mutations in *KRAS* codon 12 and 13 were 78.2% and 83.0%, respectively, compared with 90.3% for that of patients who had a wild-type *KRAS*. (**Figure 2A**) With both log-rank Kaplan-Meier analysis (P < 0.001) and Cox regression univariate analysis (HR = 2.495, 95% CI: 1.741-3.575, P < 0.001) and multivariate analysis (HR = 2.846, 95% CI: 1.967-4.118, P < 0.001) patients with *KRAS* codon 12 mutations had a worse OS relative to that of patients with wild-type *KRAS* (**Figure 2B**; **Table 3**). In contrast, mutations in *KRAS* codon 13 were not associated with a worse prognosis compared with that of wild-type *KRAS* (P > 0.05).

On further analysis of the seven most common mutations in *KRAS* codons 12 and 13, the G12D and G12V mutations were at the site most associated with worsened long-term prognosis. Of note, the risk of death in patients with mutations G12D (HR = 2.802, 95% CI: 1.793-4.381, P < 0.001) and G12V (HR = 3.698, 95% CI: 2.269-6.027, P < 0.001) were 2.802 and 3.698 times that of patients with a wild-type *KRAS* (**Figure 2C** and **2D**; **Table 3**). There was no significant difference in the prognosis between patients with *KRAS* mutations at other sites and those with wild-type *KRAS* (P > 0.05).

Progression free survival

The 5-year PFS among patients with mutated *KRAS* was 74.5%, compared with 85.9% for that of patients with wild-type *KRAS* (P < 0.001). Patients with *KRAS* mutations had worse PFS relative to that of patients with wild-type *KRAS* (**Figure 2E**). Specifically, the 5-year PFS of patients with mutations in *KRAS* codons 12 and 13 were 72.8% and 79.7%, respectively, compared with 85.9% for patients who had a wild-type *KRAS*. According to Kaplan-Meier analysis (log-rank, P < 0.001) and both Cox regression univariate analysis (HR = 1.867, 95% CI: 1.349-2.584, P < 0.001) and multivariate analysis (HR = 2.011, 95% CI: 1.450-2.789, P < 0.001), patients with *KRAS* codon 12 mutations had a worse PFS compared with that of patients with wild-type *KRAS* (**Figure 2F**; **Table 3**). In contrast, mutations in *KRAS* codon 13 were not associated with progression of disease compared with that of patients with a wild-type *KRAS* (P > 0.05).

Further analysis of the seven most common mutations in *KRAS* codons 12 and 13 revealed that the G12D and G12V mutations were the site of mutations most associated with progression of disease. Specifically, the risk of disease progression in patients with mutations G12D (HR = 2.079, 95% CI: 1.396-3.099, P < 0.001) and G12V (HR = 2.408, 95% CI: 1.517-3.822, P < 0.001) were 2.079 and 2.408 times, respectively, that of patients with wild-type *KRAS* (**Figure 2G** and **2H**; **Table 4**). There was no significant difference in the progression of disease between patients with *KRAS* mutations at other sites and those with wild-type *KRAS* (P > 0.05).

Discussion

The development of CRC is a complex process regulated by several genes. Abnormal signaling of pathways caused by gene mutation are involved in the process that leads to the dysregulation of intestinal epithelial cell proliferation, differentiation, and apoptosis. It has been confirmed that mutations in *KRAS* are key molecular events in the development of CRC with about 30%-45% of patients with CRC having a *KRAS* mutation [14-18]. However, there are many mutation sites in *KRAS* and the biological effects caused by mutations at the different sites are still controversial. To date, more than 3,000 *KRAS* mutation sites have been reported [19] with the most common mutations being mainly concentrated in codons 12 and 13 of exon 2. Among the 1164 Chinese patients with CRC in the current study, the total mutation rate of *KRAS* was 42.1%, which was consistent with most reports. All mutation forms were missense mutations with the base change mainly being G > A. Among the seven most common mutation sites in *KRAS*, the mutation

Table 3. Univariate and multivariate Cox proportional hazard analysis for overall survival and progression free survival

Prognostic Factor	Overall Survival				Progression Free Survival			
	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Gender								
Female	1 (Referent)				1 (Referent)			
Male	1.109 (0.785-1.566)	0.558	...		1.051 (0.770-1.434)	0.755	...	
Age								
< 60 y	1 (Referent)		1 (Referent)		1 (Referent)			
≥ 60 y	1.636 (1.130-2.370)	0.009	1.591 (1.095-2.313)	0.015	1.593 (1.142-2.222)	0.006	1.567 (1.122-2.188)	0.008
Tumor size								
< 5 cm	1 (Referent)				1 (Referent)			
≥ 5 cm	0.892 (0.626-1.270)	0.525	...		0.848 (0.614-1.170)	0.314	...	
Tumor site								
Rectum	1 (Referent)				1 (Referent)			
Right colon	1.008 (0.641-1.586)	0.973	...		0.859 (0.564-1.308)	0.479	...	
Left colon	1.378 (0.940-2.022)	0.101	...		1.275 (0.904-1.800)	0.167	...	
Pathological type								
Protrude	1 (Referent)		1 (Referent)		1 (Referent)		1 (Referent)	
Infiltrative	3.516 (1.733-7.131)	< 0.001	2.704 (1.316-5.557)	0.007	3.077 (1.534-6.172)	0.002	2.661 (1.312-5.395)	0.007
Ulcerative	1.747 (1.176-2.595)	0.006	1.617 (1.077-2.427)	0.020	1.956 (1.359-2.815)	< 0.001	1.804 (1.246-2.611)	0.002
Histological type								
Tubular adenocarcinoma	1 (Referent)		1 (Referent)		1 (Referent)			
Mucinous adenocarcinoma	0.800 (0.419-1.528)	0.499	0.734 (0.382-1.413)	0.355	0.773 (0.429-1.395)	0.393	...	
Others	2.009 (1.052-3.835)	0.034	2.225 (1.141-4.342)	0.019	1.787 (0.968-3.302)	0.064	...	
T stage								
T1/T2	1 (Referent)				1 (Referent)			
T3/T4	2.387 (1.253-4.549)	0.008	...		2.251 (1.277-3.968)	0.005	...	
N stage								
N0	1 (Referent)		1 (Referent)		1 (Referent)		1 (Referent)	
N1-2	2.584 (1.793-3.723)	< 0.001	2.089 (1.432-3.047)	< 0.001	2.173 (1.578-2.994)	< 0.001	1.822 (1.310-2.532)	< 0.001
M stage								
M0	1 (Referent)		1 (Referent)		1 (Referent)		1 (Referent)	
M1	2.565 (1.706-3.857)	< 0.001	2.114 (1.394-3.206)	< 0.001	2.283 (1.557-3.348)	< 0.001	1.905 (1.289-2.817)	0.001
KRAS Status								
Wild type	1 (Referent)		1 (Referent)		1 (Referent)		1 (Referent)	
All codon 12 mutants	2.495 (1.741-3.575)	< 0.001	2.846 (1.967-4.118)	< 0.001	1.867 (1.349-2.584)	< 0.001	2.011 (1.450-2.789)	< 0.001
All codon 13 mutants	1.537 (0.839-2.817)	0.164	1.776 (0.961-3.281)	0.067	1.450 (0.857-2.453)	0.166	1.564 (0.921-2.654)	0.098
CEA								
< 5 ng/ml	1 (Referent)				1 (Referent)			
≥ 5 ng/ml	2.079 (1.452-2.976)	< 0.001	...		1.795 (1.307-2.464)	< 0.001	...	
CA19-9								
< 27 U/ml	1 (Referent)				1 (Referent)			
≥ 27 U/ml	1.341 (0.948-1.896)	0.097	...		1.247 (0.909-1.710)	0.171	...	

CEA, carcinoembryonic antigen; CA 19-9, carbohydrate antigen 19-9; HR, hazard ratio; 95% CI, 95% confidence interval.

Table 4. Univariate and multivariate Cox proportional hazard analysis for overall survival and progression free survival according to specific *KRAS* mutations

KRAS	Overall Survival				Progression Free Survival			
	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P	Univariate HR (95% CI)	P	Multivariate HR (95% CI)	P
Wild type	1 (Referent)		1 (Referent)		1 (Referent)		1 (Referent)	
G12D	2.566 (1.656-3.975)	< 0.001	2.802 (1.793-4.381)	< 0.001	2.039 (1.371-3.032)	< 0.001	2.079 (1.396-3.099)	< 0.001
G12V	3.010 (1.860-4.870)	< 0.001	3.698 (2.269-6.027)	< 0.001	2.102 (1.328-3.326)	0.002	2.408 (1.517-3.822)	< 0.001
G12C	1.705 (0.617-4.710)	0.304	1.780 (0.638-4.965)	0.270	1.198 (0.438-3.274)	0.725	1.186 (0.432-3.256)	0.740
G12S	1.522 (0.476-4.867)	0.479	1.639 (0.507-5.296)	0.409	1.139 (0.359-3.610)	0.825	1.371 (0.431-4.358)	0.593
G12A	1.367 (0.333-5.609)	0.664	1.834 (0.443-7.587)	0.402	0.951 (0.234-3.873)	0.944	1.164 (0.285-4.760)	0.832
G12R	4.439 (0.614-32.111)	0.140	6.076 (0.823-44.830)	0.077	3.006 (0.418-21.629)	0.274	3.985 (0.547-29.033)	0.172
G13D	1.538 (0.839-2.818)	0.164	1.774 (0.961-3.278)	0.067	1.450 (0.857-2.454)	0.166	1.567 (0.923-2.660)	0.096

HR, hazard ratio; 95% CI, 95% confidence interval.

rate of G12D was the highest (34.5%) followed by G12V (22.3%).

Different relationships between *KRAS* mutations, clinicopathologic characteristics, and the prognosis for CRC have been reported. Although several previous studies have demonstrated differences between the prognostic associations of *KRAS* mutations in codon 12 and 13, results are conflicting and none of the studies on the relationship between specific site mutations, clinicopathologic characteristics, and the prognosis of CRC have been large-scale studies with a sample size more than 300. Therefore, we evaluated specific mutations in *KRAS* and analyzed the relationships between the mutations and the clinicopathologic characteristics and the prognosis of CRC.

We found that the *KRAS* mutation rate in female Chinese CRC patients was higher than that in male patients, suggesting that the RAS signaling pathway may be affected by estrogen, which resulted in a gender-associated difference in the *KRAS* mutation rate. Recently, it was reported that *KRAS* mutations were related to the primary site of the tumor. Both the work from Li et al [20] and our research indicate that *KRAS* mutations are more likely to be found in the proximal colon. After further analysis, we found that the mutation rate for codon 13 in colon cancer was higher than that of rectal cancer, which was consistent with the study of Sylvester et al [21]. In addition, several previous studies have shown that mucinous adenocarcinoma has a worse prognosis than other histological types of CRC. It is also believed that the long-term survival rate of patients with elevated pre-operative serum levels of CEA and CA19-9 is lower than that of patients with normal levels. In our current study, patients with mucinous adenocarcinoma had the highest *KRAS* mutation rate among the different histologic types. In addition, patients with elevated pre-operative levels of serum CEA had higher *KRAS* mutation rates than that of patients without elevated levels. This was also the case for serum CA19-9. Both these findings suggested that *KRAS* may serve as a prognostic indicator.

Similar to previous studies [22-24], our current study indicated that CRC patients with *KRAS* mutations had a significant increased risk of postoperative tumor recurrence and death compared with that of patients with a wild-type KRAS. However, the impact of *KRAS*-specific codons and even specific sites within the gene on the prognosis of CRC patients is still not clear. Specifically, we found that the postoperative long-term survival rate of patients with a *KRAS* codon 12 mutation was significantly lower than that of patients with wild-type *KRAS*. In contrast, patients with a mutation in codon 13 of *KRAS* and patients with a wild-type *KRAS* showed no significant differences, which held true for recurrence as well. According to the results from cell experiments by Guerrero et al [25], codon 12 mutations increase the activation of the AKT and B-cell lymphoma 2 (bcl-2) pathways and decrease the activity of the Ras homolog gene family, member A (RhoA) pathway, thereby increasing the threshold of apoptosis. Moreover, codon 12 mutations have biological properties, such as anchorage-independent growth and cell-cell contact deregulation. These findings suggest that while they were both *KRAS* mutations, codon 12 and 13 mutations represent different tumor clones

and cannot be generalized. *KRAS* codon 12 mutations conferred a more aggressive tumor phenotype compared to that of the codon 13 mutations. Thus, we may be able to determine the prognosis of CRC patients by evaluating the mutation subtype in *KRAS* and develop different treatment strategies according to the particular genetic status.

Notably, of the seven most common *KRAS* codon 12 and codon 13 mutations, mutations G12D and G12V were independent risk factors for poor prognosis and disease progression in CRC patients. This suggested that even though they were both located in codon 12, the mechanism for of the distinctive mutation sites affecting the biological behavior of tumors was different. It is well known that RAS protein expressed by *KRAS* has GTPase activity, but this hydrolase activity is extremely weak; thus, the stimulation of GTPase activating proteins (GAPs) is required to catalyze the process to promote the hydrolysis of GTP. Normally, the binding of RAS to GDP or GTP is in dynamic equilibrium, which allows the functional signals of RAS that regulate normal growth to be transmitted and correctly interpreted. The mutation of codon 12 may cause different degrees of damage to the balance. When the mutation is G12D, the glycine located near the catalytic site of the RAS protein is replaced with aspartic acid, which may attenuate the intrinsic GTPase activity of RAS and may also impair the stability of RAS binding with GAPs, thereby causing elevated levels of RAS-GTP in cells. Eventually, a sustained mitogenic signal for cell growth is delivered. Hunter et al [26] confirmed that the intrinsic hydrolysis rate of G12D is much smaller than that of wild-type *KRAS*. Even under the stimulation of GAPs, the hydrolysis rate of G12D does not obviously increase, while wild-type *KRAS* is able to increase by several times. Therefore, the G12D mutation not only reduces its own hydrolase activity, but also reduces its sensitivity to GAPs, thereby activating signaling pathways and promoting tumor proliferation.

The effect of the G12V mutation on the RAS signaling pathway is not the same as that of G12D. The G12V mutation is able to cause a decrease in its own GTPase activity and a decrease also in the affinity for the GTPase activating protein, thereby preventing the activation of the GTPase and causing an increase in the level of RAS-GTP in cells [27, 28]. A recent study using an *in vitro* cell assay [29] indicated that primary tumors with the G12V mutation display significantly fewer apoptotic cells than other subtypes and the number of tumor buds was significantly higher than that of other subtypes. Furthermore, the G12V mutation is able to enhance the activity of primary tumor cells and leads to overexpression of proteins such as C-X-C chemokine receptor type 4 (CXCR4) and vascular endothelial growth factor A (VEGFA) and the abnormal activation of the AKT signaling pathway. Ultimately, the G12V mutation destroys the natural apoptosis process in tumor cells. Thus, whether it was a G12D mutation or a G12V mutation in CRC, the tumor phenotype was more aggressive than that of other subtypes. This also demonstrated that different mutations, even in a single gene, may shape distinctive biologic behaviors, which further emphasizes the importance of tumor heterogeneity in the diagnosis and treatment of cancer.

Despite these positive findings, there were several limitations to the present study. First, because it was a retrospective study, the sample population was limited, which may have introduced some selection bias. Second, although patients who received neoadjuvant therapy or anti-epidermal growth factor receptor agents were excluded from the study, the unknown remote use of those agents cannot be absolutely excluded. Finally, we did not investigate the less common mutations in *KRAS* codons 61 and 146; these should be evaluated in future studies.

In conclusion, among the seven most common mutations in *KRAS*, G12D, G12V, and G13D mutations were the most prevalent. Not all mutations of KRAS predict poor prognosis in patients with CRC. Only G12D and G12V mutations in codon 12 of *KRAS* were independent prognostic factors of worse OS and PFS for CRC patients. The determination of specific mutations in *KRAS* may help clinicians develop personalized treatment plans and follow-up strategies for patients with CRC and may even provide a reference for molecular typing of CRC.

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Disclosure of conflict of interest

None.

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References

[1] Khosravi-Far R and Der CJ. The ras signal transduction pathway. Cancer Metastasis Rev 1994; 13: 67-89.

[2] Fearon ER and Vogelstein B. A genetic model for colorectal tumorigenesis. Cell 1990; 61: 759-767.

[3] McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, Salisbury JL, Mazzarino MC, Stivala F and Libra M. Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul 2006; 46: 249-279.

[4] Downward J. Ras signalling and apoptosis. Curr Opin Genet Dev 1998; 8: 49-54.

[5] Zlobec I, Bihl MP, Schwarb H, Terracciano L and Lugli A. Clinicopathological and protein characterization of BRAF- and K-RAS-mutated colorectal cancer and implications for prognosis. Int J Cancer 2010; 127: 367-380.

[6] Fariña-Sarasqueta A, van Lijnschoten G, Moerland E, Creemers GJ, Lemmens VE, Rutten HJ, van den Brule AJ. The BRAF V600E mutation is an independent prognostic factor for survival in stage II and stage III colon cancer patients. Ann Oncol 2010; 21: 2396-2402.

[7] Richman SD, Seymour MT, Chambers P, Elliott F, Daly CL, Meade AM, Taylor G, Barrett JH and Quirke P. KRAS and BRAF mutations in advanced colorectal cancer are associated with poor prognosis but do not preclude benefit from oxaliplatin or irinotecan: results from the MRC FOCUS trial. J Clin Oncol 2009; 27: 5931-5937.

[8] Samowitz WS, Curtin K, Wolff RK, Tripp SR, Caan BJ and Slattery ML. Microsatellite instability and survival in rectal cancer. Cancer Causes Control 2009; 20: 1763-1768.

[9] Barault L, Charon-Barra C, Jooste V, de la Vega MF, Martin L, Roignot P, Rat P, Bouvier AM, Laurent-Puig P, Faivre J, Chapusot C and Piard F. Hypermethylator phenotype in sporadic colon cancer: study on a population-based series of 582 cases. Cancer Res 2008; 68: 8541-8546.

[10] Ogino S, Meyerhardt JA, Irahara N, Niedzwiecki D, Hollis D, Saltz LB, Mayer RJ, Schaefer P, Whittom R, Hantel A, Benson AB 3rd, Goldberg RM, Bertagnolli MM, Fuchs CS; Cancer and Leukemia Group B; North Central Cancer Treatment Group; Canadian Cancer Society Research Institute; Southwest Oncology Group. KRAS mutation in stage III colon cancer and clinical outcome following intergroup trial CALGB 89803. Clin Cancer Res 2009; 15: 7322-7329.

[11] Chen J, Ye Y, Sun H and Shi G. Association between KRAS codon 13 mutations and clinical response to anti-EGFR treatment in patients with metastatic colorectal cancer: results from a meta-analysis. Cancer Chemother Pharmacol 2013; 71: 265-272.

[12] Peeters M, Douillard JY, Van Cutsem E, Siena S, Zhang K, Williams R and Wiezorek J. Mutant KRAS codon 12 and 13 alleles in patients with metastatic colorectal cancer: assessment as prognostic and predictive biomarkers of response to panitumumab. J Clin Oncol 2013; 31: 759-765.

[13] Tejpar S, Celik I, Schlichting M, Sartorius U, Bokemeyer C and Van Cutsem E. Association of KRAS G13D tumor mutations with outcome in patients with metastatic colorectal cancer treated with first-line chemotherapy with or without cetuximab. J Clin Oncol 2012; 30: 3570-3577.

[14] Brink M, de Goeij AF, Weijenberg MP, Roemen GM, Lentjes MH, Pachen MM, Smits KM, de Bruine AP, Goldbohm RA and van den Brandt PA. K-ras oncogene mutations in sporadic colorectal cancer in the netherlands cohort study. Carcinogenesis 2003; 24: 703-710.

[15] Karapetis CS, Khambata-Ford S, Jonker DJ, O'Callaghan CJ, Tu D, Tebbutt NC, Simes RJ, Chalchal H, Shapiro JD, Robitaille S, Price TJ, Shepherd L, Au HJ, Langer C, Moore MJ and Zalcberg JR. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008; 359: 1757-1765.

[16] Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, Patterson SD and Chang DD. Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 2008; 26: 1626-1634.

[17] Sundstrom M, Edlund K, Lindell M, Glimelius B, Birgisson H, Micke P and Botling J. Kras analysis in colorectal carcinoma: analytical aspects of pyrosequencing and allele-specific PCR in clinical practice. BMC Cancer 2010; 10: 660.

[18] Gao J, Li YY, Sun PN and Shen L. Comparative analysis of dideoxy sequencing, the KRAS StripAssay and pyrosequencing for detection of KRAS mutation. World J Gastroenterol 2010; 16: 4858-4864.

[19] Lea IA, Jackson MA, Li X, Bailey S, Peddada SD and Dunnick JK. Genetic pathways and mutation profiles of human cancers: site- and exposure-specific patterns. Carcinogenesis 2007; 28: 1851-1858.

[20] Li W, Qiu T, Zhi W, Shi S, Zou S, Ling Y, Shan L, Ying J and Lu N. Colorectal carcinomas with KRAS codon 12 mutation are associated with more advanced tumor stages. BMC Cancer 2015; 15: 340.

[21] Sylvester BE, Huo D, Khramtsov A, Zhang J, Smalling RV, Olugbile S, Polite BN and Olopade OI. Molecular analysis of colorectal tumors within a diverse patient cohort at a single institution. Clin Cancer Res 2012; 18: 350-359.

[22] Andreyev HJ, Norman AR, Cunningham D, Oates JR and Clarke PA. Kirsten ras mutations in patients with colorectal cancer: the multicenter "RASCAL" study. J Natl Cancer Inst 1998; 90: 675-684.

[23] Conlin A, Smith G, Carey FA, Wolf CR and Steele RJ. The prognostic significance of K-ras, p53, and APC mutations in colorectal carcinoma. Gut 2005; 54: 1283-1286.

[24] Kadowaki S, Kakuta M, Takahashi S, Takahashi A, Arai Y, Nishimura Y, Yatsuoka T, Ooki A, Yamaguchi K, Matsuo K, Muro K and Akagi K. Prognostic value of KRAS and BRAF mutations in curatively resected colorectal cancer. World J Gastroenterol 2015; 21: 1275-1283.

[25] Guerrero S, Casanova I, Farre L, Mazo A, Capella G and Mangues R. K-ras codon 12 mutation induces higher level of resistance to apoptosis and predisposition to anchorage-independent growth than codon 13 mutation or proto-oncogene overexpression. Cancer Res 2000; 60: 6750-6756.

[26] Hunter JC, Manandhar A, Carrasco MA, Gurbani D, Gondi S and Westover KD. Biochemical and structural analysis of common cancer-associated KRAS mutations. Mol Cancer Res 2015; 13: 1325-1335.

[27] Al-Mulla F, Milner-White EJ, Going JJ and Birnie GD. Structural differences between valine-12 and aspartate-12 ras proteins may modify carcinoma aggression. J Pathol 1999; 187: 433-438.

[28] Bollag G and McCormick F. Intrinsic and GTPase-activating protein-stimulated ras GTPase assays. Methods Enzymol 1995; 255: 161-170.

[29] Alamo P, Gallardo A, Di Nicolantonio F, Pavon MA, Casanova I, Trias M, Mangues MA, Lopez-Pousa A, Villaverde A, Vazquez E, Bardelli A, Cespedes MV and Mangues R. Higher metastatic efficiency of KRas G12V than KRas G13D in a colorectal cancer model. FASEB J 2015; 29: 464-476.