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Original Article

Prognosis and clinical characteristics of colorectal cancer patients with KRAS gene mutation: a 5-year follow-up study

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Abstract: To investigate the correlation of KRAS gene mutation and surgery for clinical characteristics and prognosis in patients with colorectal cancer under a 5-year follow-up, clinical data of 277 patients with colorectal cancer treated from January 2010 to December 2010 were collected. Patients with KRAS gene mutation were detected by direct sequencing and followed up for 5 years. KRAS gene mutation effect on clinicopathological factors and univariate and multivariate survival were analyzed. Among 277 patients, 109 (39.8%) indicated KRAS gene exon 2 mutation, among which 12 colon mutations showed the highest frequency. Furthermore, KRAS gene mutation was revealed to have a correlation with the expression of EGFR, primary tumor site, and multiple metastases of tumor. In follow-up, the average survival time of patients carrying wild-type and mutant-type was 49.9 months and 50.7 months, respectively. Univariate analysis showed that high TNM stage, advanced age, pulmonary metastasis, hepatic metastasis, and multiple metastases were associated with poor postoperative prognosis in patients with colorectal cancer. Multivariate analysis showed that advanced age and high TNM stage were independent risk factors for postoperative outcome in patients with colorectal cancer. The median OS in IV patients after surgery still reached 39.6 months, and some patients survived. In conclusion, KRAS gene mutation in colorectal cancer patients is closely related to EGFR expression, primary tumor site and multiple metastasis, and the survival time of advanced patients is prolonged.

Keywords: KRAS gene, colorectal cancer, clinical feature, postoperative prognosis

Introduction

Carcinoma of colon and rectum is the common malignant tumor in gastrointestinal tract, ranking the third and the second regarding the incidence rate of malignant tumors and the death rate in the world, respectively, and the annual increase of new cases is about 1,200,000. Its incidence and mortality are second only to gastric cancer, esophageal cancer, and primary liver cancer. In recent years, as people changed eating habits, the incidence and mortality of colorectal cancer in China also showed a rapid upward trend. In China, colorectal cancer now ranks the third with the highest mortality rate [1].

Some studies have reported that colorectal cancer may be related to the activation of EGFR signaling. KRAS is a proto-oncogene acting as an important molecule in the downstream signaling pathway of EGFR [2]. The EGFR signaling pathway plays an important role in the development of colorectal cancer.KRAS mutations continue to activate the EGFR signaling pathway [3-5]. However, there are few studies focused on the influence of KRAS gene mutation combined with tumor resection in other pathological factors and prognosis of colorectal cancer patients. The effect of Kras on the prognosis of patients is in consistent. With respect to the above, our hospital conducted a clinical study for 5 years since 2010, to analyze the relationship between KRAS gene mutation and other pathological factors and postoperative prognosis in 277 colorectal cancer patients, to provide a basis for clinical treatment of patients with colorectal cancer after operation.

Table 1. Clinical characteristics of Kras gene mutation in patients with colorectal cancer

Clinical finding	n	Kras genotypes		*P* value
		Wild-type	Mutant-type	
Gender				
Male	182	114	68	
Female	95	54	41	0.408
Age				
≥ 60 years	167	99	68	
< 60 years	110	69	41	0.489
EGFR				
0-+	201	127	72	
++-+++	63	27	36	0.003
P53				
0-+	125	74	51	
++-+++	139	82	57	0.973
Tumor sites				
Right colon	66	29	37	
Left colon	68	43	25	0.008
Rectum	134	89	45	
Differentiation degree				
Poor	30	19	11	
Moderate-high	228	137	91	0.773
Tumor stage				
I	41	27	14	
II	95	55	40	
III	94	60	34	0.714
IV	47	26	21	
Hepatic metastasis				
Hepatic metastasis	14	7	7	0.740
Others	33	15	18	
Without metastasis	230	144	86	
Pulmonary metastasis				
Pulmonary metastasis	5	3	2	
Others	42	19	23	0.315
Without metastasis	230	144	86	
Multiple metastasis				
Multiple metastasis	17	5	12	
Others	30	17	13	0.022
Without metastasis	230	144	86	
Lymph node metastasis				
0-3	229	137	92	
> 3	48	31	17	0.483

Materials and methods

Patients selection

During January 2010 to December 2010, there were 277 patients with colorectal cancer who underwent surgical resection in our department of gastrointestinal surgery. Surgical procedures included traditional laparotomy and laparoscopic assisted radical surgery. Only cancer tissues ($3 \times 3 \times 5$ mm^3) on the original site were collected in the included colorectal cancer patients and avoided any focus of necrosis in the cancer center. Specimens were fixed, embedded in paraffin; they were histologically diagnosed by two pathological experts from the pathology department of our hospital, and the routine pathological examination was used to identify the types of diagnosis and staging. International Union Against Cancer (UICC)/American Joint Committee on Cancer (AJCC) TNM staging modified in 2003 were applied for colorectal cancer staging. The study was approved by the ethics committee of Guangdong General Hospital, Guangzhou, China. Written informed consent was obtained from all patients.

EGFR immunohistochemistry (IHC)

Immunohistochemical PV9000 two step method was used for IHC. Conventional paraffin specimens of 4 μm thick slices were immersed in xylene for 15 min, each concentration of ethanol (100%, 85% and 75%) for 5 min, then the slides were dewaxing and incubated with 3% H_2O_2 for 25 min, and then blocked with 3% bovine serum albumin (BSA) for 30 min at 37°C. The slides were incubated with primary antibody EGFR overnight at 4°C. The slides were washed with PBS and incubated with HRP-conjugated secondary antibodies (1:5000; Abcam) for 60 min. After washing in PBS for 5 min, slides were incubated with fresh DAB Chromogen Solution for 10 min and nuclear counterstained with hematoxylin for 5 min. Images were captured under a microscope (Olympus, Tokyo, Japan). Each batch was set up with positive pictures, and PBS was used as negative control. EGFR was expressed in the cell membrane, and cytoplasm also had a little coloring. The results of immunohistochemistry were scored: (-); the

Table 2. Clinical characteristics of Kras gene mutation in patients with rectal cancer

Clinical finding	n	Kras genotypes		*P* value
		Wild-type	Mutant-type	
Gender				
Male	92	61	31	
Female	38	24	14	0.441
Age				
≥ 60 years	51	35	16	
< 60 years	79	50	29	0.333
EGFR				
0 - +	104	73	31	
++ - +++	26	12	14	0.020
P53				
0 - +	66	43	23	
++ - +++	64	42	22	0.551
Differentiation degree				
Poor	20	13	7	
Moderate-high	110	72	38	0.578
Tumor stage				
I	22	16	6	
II	35	24	11	
III	51	31	20	0.738
IV	21	13	8	
Hepatic metastasis				
Hepatic metastasis	5	3	2	0.792
Others	17	10	7	
Without metastasis	108	72	36	
Pulmonary metastasis				
Pulmonary metastasis	4	3	1	
Others	18	10	8	0.603
Without metastasis	108	72	36	
Multiple metastasis				
Multiple metastasis	9	2	7	
Others	15	11	4	0.017
Without metastasis	106	72	34	
Lymph node metastasis				
0-3	103	69	34	
> 3	26	15	11	0.374

number of positive cells was less than 10%, light yellow as (+); 10% ~ 50%, pale brown as (+ +); > 50%, brown as (+ ++).

DNA extraction, amplification and sequencing

In this study, the KRAS gene mutation detection method using the currently accepted direct sequencing [6]. Goelz extraction method was used to extract DNA from paraffin embedded tissue by DNA template. The design and synthesis of primers: according to the NCBI gene database, we designed primers according to the detected loci.

The upstream primer sequence was 5'-GGCCTGCTGAAAATGACTGA-3' and the downstream primer sequence was 'GTCCTGCACCAGTAATATGC-3'. In the set of the PCR instrument for amplification reaction: pre denaturation of 90°C, for about 5 minutes; denaturation of the temperature of 90°C, for about 40 seconds; annealing at a temperature of 50°C, for a period of 40 seconds; extending to a temperature of 70°C for about 40 seconds; a total of 30 cycles; an extension of a temperature of 70°C and a minute; a preservation of a temperature of 4°C. The PCR amplification products were observed for 45 minutes after 80 V electrophoresis. The results were observed under the gel imaging system. The PCR amplification products were purified and sequenced using pyrosequencing.

Statistical analysis

Statistical analysis in the present study was performed using SPSS20.0 software. By applying a Chi-square test, the relationship between the genetic variation of KRAS gene and clinicopathological features in colorectal cancer patients who underwent surgical excision were then compared (such as gender, age, TNM stage, tumor differentiation, metastasis, primary tumor site, lymph node metastasis). Kaplan-Meier survival curve was used to evaluate the relationship between clinicopathological features and survival outcomes in different patients with colorectal cancer. Significance test used Log-rank test analysis. Multivariate survival analysis was further conducted with Cox regression model, to evaluate the effect of various factors on prognosis of colorectal cancer. $P < 0.05$ was defined as significant.

Results

General information

There was a total of 277 cases of patients underwent surgical treatment in the present

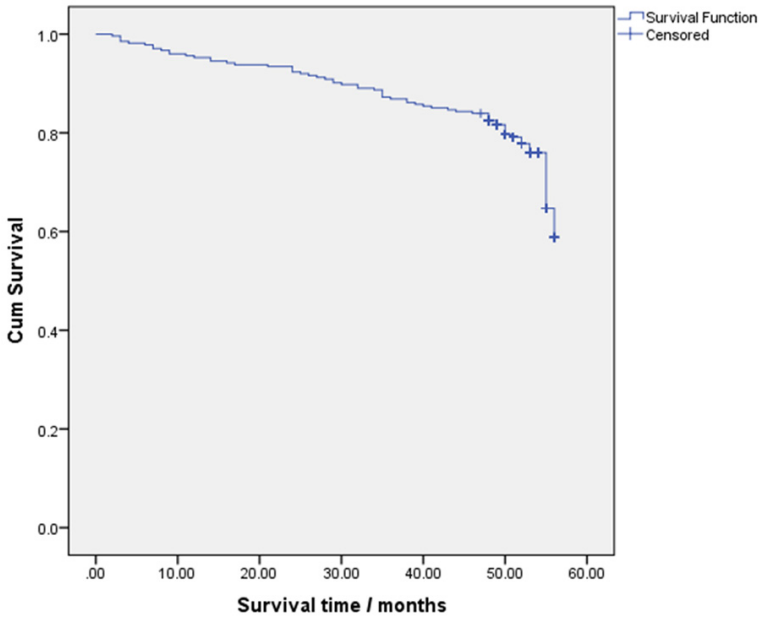

Figure 1. Overall survival curve of 277 colorectal cancer patients.

study (male: 182, female: 95), with the median age of 62 years old (ranging from 26 to 85 years old). The follow-up period lasted for 5 years, and the 5-year follow-up rate was 88.8%. There were no statistical differences regarding gender, age and other baseline data in patients grouped according to different KRAS genotypes (P > 0.05), as shown in **Table 1**. Patients were divided into two groups based on the expression level of EGFR, there were 201 cases and 63 cases in grade EGFR 0-+ and grade EGFR++-+++, respectively; in view of the results of P53 expressions, 12 cases were classified in grade P53 0-+ and 139 cases in grade P53 ++-+++; patients were grouped into three groups, with 30 cases in the poor differentiation group, and 228 cases in moderate-high differentiation group; meanwhile, according to the criteria of TNM staging after operation, there were 41 patients of stage I, 95 patients of stage II, 94 patients of stage III, and 47 patients of stage IV; patients were divided into three groups based on hepatic metastasis, there were 14 cases found to have hepatic metastasis, 33 cases with other metastasis types and 230 cases showed no metastasis; on the basis of pulmonary metastasis, 5 patients were shown to have pulmonary metastasis, 42 patients indicated other types of metastasis, and 230 patients revealed no metastasis; on the basis of multiple metastases, 17 patients were shown to have multiple metastasis, 30 patients had other types of metastasis, and 230 patients revealed no metastasis; in light of lymph node metastasis, there were 48 cases with over three lymph node metastases, and 229 cases with 0-3 lymph node metastasis. The details are shown in **Table 1**.

Genotyping

KRAS had point mutations; about 90% mutation loci of KRAS gene located at codon 12 and codon 13 of exon 2. As for the incorporated 277 patients with colorectal cancer, there were 109 patients showed KRAS gene mutation, and the mutation rate was 39.8%, among which the frequency of gene mutation was 92.7% and 6.3% at codon 12 and codon 13, respectively. Co-mutation frequency at codon 12 and codon 13 was 1.0%.

Relationship between genetic mutation of KRAS gene and clinical pathological features in patients with colorectal cancer

There were significant correlations of KRAS genetic mutation with EGFR expression at the tumor site, primary tumor site in patients with colorectal cancer and multiple metastasis.Due to a high proportion of patients with rectal cancer, we evaluated the positioning of the individual parts of rectal cancer, KRAS gene mutation was revealed to have strong correlation with the expression of EGFR (72 cases of EGFR 0-+ vs 36 cases of EGFR ++-+++, P < 0.05), primary tumor site (37 cases in the right colon vs 25 cases in the left colon vs 45 cases in the rectum, P < 0.05) and multiple metastasis of tumor (12 cases of multiple metastasis vs 13 cases of other types of metastasis vs 84 cases without metastasis, P < 0.05), indicating a statistical difference. However, as shown in the table, there was no statistical difference of KRAS gene mutation with other pathological factors, such as gender, age, clinical tumor markers, TNM stage, differentiation degree,

Table 3. Univariate analysis of prognostic factors in patients with colorectal cancer by Kaplan-Meier

Factors	n	3-year survival rate (%)	5-year survival rate (%)	*Average survival month*	*P* value
Gender					
Male	179	85.5%	60.5%	49.7	
Female	95	90.5%	56.2%	51.2	0.596
Age					
≥ 60 years	107	89.7%	87.5%	51.9	0.002
< 60 years	167	84.4%	48.3%	49.2	
KRAS genotypes					
Wild-type	166	84.9%	60.6%	49.9	0.649
Mutant-type	108	89.8%	58.1%	50.7	
EGFR					
0 - +	199	87.4%	59.5%	50.6	0.349
++ - +++	60	83.3%	44.1%	48.4	
P53					
0 - +	122	89.3%	51.3%	50.8	0.800
++ - +++	137	83.9%	73.2%	49.5	
Tumor sites					
Right colon	65	76.3%	56.3%	48.8	0.428
Left colon & rectum	198	85.4%	61.2%	52.8	
Differentiation degree					
Poor	28	89.3%	42.9%	51.0	0.446
Moderate-high	225	85.8%	58.3%	49.9	
Tumor stage					
I	41	95.0%	92.5%	53.9	0.001
II	95	91.6%	84.0%	51.5	
III	94	88.4%	82.8%	50.3	
IV	47	70.2%	61.7%	39.6	
Hepatic metastasis					
Hepatic metastasis	14	64.3%	57.1%	39.2	
Others	33	75.8%	66.7%	44.8	0.001
Without metastasis	226	90.9%	84.6%	51.2	
Pulmonary metastasis					
Pulmonary metastasis	5	80.0%	60.0%	47.8	0.001
Others	42	69.0%	64.3%	42.2	
Without metastasis	226	91.5%	69.4%	51.2	
Multiple metastasis					
Multiple metastasis	17	71.4%	67.9%	43.7	0.001
Others	30	68.4%	57.9%	42.4	
Without metastasis	226	90.9%	84.6%	51.2	
Lymph node metastasis					
0-3	226	87.2%	70.6%	50.6	0.052
> 3	47	85.1%	29.5%	48.6	

hepatic metastasis, pulmonary metastasis and lymph node metastasis.

Relationship between genetic mutation of KRAS gene and clinicopathological features in patients with rectal cancer

Taking into account the number of patients with rectal cancer in this study, we further investigated the relationship between KRAS gene mutation and clinicopathological factors in rectal cancer patients. As shown in **Table 2**, there were statistical differences that KRAS genetic mutation was indicated to have close relationship with the expression of EGFR in rectal cancer patients (31 cases of EGFR 0-+ vs 14 cases of EGFR +-++, $P < 0.05$) and multiple metastasis (7 cases with multiple metastasis vs 4 cases with other metastasis vs 34 cases without metastasis, $P < 0.05$), but revealed no statistical difference with other pathological factors, including gender, age, clinical tumor markers, TNM stage, differentiation degree, hepatic metastasis, pulmonary metastasis and lymph node metastasis.

Analysis of prognostic factors in colorectal cancer patients

Follow-up results of the 277 cases of patients revealed 179 patients were survived and 31 patients were lost, with a median overall survival (OS) period of 51 months, the 3-year and 5-year survival rate was 86.9% and 58.8%, respectively (**Figure 1**).

Results of univariate analysis of Kaplan-Meier methods (**Table 3**) indicated that the average survival time of patients carrying wild-type and mutant-type was 49.9 months and 50.7 months, respectively, showing no apparent statistical difference ($P > 0.05$; **Figure 2**).

The relationship of other clinicopathologic factors and postoperative prognosis survival were as follows: TNM stage was found to be correlated with postop-

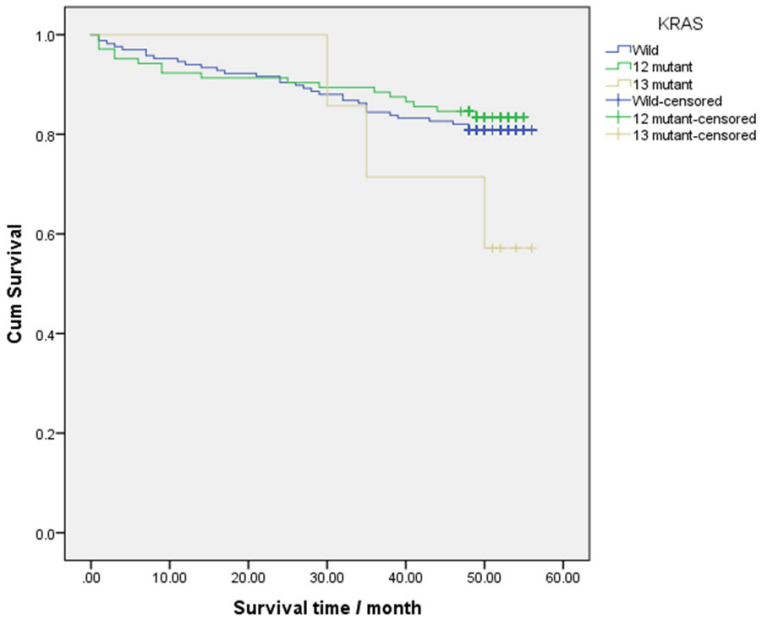

Figure 2. Overall survival curve under different genotypes of KRAS gene.

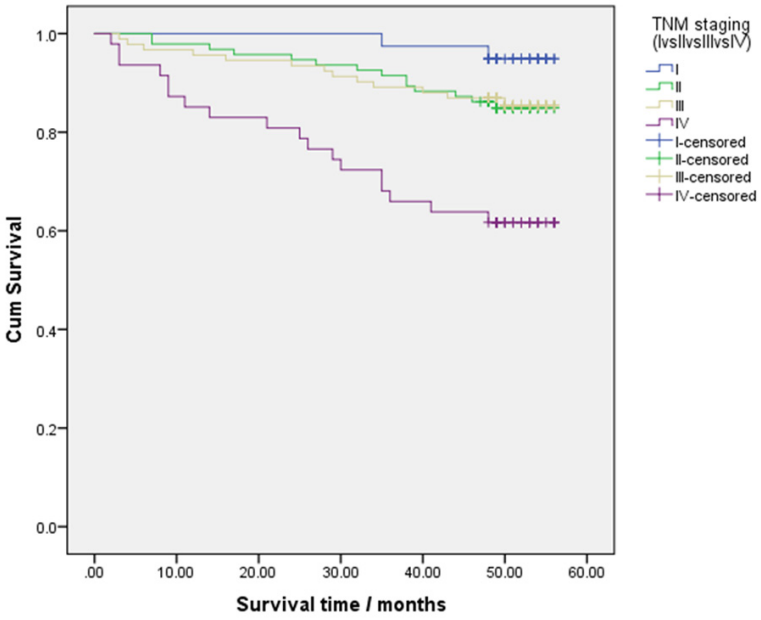

Figure 3. Overall survival curve under different TNM stages.

erative prognosis, the median survival time was 55.2 months, 51.9 months, 50.7 months and 39.6 months in stage I patients, stage II patients, stage III patients and stage IV patients, respectively, indicating statistical differences ($P < 0.05$; **Figure 3**). In addition, there was statistical difference of the mean survival time of patients of various ages (51.9 months in patients with age ≥ 60 years vs 49.2 months in patients with age < 60 years; $P < 0.05$; **Figure 4**). It was observed that metastasis of cancer cells had a significant impact on postoperative prognosis of patients with colorectal cancer; Kaplan-Meier univariate survival analysis results indicated that hepatic metastasis ($P < 0.05$; **Figure 5**), pulmonary metastasis ($P < 0.05$; **Figure 6**) and multiple metastasis ($P < 0.05$; **Figure 7**) were associated with poor postoperative prognosis.

Cox Proportional Hazard Model for multivariate survival analysis (as shown in **Table 4**) indicated that advanced age and high TNM stage were independent risk factors influencing the prognosis ($P = 0.017$, $P = 0.005$). Compared with other patients (< 60 years), advanced patients (≥ 60 years) with colorectal cancer had relatively poor prognostic outcomes. The relative risk of death in stage I-III patients was 0.351 relative to stage IV patients, which meant that the risk of death was lower by 64.9% in patients with stage I-III.

Discussion

KRAS gene is suggested to be correlated with intracellular signal transduction. More importantly, KRAS is the major transduction pathway in the EGFR signaling pathway. KRAS gene mutation can induce the proliferation, angiogenesis, invasion, and metastasis of tumor cells, and abrogate the normal regulation of the EGFR signaling pathway. Mutation of KRAS gene affects the effect of EGFR inhibitor. Previous evidence has shown that the mutation rate of KRAS gene was 20-60% in colorectal cancer [7]. Some researchers believe that

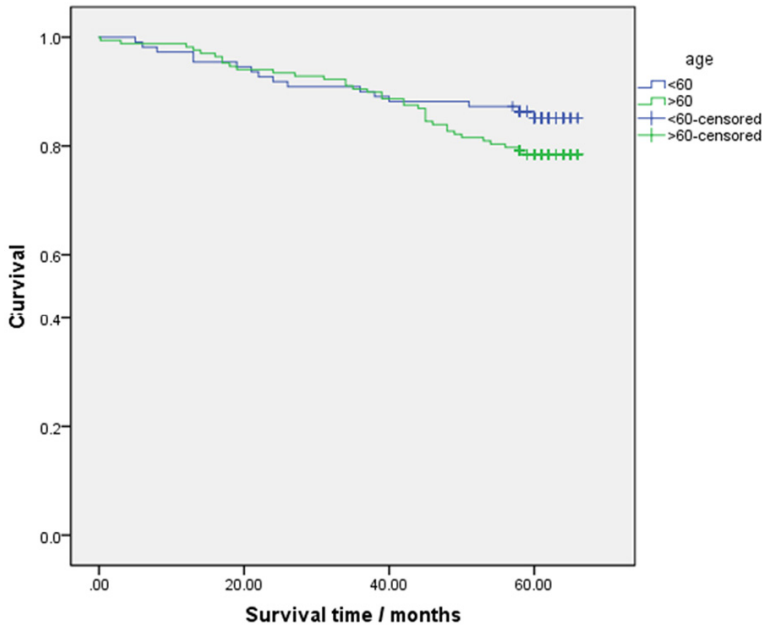

Figure 4. Overall survival curve under different age-stratified groups.

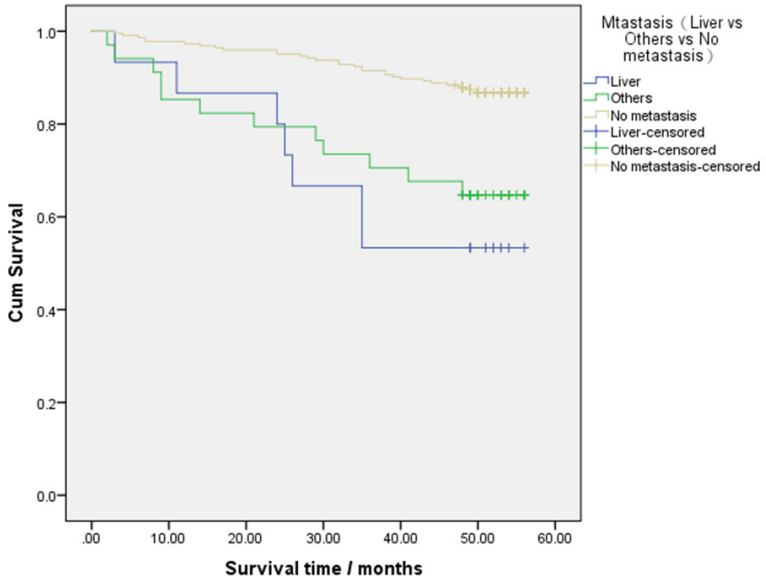

Figure 5. Overall survival curve comparison of colorectal cancer patients with/without hepatic metastasis.

mutation of KRAS gene in colorectal cancer may serve as a prognostic and therapeutic indicator [6, 8-11]; there are, of course, reports of opposite conclusions [12-15].

We found that our research largely differed from most of the above research. We selected tumor resection tissues in patients after operation. The group's physical condition, tolerance, and life expectancy might be longer, so the survival time of patients was therefore relatively longer. Especially for advanced patients, we found that the overall survival of these patients was well demonstrated after surgical treatment, suggesting the potential benefit of surgical treatment in advanced patients. Studies have also shown that, in improving patients' survival rate and survival time, tumor resection was often more effective than simple chemotherapy [16].

The results of this study showed that the mutation frequency of the KRAS gene was higher in Chinese colorectal cancer patients than that has been reported in the world (30-35%), namely 39.8% [8], and the mutation at codon 12 was more common [17]. The chi square test showed that KRAS gene mutation was correlated with EGFR expression, tumor location and tumor metastasis, suggesting that patients with higher EGFR expression, with right colon cancer, and with multiple metastases were more susceptible to KRAS mutation. In addition, KRAS gene mutation was not related to other pathological factors such as sex, age, P53 expression, TNM stage, differentiation degree and lymph node metastasis. Our study confirmed previous related research that higher KRAS mutation rate and mutation status were not associated with gender and age in right colon cancer patients [8, 9], and for the first time identified the relationship between KRAS mutation and multiple metastasis of tumor. Furthermore, this was the first time that KRAS gene mutations in patients with rectal cancer and other pathological factors were analyzed. Analysis results documented that KRAS gene

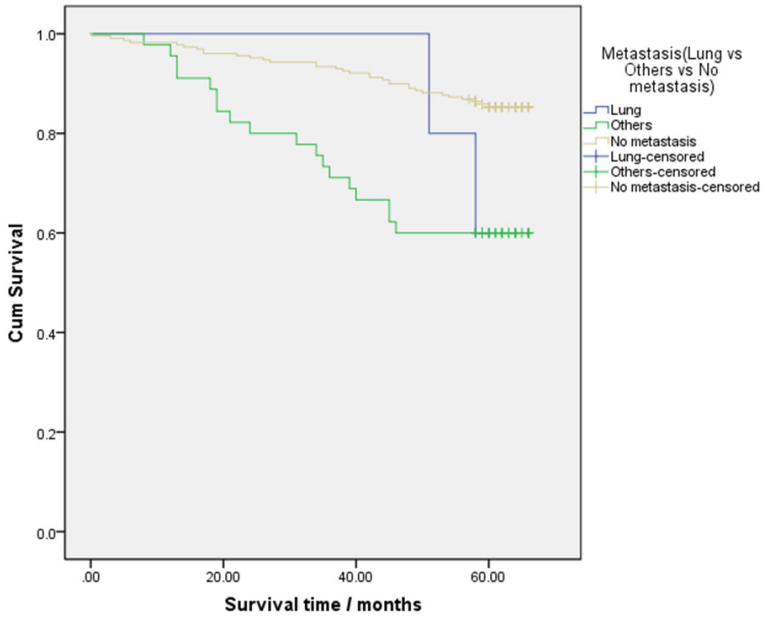

Figure 6. Overall survival curve comparison of colorectal cancer patients with/without pulmonary metastasis.

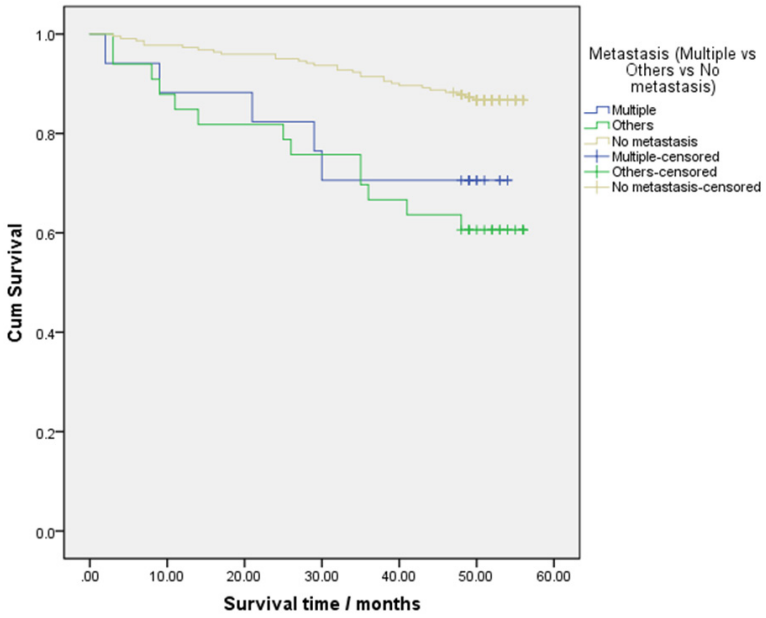

Figure 7. Overall survival curve comparison of colorectal cancer patients with/without multiple metastasis.

mutation was related to EGFR expression and tumor metastasis, and this was consistent with the overall outcome of included patients in our study.

Univariate analysis showed no significant difference in overall survival of patients with wild-type and mutant-type KRAS colorectal cancer. In a parallel study of stage II-III colorectal cancer [12], Roth supported that KRAS gene mutation did not possess predictive value of postoperative prognosis in patients with colorectal cancer. Several retrospective clinical studies on KRAS gene mutation [13, 14, 18] have yielded similar results. However, the mainstream view is that KRAS gene mutation has some influence in the prognosis of patients with colorectal cancer.

Nevertheless, there is another opinion about the two different views in academic circles at present. Some scholars believe that the detection of KRAS gene mutation depends on the sensitivity and specificity of detection methods, as well as sample size and environmental factors [19, 20], all of which may affect the final results of KRAS mutation rate. In this study, we thought it might be correlated with the following reasons: (1) heterogeneity of the study population, for example, the lacking of the study population of early cases; (2) the limitation of detection methods of KRAS gene mutation, false negative results was more likely to obtain by direct sequencing method in KRAS wild-type patients; (3) the difference in the amount of KRAS mutation (Mutant abundance) might affect the prognosis of patients [21], under low sensitivity detection, patients with low mutant abundance were detected as wild-type, resulting in inaccurate results.

Additionally, in the European Cancer Conference 2013, a collaborative survey team of the German Association for Cancer Medicine sup-

Table 4. COX regression analysis of prognosis in patients with colorectal cancer

Factors	β	SE	Wald	df	*P*	RR	95.0% CI for RR	
							Lower	Lower
Gender	0.000	0.000	3.129	1	0.077	1.000	1.000	1.001
Age	-0.902	0.379	5.667	1	0.017	0.406	0.193	0.853
KRAS gene mutation	-0.014	0.032	0.203	1	0.653	0.986	0.926	1.049
EGFR expression	0.625	0.333	3.516	1	0.061	1.869	0.972	3.592
P53 expression	0.025	0.309	0.007	1	0.936	1.025	0.559	1.879
Differentiation degree	-0.151	0.453	0.110	1	0.740	0.860	0.354	2.092
TNM stage	-0.934	0.221	3.128	1	0.005	0.351	0.191	0.541
Hepatic metastasis	-0.005	0.147	0.001	1	0.974	0.995	0.746	1.328
Pulmonary metastasis	0.002	0.007	0.105	1	0.746	1.002	0.989	1.016
Lymph node metastasis	0.000	0.000	0.311	1	0.577	1.000	0.999	1.001

ported that detection of mutations in the KRAS gene alone was not sufficient for the selection of key therapeutic strategies for patients with colorectal cancer. Currently, the latest advances in research believe that at the same time of detecting KRAS, detection of NRAS and BRAF genes are more conducive to making treatment programs benefit more colorectal cancer patients [22].

Further analysis of other pathological factors in colorectal cancer patients with relevant prognostic factors found that TNM staging, age and tumor metastasis were closely related to poor prognosis in patients with colorectal cancer. Cox Proportional Hazard Model for multivariate survival analysis indicated that age and TNM stage were independent risk factors influencing the prognosis of patients.

To sum up, based on the results of 5-year follow-up, KRAS gene mutation is observed to be correlated with EGFR expression, primary tumor site and multiple metastasis of tumors. To carry out necessary surgical treatment if conditions allow to prolong the survival outcome of advanced colorectal cancer, however, KRAS is not completely elucidated with regards to prognosis.

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Disclosure of conflict of interest

None.

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