

## Original Article

# Intratumoral heterogeneity of KRAS mutations in patients with colorectal cancer and metastatic livers in southwest China

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**Abstract:** Introduction: KRAS mutations are the most common somatic alterations in colorectal cancer (CRC) patients. Epidermal growth factor receptor (EGFR) antibody therapies are effective in 50% of the CRC patients with wild-type KRAS. To confirm the possible causes of the therapeutic failure, we examined KRAS mutations and analyzed their intratumoral heterogeneity in different areas of the primary tumors and the metastatic liver lesions. Methods: The sequences of exon 2 of KRAS were evaluated by direct sequencing of samples from 26 CRC patients, including 2 patients with colorectal liver metastasis. Tumor tissues were macrodissected from five different areas in primary CRC tumors and two different areas of metastatic liver lesions. Results: KRAS mutations were detected in 26.9% of the primary tumors. By comparing the different areas of primary tumors and liver metastasis, the intratumoral heterogeneity of KRAS mutations was observed in 11.5% of the primary tumors, but not in patients with liver metastasis. This study is the first to report the intratumoral heterogeneity of KRAS mutations in CRC patients from Southwest China, although our relatively small sample size might not provide sufficient statistical power. Conclusions: The failure of EGFR antibody therapies in CRC patients with wild-type KRAS might be attributed to the false-negative sequencing results caused by intratumoral heterogeneity. Considering the high rates of heterogeneity among primary tumors, the different parts of tumors should be tested to correctly predict the KRAS mutations.

**Keywords:** Intratumoral heterogeneity, colorectal cancer, KRAS, southwest China

## Introduction

Colorectal cancer (CRC) is the third most common cancer and has become the fourth leading cause of cancer mortality in China [1, 2]. Targeted therapy was introduced to oncology in recent years; to date, this form of therapy represents an important approach of clinical anti-cancer therapies. Several recent studies have demonstrated that CRC patients with wild-type KRAS significantly benefit from monoclonal antibodies [3-9]. Epidermal growth factor receptor (EGFR) is a member of the tyrosine kinase receptor and belongs to the ErbB family. EGFR has a key role in the development and progression of CRC. Two main EGFR-dependent

signaling pathways are the RAS/RAF/MAPK and PI3K/AKT pathways, which regulate cell growth, proliferation, differentiation, invasion, and migration [10-13]. The KRAS oncogene can regulate cell growth via the MAPK signaling pathway [14, 15]. However, with KRAS mutations, the blocking of EGFR pathways disrupts the downstream signaling pathways [16, 17]. Numerous studies have demonstrated that KRAS mutations are the most common somatic alterations in CRC patients with frequencies of 30%-60% [5, 18, 19]. These mutations have proved to be useful predictive markers of the patient's response to EGFR-targeted therapies [3, 20-25]. In various clinical studies with cetuximab or panitumumab therapy, only patients

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with wild-type KRAS responded to therapy, and these patients accounted for approximately 40%-60% of all patients [5, 22-24]. KRAS mutations occurred in approximately 80% of the patients in exon 2 at codons 12 and 13, but in less than 5% in exons 3 and 4 [26]. These somatic missense mutations can partially explain the lack of response to EGFR-targeted therapies. Furthermore, we considered that a possible reason for the unexpected therapeutic failure may be the intratumoral heterogeneity, which causes false-negative predictions of KRAS mutations in diagnostic samples. Some patients with wild-type KRAS could be considered as false-negative because of erroneous examination. Several previous studies have shown the heterogeneity of KRAS mutations by comparing primary tumors with their corresponding metastases [27-30]. However, these studies could not collect different regions of the metastatic tumor, and the intratumoral heterogeneity of KRAS mutations cannot be detected in metastatic tumor tissues.

Therefore, further research is needed to elaborate the intratumoral heterogeneity of the metastasis of tumor tissues. In the present study, we detected the KRAS mutations heterogeneity in different regions of tumor tissues in CRC patients from Southwest China. Furthermore, we evaluated the intratumoral heterogeneity of KRAS mutations in metastatic liver lesions.

### Materials and methods

#### *Patients and specimens*

Written informed consent was obtained from the patients for the publication of this study and any accompanying images. Tumor specimens were obtained (with informed consent) from newly diagnosed CRC patients who underwent surgical resection. The patients underwent surgical resection and were histopathologically diagnosed with CRC at the First People's Hospital of Yunnan Province (Kunming, China) from 2013 to 2014. The patients had received no prior treatment for their disease, including chemotherapy or radiotherapy. All cases were collected regardless of the surgical stage or histological grade. Each patient had a companion normal tissue specimen that was taken from >5 cm from the tumor. Tumor specimens were selected from 5 of different tumor areas and 2 of different metastatic liver areas.

All samples from surgical resection specimens were stored at -80°C until future use. The study was approved by the Institutional Ethics Committee of the First People's Hospital of Yunnan Province, and was conducted by Chinese law for the use of human tissue for research.

#### *DNA preparation*

Samples for DNA extraction were required to contain an average of 70% tumor cells with less than 20% necrosis for inclusion in the study [31-33]. Two experienced pathologists verified the diagnosis in H&E-stained paraffin sections. All genomic DNA was respectively extracted from the primary cancerous and normal tissues via the standard phenol/chloroform method. The extracted genomic DNA was dissolved in a total volume of 100 µL sterile water and stored at -80°C.

#### *DNA amplification and sequencing analysis*

For each specimen, KRAS was amplified in exon 2 by polymerase chain reaction (PCR). PCR was performed in a total volume of 50 µL with 50 ng genomic DNA as template. Each mixture contained 0.2 µmol/L of primers, 0.2 mmol/L of the dNTP Mix, and 0.25 units of *Taq* DNA polymerase (Takara). The Fwd (5'-TAAGCGTCGATGGAGGAG-3') and Rev (5'-TCT-GAAATGTACCTTGGGTT-3') primer sequences were designed in the present study. Primers were purchased from Genaray Biotechnology (Shanghai, China).

The amplification reactions were as follows: an initial denaturation cycle of 95°C for 5 min, followed by 30 cycles of denaturation (98°C for 30 s), annealing (57°C for 30 s), and elongation (72°C for 1 min), with a final extension cycle at 72°C for 5 min. Mutation analysis of KRAS in exon 2 was detected by direct sequencing of the amplified PCR products at Genewiz Biotechnology (ABI 3730xL Genetic Analyzer; Suzhou, China) with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). The sequencing data analysis was performed with DNASTar SEQMAN software.

#### *Statistical analysis*

All statistical analyses were performed with the SPSS statistical package. We used Fisher's exact test to determine the correlation between the KRAS mutations in exon 2 and the clinico-

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**Table 1.** Patient characteristics and correlations between KRAS mutation in exon 2 and clinicopathological parameters in CRC (N=26)

Characteristic	ALL	Wild-type	KRAS Mutation	P-value
	N	N (%)	N (%)	
Gender				0.378
Male	13	11 (57.9)	2 (28.6)	
Female	13	8 (42.1)	5 (71.4)	
Age				0.661
<65 years	17	13 (68.4)	4 (57.1)	
≥65 years	9	6 (31.6)	3 (42.9)	
Tumor diameter				0.391
<5	12	10 (52.6)	2 (28.6)	
≥5	14	9 (47.4)	5 (71.4)	
Primary site				1.000
Colon	13	10 (52.6)	3 (42.9)	
Rectum	13	9 (47.4)	4 (57.1)	
Differentiation				0.095
Well	15	13 (68.4)	2 (28.6)	
Poor	11	6 (31.6)	5 (71.4)	
Metastases				0.095
M (-)	15	13 (68.4)	2 (28.6)	
M (+)	11	6 (31.6)	5 (71.4)	

**Table 2.** Frequency and type of KRAS mutations in exon 2 in CRC (N=26)

	Nucleotide change	Amino acid change	Case (%)
Codon 12	c.35G>A	p.G12D	3 (11.5)
	c.35G>T	p.G12V	1 (3.8)
Codon 13	c.38G>A	p.G13D	3 (11.5)

pathological parameters. All differences were considered statistically significant if the *P* value was less than 0.05 ( $P < 0.05$ ).

### Results

#### *Patient characteristics*

Among the 26 patients included in this study, 13 patients (50%) were males, and 13 patients (50%) were females, including the 2 CRC patients with liver metastasis. The median age was 61.3 years old and ranged from 18 years old to 80 years old. The primary tumor sites were colon (13 of 26 patients, 50%) and rectum (13 out of 26 patients, 50%). The tumor diameters were less than 5 cm for 12 patients (46.2%), whereas 14 patients (53.8%) had ≥5

cm. Only 2 patients (7.7%) had liver metastases among the 11 metastatic cases. We found no association between the KRAS mutations in exon 2 and the various clinicopathological features. The specific CRC patient characteristics are shown in **Table 1**.

#### *Frequency and type of KRAS mutations in exon 2*

Among the 26 CRC patients (130 primary tumors and 4 liver metastases), KRAS mutations in exon 2 were detected in 5 different tumor areas and 2 different liver metastases areas by direct sequencing. The frequency of KRAS mutations in primary tumors was 26.9% (7/26). The mutations caused 4 cases at codon 12, and 3 cases at codon 13. No point mutations were tested in exon 2 at other codons. The mutations were G12D or G13D in 3 cases, but were G12V in 1 case. We found no KRAS mutations in exon 2 in 2 patients with liver metastases. The detected specific nucleotide and codon changes are listed in **Table 2**. The representative sequencing analyses of wild-type KRAS and G13D are shown in **Figure 1**.

#### *Intratumoral heterogeneity analysis of KRAS mutations in exon 2*

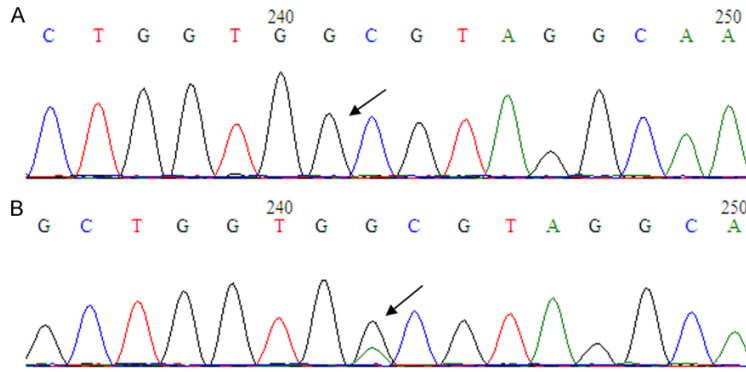
Among the 7 CRC patients with KRAS mutations, we performed a heterogeneity analysis of KRAS mutations in exon 2. We identified the discordant KRAS mutations in exon 2 among the 5 different primary tumor areas in 3 cases (11.5%), thereby demonstrating intratumoral heterogeneity. For 2 cases, we tested the concordant KRAS mutations in exon 2 among 2 different areas of liver metastases. Specific KRAS mutations sites in exon 2 are shown in **Table 3**.

However, we tested that not more than one amino acid changed in the discordant KRAS mutations in exon 2. Although these samples came from 5 different primary tumor areas, each CRC patient with KRAS mutations in exon 2 changed the same amino acid.

### Discussion

KRAS mutations are regarded as the most common mutations among CRC patients. By analyz-

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**Figure 1.** Sequencing analysis of KRAS at codon 13. A. Wild-type. B. Sequence data showed a mutation in codon 13 (C.38G>A) in the primary tumor.

**Table 3.** Detailed analysis of patients showing heterogeneity according to KRAS mutations in exon 2, WT, Wild-type; B, C, D, E and F, five different areas of the primary tumor; M1 and M2, two areas of liver metastases

Patients ID number	Primary tumor					Liver metastases	
	B	C	D	E	F	M1	M2
1	c.35G>A	c.35G>A	c.35G>A	c.35G>A	c.35G>A		
10	c.35G>A	c.35G>A	c.35G>A	c.35G>A	c.35G>A		
11	c.38G>A	c.38G>A	c.38G>A	c.38G>A	c.38G>A		
14	WT	c.38G>A	c.38G>A	WT	WT		
19	c.35G>T	WT	c.35G>T	WT	WT		
22	c.38G>A	c.38G>A	c.38G>A	c.38G>A	c.38G>A		
26	WT	c.35G>A	WT	WT	WT	WT	WT

ing frequency and type of KRAS mutations, our study demonstrated that the frequency of KRAS mutations frequency in exon 2 was 26.9% in 26 CRC patients from Southwest China with resectable tumors, which usually occurred at codons 12 or 13. The rate of KRAS mutations is lower in our study than in previous studies [34-36]. This difference might be attributed to the small number of patients investigated in our study. The most frequent mutation was 35G>A (G12D) at codon 12 and 38G>A (G13D) at codon 13. Our results agree with those of Martinetti et al. [37]. By evaluating the KRAS mutational status at codons 12, 13, 61, and 146, their group found that the most frequent mutation was G12D at codon 12.

Another clinically important issue is the intratumoral heterogeneity of KRAS mutations in exon 2 of patients with primary tumor. We demonstrated the intratumoral heterogeneity of KRAS

mutations in exon 2 among the 5 different primary tumors and 2 different liver metastases. Recent studies suggested that the CRC patients with wild-type KRAS showed the poor response to EGFR-targeted therapies (cetuximab and panitumumab) [20-25]. One possible reason for this therapeutic failure may be the false-negative wild-type KRAS in diagnostic samples because of the intratumoral heterogeneity. In fact, only a single tumor sample could not replace the whole tumor and could result in false diagnoses in the clinic. In the present study, the intratumoral heterogeneity of KRAS mutations in exon 2 was detected in 11.5% of all cases with primary tumors. Our results are in accordance with those of Baldus et al. [38] examined KRAS status in primary tumor. By comparing the KRAS status in tumor centers and invasive fronts, their group detected KRAS intratumoral heterogeneity, which was found in 8% of all cases.

In contrast to our results, Losi et al. [39] analyzed 25 CRC patients with KRAS status and observed a 36% rate of the intratumoral heterogeneity in terms of KRAS mutations in the primary tumor, including early and advanced CRC. The intratumoral heterogeneity (36%) was much higher than in our results. This difference might be attributed to the fact that the researchers selected further samples from every tumor. That is, 9-14 areas were selected, depending on the size of the tumor, whereas we only selected 5 areas from every tumor.

To the best of our knowledge, this study is the first to analyze different areas of the metastatic tumor (liver) in Southwest China. We examined KRAS mutations in exon 2 in two different areas of liver metastatic lesions. However, we found no KRAS mutations in exon 2 in the liver metastatic lesions. This result indicated that the metastatic liver has no heterogeneity, which is



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in line with the result of a previous report [38]. In addition, we did not examine any significant association between the KRAS mutations in exon 2 and the various clinicopathological features. This result might be partially because of our small sample size.

However, our study has several limitations. We only identified the intratumoral heterogeneity of KRAS mutations in exon 2. Because CRC arises through the accumulation of several genetic alterations, mutations of other EGFR-dependent signaling molecules, such as BRAF and PIK3CA, could also lead to the poor response to EGFR-targeted therapies [40-44]. However, whether BRAF or PIK3CA mutations show intratumoral heterogeneity is unknown in Southwest China. Another limitation of the study is that whether other metastatic lesions show intratumoral heterogeneity of KRAS mutations remains indistinct. Usually, several CRC patients in clinical practice have multiple synchronous or metachronous metastases (for example, lung and brain). However, we only studied the intratumoral heterogeneity of the liver metastatic lesions. Therefore, this aspect needs to be clarified in prospective studies. Finally, we selected a relatively small sample size ( $N=26$ ), with only 2 CRC patients having liver metastatic lesions. We are currently enlarging our sample size with patients from other hospitals and centers. We believe that the intratumoral heterogeneity of KRAS mutations and the response to anti-EGFR therapies will also need to be demonstrated in future studies.

### Conclusions

Our study observed the concordance rate of the frequency and type of KRAS mutations in primary tumors. We confirmed the intratumoral heterogeneity of KRAS mutations in exon 2 of CRC patients in Southwest China. Therefore, the different tumoral areas should be tested for KRAS mutations to correctly predict the KRAS status in tumors with heterogeneous KRAS status. This information is important in the clinical setting to avoid anti-EGFR therapy for KRAS mutant lesions.

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### Disclosure of conflict of interest

None.

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