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Original Article

Correlation between clinicopathological features and *KRAS*, *NRAS*, and *BRAF* mutation status in Chinese colorectal cancer patients

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Abstract: This study was retrospectively performed to analyze correlations between clinicopathological features of colorectal cancer (CRC) and mutations in *KRAS*, *NRAS*, and *BRAF* in Chinese patients, and to assess the importance of detecting additional mutations in *KRAS* exons 3 and 4 and *NRAS* in patients with CRC. *RAS* (*KRAS* and *NRAS*) and *BRAF* mutations were detected in 715 and 655 patients respectively. The mutation rate of *RAS* (*KRAS* or *NRAS*) was 45.6% (326/715). *KRAS* exon 2 mutations were evaluated in 36.6% of patients (262/715). Additional mutations in *RAS* exons occurred in 9.0% of patients (64/715), including *KRAS* exons 3 and 4 in 5.6% (40/715) and *NRAS* exons 2, 3, or 4 in 3.4% (24/715). Among 453 patients with wild-type *KRAS* exon 2, 14.1% (64/453) had other mutations in *RAS* exons. The most frequent sites of mutations were codons 12, 13, 61, and 146 in *KRAS* and codons 12 and 61 in *NRAS*. The mutation rate of *BRAF* (exon 15) was 4.0% (26/655), and the most frequent mutation site was codon 600. Among 440 patients with CRC who had a primary tumor resection at our center, those with mucinous or signet ring cell CRC were more likely to harbor *KRAS* mutations than those with adenocarcinoma (62.7% vs. 43.6%, P=0.006 and 59.3% vs. 39.6%, P=0.004, respectively). Female patients had a higher *BRAF* (exon 15) mutation rate than male patients (5.1% vs. 1.1%, P=0.017). Detection of both *KRAS* and *NRAS* mutations is useful for selecting patients who will benefit from anti-EGFR monoclonal antibody therapy. *KRAS* mutations were more frequent in patients with mucinous adenocarcinoma/signet ring cell CRC, whereas *BRAF* mutations were more common in female patients with CRC.

Keywords: KRAS, NRAS, BRAF, mutations, colorectal cancer

Introduction

KRAS is frequently mutated in metastatic colorectal cancer (mCRC). Previous randomized, controlled trials indicated that patients with mutations in *KRAS* exon 2 do not benefit from anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibody (mAb) therapy [1, 2]. Recently, the retrospective PRIME trial showed that other mutations in *RAS* (exons 2, 3, and 4 in *NRAS* and exons 3 and 4 in *KRAS*) are also associated with decreased responses to anti-EGFR mAb therapy. Thus, detection of *RAS* family mutations (*KRAS* and *NRAS*) is recommended in patients with mCRC [3, 4]. Patients with CRC who do not have mutations in both *KRAS* and *NRAS* appear to benefit from anti-EGFR mAb therapy [3, 5]. Therefore, analysis of the biological features of CRC specimens may be important prior to cetuximab treatment. Moreover, mutations in *KRAS*, *NRAS*, and *BRAF* have been reported in large cohorts of Chinese CRC patients. In this study, we retrospectively analyzed mutations in *KRAS*, *NRAS*, and *BRAF* in 715 Chinese patients with CRC to explore the distribution of these gene mutations and their correlations with clinical pathological features.

Materials and methods

Patient specimens

Patients (N=715) diagnosed with CRC and underwent *RAS* mutation analysis from January

Table 1. Primers for *KRAS, NRAS*, and *BRAF*

Gene name	Forward primer	Reverse primer	Size
KRAS			
EXON2	5'-AGG CCT GCT GAA AAT GAC TG-3'	5'-TCA AAG AAT GGT CCT GCA CC-3'	173 bp
EXON3	5'-CTGTGTTTCTCCCTTCTCAGG-3'	5'-TGCATGGCATTAGCAAAGAC-3'	281 bp
EXON4	5'-TGACAAAAGTTGTGGACAGGT-3'	5'-TGTTACTTACCTGTCTTGTCTTTGC-3'	247 bp
NRAS			
EXON2	5'-CAGGTTCTTGCTGGTGTGAA-3'	5'-CACTGGGCCTCACCTCTATG-3'	144 bp
EXON3	5'-CCCCAGGATTCTTACAGAAAA-3'	5'-CCCCATAAAGATTCAGAACACA-3'	244 bp
EXON4	5'-AGGGAGCAGATTAAGCGAGT-3'	5'-CAAACTCTTGCACAAATGCTG-3'	198 bp
BRAF			
EXON15	5'-GCTTGCTCTGATAGGAAAATGAG-3'	5'-GTAACTCAGCAGCATCTCAGG-3'	237 bp

2014 to September 2015 at Fudan University Shanghai Cancer Center (FUSCC) were included in our study. Inclusion criteria were (1) the diagnosis of CRC as a single primary tumor, (2) definite histotype (adenocarcinoma, mucinous, or signet ring cell), (3) and available data for age, gender, and tumor location (colon or rectum). Mutational analyses of *KRAS* (exons 2, 3, and 4) and *NRAS* (exons 2, 3, and 4) were performed in all patients. In addition, samples from 655 patients were evaluated for mutations in *BRAF* exon 15. Stage of disease was recorded for 440 patients who received primary tumor resection at FUSCC based on the American Joint Committee on Cancer (AJCC) tumor-node-metastasis staging (TNM) system (7th edition, 2010). Our study was approved by the Ethical Committee and Institutional Review Board of FUSCC. All patients signed informed consent forms before inclusion in this study.

DNA extraction

Genomic DNA was extracted from formalin-fixed paraffin-embedded CRC tissue. A standard xylene-phenol protocol was used to dissolve paraffin. Tissue specimens (4-5 mm) were digested with proteinase K. Genomic DNA was extracted using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration and quality were determined on a Nanodrop spectrophotometer (ND-1000, Thermo-Fisher Scientific, Wilmington, DE, USA).

Direct sequencing of RAS and BRAF

PCR amplification and direct sequencing of exons 2, 3, and 4 of *KRAS*, exons 2, 3, and 4 of *NRAS*, and exon 15 of *BRAF* were performed. Primers for *KRAS, NRAS*, and *BRAF* are shown in **Table 1**. The following PCR conditions were used: 94°C for 10 minutes, then 38 cycles for denaturing at 94°C for 45 seconds, annealing at 60°C for 45 seconds, extension at 72°C for 45 seconds, and final extension at 72°C for 7 minutes. PCR products were purified using a QIAquick gel extraction kit (Qiagen, Germany) and were used to prepare sequencing reactions. Sequencing was performed with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the following PCR conditions: 94°C for 1 minute, 24 cycles of denaturing at 94°C for 10 seconds, annealing at 50°C for 5 seconds, extension at 60°C for 1 minute, and final extension at 72°C for 5 minutes. Sequenced PCR products were purified and analyzed on an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Statistical analyses

Chi-square or Fisher's exact tests were performed for categorical variables. All statistical analyses were performed with SPSS for Windows version 22 (IBM Corp, Armonk, NY, USA). Two-sided $P<0.05$ was recognized as being statistically significant.

Results

Clinical characteristics of patients harboring RAS and BRAF mutations

Clinical characteristics of all the 715 patients are shown in **Table 2**. The mean age of the 715 patients included in this study was 58 years old (range, 15-87 years), with 419 (58.6%, 419/715) men and 296 (41.4%, 296/715)

Table 2. Clinicopathological and genetic features of CRC patients

Variable	N	%
Sex		
Male	419	58.6
Female	296	41.4
Age		
≥60	391	54.7
<60	324	45.3
Tumor histology variant		
Adenocarcinoma	608	85.0
Mucinous adenocarcinoma/signet ring cell cancer	107	15.0
Tumor location		
Colon	408	57.1
Rectum	307	42.9
RAS		
Mutation	326	45.6
Wild-type	389	54.4
KRAS		
Mutation	302	42.2
Wild-type	413	57.8
NRAS		
Mutation	24	3.4
Wild-type	691	96.6
*BRAF**		
Mutation	26	4.0
Wild-type	629	96.0
KRAS exon 2		
Mutation	262	36.6
Wild-type	453	63.4

*655 of 715 patients were analyzed for both *RAS* and *BRAF* mutations. CRC, colorectal cancer.

women. The *RAS* mutation rate was 45.6% (326/715), with *KRAS* mutations more common (42.2%, 302/715) than *NRAS* mutations (3.4% 24/715). Among 655 patients who were analyzed for *BRAF* mutations, mutation rate found in *BRAF* exon 15 was 4.0% (26/655).

KRAS, NRAS, and BRAF mutations in patients with CRC

The distribution of *KRAS* mutations among 715 CRCs is displayed in **Table 3**. The most common mutation was in *KRAS* exon 2 (36.6%, 262/715) at codons 12 and 13. Among patients who did not have a mutation in *KRAS* exon 2, 14.1% (64/453) had mutations in *KRAS* exons 3 or 4 or in *NRAS*. Forty patients had mutations in *KRAS* exons 3 and 4. The most common mutation sites of *KRAS* exons 3 and 4 were codon 61 (47.5%, 19/40) and codon 146 (32.5%, 13/40) respectively. Common amino acid changes were Q61H>Q61L>Q61R>Q61K in codon 61 of *KRAS* exon 3 and A146T> A146V in codon 146 of *KRAS* exon 4.

The distribution of *NRAS* and *BRAF* mutations in 715 CRCs is displayed in **Table 4**. Twenty-four patients had mutations in *NRAS*. The most common sites of mutations were codon 61 in exon 3 (37.5%, 9/24) and codon 12 in exon 2 (29.2%, 7/24). Common amino acid changes were Q61K> Q61L>Q61R and Q61H in codon 61 of *NRAS* exon 3 and G12D and G12V>G12C in codon 12 of *NRAS* exon 4.

NRAS exon 4 mutations were rare (8.3%, 2/24) compared with mutations in exons 2 and 3. Other uncommon mutations in *KRAS* exons 3 and 4 and in *NRAS* are presented in **Table 3**.

Among the 655 patients with *BRAF* mutations, 26 had a mutation in *BRAF* exon 15. The most common site of mutation was codon 600 (76.9%, 20/26). Other sites of mutations included codons 601, 594, and 559 (23.1%, 6/26).

Two patients harbored mutations in both *KRAS* and *NRAS*, and only one patient harbored mutations in both *KRAS* and *BRAF*.

Associations between RAS or BRAF mutations and clinicopathological features

Associations between *KRAS*, *NRAS*, or *BRAF* mutations and the clinicopathological features of patients are presented in **Table 5**. Patients with mucinous or signet ring cell CRC were more likely to harbor *KRAS* mutations compared with patients with adenocarcinoma (mucinous or signet ring cell cancer vs. adenocarcinoma cancer, 62.7% vs. 43.6%, P=0.006 and 59.3% vs. 39.6%, P=0.004, respectively). No statistical significance was observed between *KRAS* mutations and other clinicopathological features. All clinicopathological fea-

Table 3. Detailed distribution of *KRAS* mutations in CRC

Mutation hotspot	Number of mutations	Percentage (%)
KRAS	302	42.2
EXON2	262	36.6
Codon12	196	27.4
G12D	100	14
G12V	58	8.1
G12C	11	1.5
G12S	14	2.0
G12A	11	1.5
G12R	2	0.28
Codon13	64	9.0
G13D	63	8.8
G13R	1	0.14
Codon14	1	0.14
V14I	1	0.14
Codon22	1	0.14
Q22K	1	0.14
EXON3	21	2.9
Codon59	1	0.14
Codon61	19	2.7
Q61H	10	1.4
Q61L	5	0.7
Q61R	3	0.4
Q61K	1	0.14
Codon76	1	0.14
G76E	1	0.14
EXON4	19	2.7
Codon117	5	0.7
K117D	4	0.6
K117N	1	0.14
Codon131	1	0.14
Codon146	13	1.8
A146T	10	1.4
A146V	3	0.4

Table 4. Detailed distribution of *NRAS* and *BRAF* mutations in CRC

NRAS	24	3.4
EXON2	11	1.5
Codon12	7	1.0
G12D	3	0.4
G12C	1	0.14
G12V	3	0.4
Codon13	3	0.4
G13D	1	0.14
G13R	2	0.3
Codon22	1	0.14
EXON3	11	1.5
Codon59	1	0.14
Codon60	1	0.14
Codon61	9	1.3
Q61H	1	0.14
Q61L	2	0.3
Q61K	5	0.7
Q61R	1	0.14
EXON4	2	0.3
Codon117	1	0.14
Codon142	1	0.14
BRAF EXON15	26	3.6
Codon600	20	2.8
Codon601	3	0.4
Codon594	2	0.3
Codon559	1	0.14

tures appeared to be unrelated to *NRAS* mutations.

Female patients had a higher *BRAF* mutation rate compared with male patients (female vs. male, 5.1% vs. 1.1%, P=0.017). However, age, histological type, tumor location, and TNM stage did not significantly correlate with the presence of a *BRAF* mutation.

Discussion

Recent studies showed that mutations in *RAS* family members (*NRAS* mutations and *KRAS* mutations outside exon 2) are associated with resistance to anti-EGFR mAb therapy [6]. Sorich et al. analyzed nine randomized, controlled trials comprising a total of 5948 patients with CRC, finding that approximately 20% patients with wild-type *KRAS* exon 2 harbored another *RAS* mutation [7]. They concluded that patients with CRC and any type of *RAS* mutation are unlikely to benefit from anti-EGFR mAb therapy [7]. In the PRIME trial, 17% (108/512) of patients had a wild-type *KRAS* exon 2 but had other mutations in *RAS* (involving *KRAS* exons 3 or 4 or *NRAS* exons 2, 3, or 4) [6]. The effects of anti-EGFR mAb therapy differ between patients who lack *RAS* mutations and those who lack mutations in *KRAS* exon 2 but have other mutations at other sites within *RAS* [6]. These studies suggested that *KRAS* exon 2 and other *RAS* mutations serve as a negative predictive factor of response to anti-EGFR mAb treatment. Therefore, detection of multiple *RAS* mutations is necessary in patients with CRC before anti-EGFR mAb treatment. In our cases,

Table 5. Association between *KRAS*, *NRAS*, and *BRAF* mutations and clinicopathological features of 440 patients who received primary tumor resection at FUSCC

Clinicopathological features	Total number N=440	*RAS* n (%)			*KRAS* n (%)			*NRAS* n (%)			*BRAF* n (%)		
		Mutation	Wild-type	*P* value	Mutation n (%)	Wild n (%)	*P* value	Mutation n (%)	Wild n (%)	*P* value	Mutation n (%)	Wild n (%)	*P* value
Age				0.116			0.153			1.000*			1.000*
<70	373	178 (47.7)	195 (52.3)		162 (43.4)	211 (56.6)		16 (4.3)	357 (95.7)		11 (2.9)	362 (97.1)	
≥70	67	25 (37.3)	42 (62.7)		23 (34.3)	44 (65.7)		2 (3.0)	65 (97.0)		2 (3.0)	65 (97.0)	
Sex				0.216			0.224			0.709			0.017*
Male	263	115 (43.7)	148 (56.3)		105 (39.9)	158 (60.1)		10 (3.8)	253 (96.2)		3 (1.1)	260 (98.9)	
Female	177	88 (49.7)	89 (50.3)		81 (45.8)	96 (54.2)		8 (4.5)	169 (95.5)		9 (5.1)	168 (94.9)	
Location				0.247			0.118			0.215			0.123
Colon	234	114 (48.7)	120 (51.3)		107 (45.7)	127 (54.3)		7 (3.0)	227 (97.0)		9 (3.8)	225 (96.2)	
Rectal	206	89 (43.2)	117 (56.8)		79 (38.3)	127 (61.7)		11 (5.3)	195 (94.7)		3 (1.5)	203 (98.5)	
Histotype				0.006			0.004			1.000*			1.000*
Adenocarcinoma	381	166 (43.6)	215 (56.4)		151 (39.6)	230 (60.4)		16 (4.2)	365 (95.8)		11 (2.9)	370 (97.1)	
Mucinous/signet ring cell	59	37 (62.7)	22 (37.3)		35 (59.3)	24 (40.7)		2 (3.4)	57 (96.6)		1 (1.7)	58 (98.3)	
TNM stage				0.467			0.917			0.113*			1.000*
I/II	134	58 (43.3)	76 (56.7)		56 (41.8)	78 (58.2)		2 (1.5)	132 (98.5)		3 (2.2)	131 (97.8)	
III/IV	306	145 (47.4)	161 (52.6)		130 (42.5)	176 (57.5)		16 (5.2)	290 (84.8)		9 (2.9)	297 (97.1)	

*Fisher test. FUSCC, Fudan University Shanghai Cancer Center.

Table 6. Distribution of gene mutations in the present study and published literature

Study	Country	No of patients	RMR (%)	KMR (%)	NMR (%)	BMR (%)
The present study	China	715	45.6	42.2	3.4	4.1 (27/655)
Nicolas et al. 2015 [8]	France	6803	49.1	44.2	4.8	
Negru et al. 2014 [9]	Greece & Romania	354	50.0	44.4	5.7	7.3
Vaughn et al. 2011 [10]	America	2121	44.1	42.4	1.2	3.7
Baldus, S. E. et al. 2010 [11]	Germany	100		41		7

RMR, *RAS* mutation rate; KMR, *KRAS* mutation rate; NMR, *NRAS* mutation rate; BMR, *BRAF* mutation rate.

14.1% (64/453) of patients with wild-type *KRAS* exon 2 had mutations in *KRAS* exons 3 or 4 or in *NRAS*. Detection of other *RAS* mutations in patients with CRC who lack mutations in *KRAS* exon 2 may help avoid unnecessary toxicities and costs related to anti-EGFR mAb therapy. We compared our data with other studies (**Table 6**) and found a similar rate of *KRAS* mutations [8-11]. However, the *NRAS* mutation rate in the United States (1.2%) was lower than that reported in other studies, including our study [11]. The total *RAS* mutation rate was similar among studies [8-11]. Therefore, the total *RAS* mutation rate in patients with CRC may not exhibit significant geographic or racial differences.

We found that the most common sites of mutations were in codons 12 (27.4%) and 13 (9.0%) in *KRAS* exon 2. In *KRAS* exons 3 and 4, the most common sites for mutations were codons 61 (2.7%), 146 (1.8%), and 117 (0.7%). Mutations in *KRAS* codons 14 (V14I), 22 (Q22K), 59, and 117 were rare (0.1%). The most common sites of mutations in *NRAS* were codon 12 in exon 2 and codon 61 in exon 3. Mutations in codons 13, 22, 59, 60, and in exon 4 (codons 117 and 146) were rare.

In the present study, we found that mucinous tumors harbored a higher *KRAS* mutation rate than did the adenocarcinomas subtype, consistent with findings from a previous study [12]. Other clinicopathological features, such as sex, age, and tumor location did not exhibit associations with *KRAS* mutations, which further supports previous findings [13].

The *BRAF* mutation rate was 4% (26/715) in our study. Compared with western studies, we found that the *BRAF* mutation rate in CRC was higher in Germany or Greece, and Romania (7% and 7.3%, respectively) than in China [8, 9, 11]. The most common mutation in *BRAF* was in codon 600. In addition, we found that seven cases harbored mutations in codons 601, 594, and 559. Interestingly, *BRAF* mutations tended to be more frequent in female patients than in male patients, which is in line with previous western population-based studies [14-16]. However, the association between *BRAF* mutations and gender was not found in other Chinese studies [17, 18]. The different results might be caused by case selection bias or regional differences.

In conclusion, detection of mutations in both *KRAS* and *NRAS* could be used to select patients who will benefit from anti-EGFR mAb therapy. This test should be a routine molecular assay performed in patients with CRC before anti-EGFR mAb therapy.

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Disclosure of conflict of interest

None.

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