

# Association of KRAS, NRAS, BRAF and PIK3CA gene mutations with clinicopathological features, prognosis and ring finger protein 215 expression in patients with colorectal cancer

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Received June 28, 2023; Accepted October 19, 2023

DOI: 10.3892/br.2023.1686

**Abstract.** The relationships of KRAS, NRAS, BRAF and PIK3CA gene mutations with the clinicopathological features and prognosis of colorectal cancer (CRC) in patient are lacking. Furthermore, the role of ring finger protein 215 (RNF215) in CRC patients with KRAS, NRAS, BRAF and PIK3CA mutations remains unclear. In the present study, 182 surgical resection specimens from patients with primary CRC for retrospective analysis, were collected. KRAS/NRAS/BRAF/PIK3CA gene mutations were confirmed by an amplification-refractory mutation system. Immunohistochemistry (IHC) was conducted to confirm KRAS, NRAS, BRAF and PIK3CA protein expression. RNF215 expression in patients with CRC was evaluated using TIMER 2.0 database and IHC. The individual mutation rates of KRAS, NRAS, BRAF and PIK3CA were 40.7% (74/182), 4.4% (8/182), 4.4% (8/182) and 3.3% (6/182), respectively. The KRAS exon 2 mutation rate was the highest (61.5%, 64/104), and these mutations mainly occurred at codons 12 and 13. KRAS/NRAS/BRAF/PIK3CA wild-type CRC patients had significantly longer overall survival and disease-free survival than mutated KRAS/NRAS/BRAF/PIK3CA CRC patients ( $P<0.05$ ). Overall, 45.4% (5/11) of patients with PIK3CA mutations had concomitant KRAS mutations. The KRAS/NRAS/BRAF/PIK3CA gene mutation rate in patients with lymph node metastasis (76.1%, 35/46) was significantly

higher than that in patients without lymph node metastasis (50.8%, 69/136) ( $P=0.0027$ ). There were no significant differences in IHC expression between patients with and without KRAS, NRAS, BRAF and PIK3CA mutations ( $P>0.05$ ). The TIMER 2.0 analysis showed that RNF215 expression was significantly higher in the mutated BRAF group than in the wild-type BRAF group in CRC ( $P<0.05$ ). In conclusion, KRAS is the most commonly mutated gene, and KRAS mutations may be a poor prognostic factor for patients with CRC. KRAS wild-type patient resistance may be related to PIK3CA gene mutations, although this needs further verification in larger cohorts. BRAF mutations may be associated with RNF215 expression in patients with CRC.

## Introduction

Colorectal cancer (CRC) is one of the most prevalent gastrointestinal malignancies worldwide. In recent years, its morbidity and mortality have gradually increased. A total of ~19 million new cases and 10 million cancer-related deaths were estimated in 2020 (1). As nearly as 65.0% of new cases and 63.6% of CRC-related deaths occurred in China, Europe and North America in 2020 (2). The 5-year survival rate of patients with CRC after surgery, radiotherapy and chemotherapy is  $>30\%$ , and it has become a serious threat to human survival (3). It was estimated that in 2010, there were 270,000 new patients with CRC diagnoses and 130,000 CRC-related deaths in China (4). By 2025, the numbers of new patient diagnoses and deaths with CRC in China are expected to reach 624,300 and 221,100, respectively (5). At present, the TNM stage of CRC remains the most important prognostic factor, but even for patients with the same TNM stage, the prognosis can differ. Additionally, in some CRC cases, there is no association between the pathological Tumor-Node-Metastasis (pTNM) stage and CRC biological and clinical behavior (6,7). Therefore, identifying additional factors that can more accurately predict the clinical course of CRC regardless of the pTNM stage has been a major research focus for numerous years.

Although CRC occurs sporadically, its occurrence has been associated with genetic variations, including chromosomal instability, microsatellite instability, and Ras/Raf/MAPK mutations. In recent years, anti-epidermal growth factor

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**Abbreviations:** ARMS, amplification-refractory mutation system; CRC, colorectal cancer; DFS, disease-free survival; EGFR, epidermal growth factor receptor; FFPE, formalin-fixed, paraffin-embedded; IFN, interferon; IHC, immunohistochemistry; KM, Kaplan-Meier; OS, overall survival; RNF215, ring finger protein 215; TMA, tissue microarray

**Key words:** colorectal cancer, KRAS, NRAS proto-oncogene, BRAF, PIK3CA, prognosis, ring finger protein 215

receptor (EGFR) monoclonal antibodies have been used for the treatment of CRC because of their ability to block downstream intracellular EGFR signaling (8). However, the therapeutic efficacy is strictly dependent on the effect of the RAS/MAPK and PI3K-PTEN-AKT pathways downstream of the EGFR pathway, which is involved in genetic integrity. EGFR has emerged as a key target in specific inhibitor therapy for CRC, and activating mutations in KRAS/NRAS are considered to be strong predictors of resistance to EGFR-targeted drugs (9). These mutations lead to the constitutive phosphorylation of RAS proteins independent of the active state of upstream EGFR proteins (10).

RAS (KRAS/NRAS) is a proto-oncogene that encodes a protein with GTPase activity that plays a role in EGFR signal transduction and self-inactivation (11). BRAF is an important component of the RAS/RAF/MAPK pathway that mediates the binding of RAF and MAPK kinase (MAPKK/MEK1/2) in signal transduction and the regulation of cell proliferation. PIK3CA encodes P110 alpha, the catalytic subunit of PI3K, which mediates the PI3K/AKT pathway and promotes cell survival. Hence, changes in PIK3CA may lead to abnormal activation of the PI3K pathway. According to De Roock *et al* (12) KRAS-, NRAS-, BRAF-, and PIK3CA-based molecular biomarkers may have prognostic value in CRC. Chemotherapy combined with wild-type RAS- and EGFR-targeted therapy can improve the prognosis of patients (13). However, mutations in the RAS factor or BRAF may activate the downstream RAS/Raf/MAPK pathway, thereby inhibiting the effects of anti-EGFR therapy (14). KRAS and NRAS mutations are predictive of cetuximab and panitumumab therapy efficacy in clinical practice, but PIK3CA mutations are not included in the current guidelines. However, Liao *et al* (15) found that regular use of aspirin was correlated with longer survival among PIK3CA-mutated patients with CRC. Therefore, it is necessary to detect mutations in RAS, BRAF, PIK3CA and other genes in patients with CRC. In addition, cetuximab and panitumumab are the main molecular targeted drugs available for patients with CRC, and they act by inhibiting an EGFR signaling pathway (PI3K/AKT/mTOR or RAS/RAF/MAPK). The use of these drugs requires analysis of the RAS mutation status, and only patients with wild-type RAS are eligible for treatment. However, <40% of KRAS wild-type patients do not respond to anti-EGFR monoclonal antibody treatment (16). This resistance can be explained by mutations in other signaling effectors downstream of EGFR, such as the BRAF, PIK3CA and NRAS genes.

Ring finger protein 215 (RNF215) is a multichannel membrane protein, and its encoding gene is located at 22q12.2. At present, to the best of the authors' knowledge, only a few studies of the RNF215 protein have been performed. Wu *et al* (17) indicated that RNF215 can bind to the NF- $\kappa$ B p65 subunit by partially inhibiting type I interferon (IFN) production and limiting the accumulation of NF- $\kappa$ B in the promoter region of IFNB1. Ma *et al* (18) suggested that high RNF215 expression was associated with poor overall survival (OS) of head/neck squamous cell carcinoma. The study by McIntosh *et al* (19) indicated that single-nucleotide polymorphism variations near RNF215 were correlated with the expression levels of the neighboring gene MTP18/SF3A1. The present preliminary study showed that the expression of RNF215 was significantly higher in CRC tumor tissues than

in normal tissues. RNF215 may contribute to the development and progression of CRC by participating in CRC-associated signaling pathways, such as the Kyoto Encyclopedia of Genes and Genomes MAPK signaling pathway, the WP RAS signaling pathway, and the WP PI3K/AKT signaling pathway (20). However, the associations between RNF215 expression and KRAS, NRAS, BRAF and PIK3CA mutations in patients with CRC have not been reported. Therefore, there is a need to further investigate the role of RNF215 in patients with CRC with KRAS, NRAS, BRAF and PIK3CA mutations.

According to CRC guidelines (<https://www.nccn.org/guidelines/nccn-guidelines>), KRAS/NRAS/BRAF gene mutation detection is recommended for patients with primary or metastatic CRC before treatment to clarify the status and guide treatment. Although PIK3CA mutation analysis is not yet recommended, PIK3CA exon 20 mutations are associated with worse prognosis in metastatic CRC patients than in wild-type patients (21). Therefore, in the present study, the occurrence of gene mutations in KRAS, NRAS, BRAF and PIK3CA in CRC patients with CRC was assessed and the correlation between mutation incidence and clinicopathological features was estimated. Furthermore, immunohistochemistry (IHC) was performed to determine whether KRAS/NRAS/BRAF/PIK3CA gene mutations in patients with CRC could be detected by IHC. In addition, RNF215 expression in patients with mutations in KRAS, NRAS, BRAF and PIK3CA was also investigated. The results of the present study suggested that drug resistance in KRAS wild-type patients may be associated with PIK3CA gene mutations, and for the first time, BRAF mutations were found to be possibly associated with RNF215 expression.

## Materials and methods

**Clinical data.** A total of 182 CRC resection specimens from Shanghai Fifth People's Hospital affiliated with Fudan University (Shanghai, China) from January 2012 to December 2016 were included. The inclusion criteria for patients with CRC were as follows: i) Patients with pathological and imaging examination data met the CRC diagnostic criteria; ii) patients had no family history of CRC; and iii) patients had favorable mental health. The exclusion criteria were as follows: i) Patients did not meet the standard CRC diagnostic criteria; ii) Patients had serious complications affecting the heart, lung, or other important organs; and iii) patients were not conscious or unable to communicate normally. Patients with drug resistance were defined as KRAS wild-type patients for whom anti-EGFR therapy (cetuximab and panitumumab) was ineffective. The present study was reviewed and approved (approval no. 2021071) by the Ethical Committee of Shanghai Fifth People's Hospital, Fudan University (Shanghai, China). Written informed consent was provided by the patients/participants who participated in the present study.

Samples were collected and evaluated by two professional pathologists according to the standard protocols of the Department of Pathology. CRC tumors were staged according to the Staging Manual of the American Joint Committee on Cancer (Eighth Edition). The pathological characteristics of patients with CRC were extracted from medical records and pathology reports.

**Mutation detection.** Archival CRC tumor tissue in formalin-fixed paraffin-embedded (FFPE) blocks was available from all patients included in the present study. All tissues were fixed with 10% formalin at room temperature (20°C) for more than 24 h. The FFPE blocks were archived at the Department of Pathology, Shanghai Fifth People's Hospital, Fudan University (Shanghai, China). Mutation analysis was performed when the pathologist observed >10% of tumor cells under the hematoxylin and eosin staining slide. DNA was extracted using a DNA FFPE Tissue kit (cat no. 20150079; Amoy Diagnostics Co., Ltd.), according to the instructions for dewaxing, lysis, digestion, repair, adsorption, elution and other steps. DNA quality and concentration were determined by spectrophotometry (OD260/OD280 ratio 1.8-2.0). Mutations in the KRAS/NRAS/BRAF/PIK3CA genes were confirmed by an amplification-refractory mutation system (ARMS) (Human KRAS/NRAS/PIK3CA/BRAF Gene Mutation Combination Detection kit; cat. no. 20153401124; Amoy Diagnostics Co., Ltd.). According to the manufacturer's instructions, the sample DNA concentration was adjusted to an appropriate concentration (10 ng/ml) for sample preparation. The real-time quantitative PCR amplification procedure is shown in Table I. The primers used in the study were part of the kit. The fluorescence channel signal was collected during the third stage of annealing at 60°C. Real-time quantitative PCR was performed, and the files were saved. Hotspot gene mutations, including those in exons 2, 3 and 4 of the human KRAS gene, exons 2 and 3 of the NRAS gene, exon 15 of the BRAF gene, and exon 20 of the PIK3CA gene, were detected (as shown in Table II). The mutational analyses were performed compared with the amplification levels of positive and negative control tests provided by the manufacturer and according to the relevant protocols.

**IHC.** All 182 FFPE colorectal tumor tissues were used for tissue chip (tissue microarray, TMA) construction, with each tumor tissue consisting of three 1.5-mm representative punches as previously described by the Kononen *et al* (22). According to the manufacturer's protocol, 3- $\mu$ m-thick TMA slides were tested on an automated Ventana benchmark machine (Roche Tissue Diagnostics; Roche Diagnostics, Ltd.). Commercially available antibodies against KRAS polyclonal, (cat no. 12063-1-AP; 1:400; Proteintech Group, Inc.), NRAS clone sp174 (cat no. ab227658; 1:100; Abcam), BRAF clone VE1 (cat no. ab228461; 1:100; Abcam), PIK3CA clone SP139 (cat no. ab135384; 1:100; Abcam) and RNF215 polyclonal, (1:300; cat. no. Ys-9264R; Shanghai Yaji Biological Technology Co., Ltd.) were used for IHC. Sections were incubated with primary antibody for 16 h at 4°C, followed by the application of a secondary antibody [ultraView Universal HRP Multimer (55  $\mu$ g/ml); cat. no. (92)760-500; Ventana Medical Systems, Inc.] for 40 min at 37°C. Finally, 3,3'-diaminobenzidine (DAB) was used as the chromogenic substrate and the sections were counterstained with hematoxylin for 1 min at 20°C. Appropriate positive and negative slide controls were included for each antibody. The sections were blocked with 5% BSA (cat. no. SW3015; Beijing Solarbio Science & Technology Co., Ltd.) for 1 h in room temperature. Full slide images were reviewed and evaluated by two gastrointestinal pathologists under a light microscope (BX45; Olympus Corporation). For

Table I. Reverse transcription-quantitative PCR amplification procedures.

Stage	Procedure	Temperature (°C)	Time	Cycles (n)
First stage	Denaturation	95	5 min	1
Second stage	Denaturation	95	25 sec	15
Third stage	Annealing	64	20 sec	31
	Extension	72	20 sec	
	Denaturation	93	25 sec	
	Annealing	60	35 sec	
	Extension	72	20 sec	

KRAS, NRAS, BRAF, PIK3CA and RNF215, samples were labeled positive if >10% of tumor cells in each batch showed cytoplasmic staining similar in intensity to that of the positive controls. Any isolated nuclear staining without cytoplasmic staining was determined to be negative. IHC staining was performed on the corresponding large tumor sections of the resected specimens by the same method to verify the TMA protein expression.

**Association between gene mutations and RNF215 expression.** RNF215 expression in KRAS-, NRAS-, BRAF-, and PIK3CA-mutated cases was first evaluated with Tumor Immune Estimation Resource (TIMER) 2.0 (<http://timer.comp-genomics.org/>) (23). Subsequently, the association between RNF215 expression and KRAS, NRAS, BRAF and PIK3CA gene mutations in the 182 CRC cases of the present study was further verified using RNF215 IHC staining.

**Statistical analysis.** Descriptive statistics were employed for the clinicopathological features of the patients. The statistical results are summarized as percentages (%). The chi-square test or Fisher's exact test was used for comparisons of clinicopathological features, IHC expression, and gene mutation results. The survival rate and statistical significance analyses were determined by the Kaplan-Meier (KM) method with the log-rank test and Gehan-Breslow-Wilcoxon test. All statistical analysis was performed using the GraphPad Prism 9.0 statistical program (GraphPad Software, Inc.). The RNF215 expression differences between wild-type and mutated CRC cases in the TIMER 2.0 database (<http://timer.comp-genomics.org/>) were determined using the Wilcoxon rank sum test. All tests were two-sided, and  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Clinicopathological features.** The clinicopathological characteristics and gene mutation results of the study population are shown in Table III. Among the 182 patients with CRC, 130 (71.4%) were males, and 52 (28.6%) were females. The age of these patients ranged from 48-95 years, with an average of 69.1 years. There were 142 patients >60 years-old and

Table II. Gene mutation detection site.

Gene name	Test section	Mutant name	Base change
KRAS	Exon 2	G12S	34G>A
		G12D	35G>A
		G12C	34G>T
		G12R	34G>C
		G12V	35G>T
		G12A	35G>C
		G13C	37G>T
		G13D	38G>A
	Exon 3	Q61L	182A>T
		Q61R	182A>G
		Q61H	183A>C
		Q61	183A>T
	Exon 4	K117N	351A>C
		K117N	351A>T
		A146T	436G>A
		A146V	437C>T
		A146P	436G>C
NRAS	Exon 2	G12D	35G>A
		G12S	34G>A
		G13R	37G>C
		G12C	34G>T
		G12V	35G>T
		G12A	35G>C
		G13V	38G>T
	Exon 3	Q61R	182A>G
		Q61K	181C>A
		Q61L	182A>T
PIK3CA	Exon 4	Q61H	183A>C
	Exon 4	A146T	436G>A
	Exon 20	H1047R	3140A>G
H1047L		3140A>T	
BRAF	Exon 15	V600E1	1799T>A
		V600K	1798,1799GT>AA (complex)
		V600E2	1799,1800TG>AA (complex)
		V600R	1798,1799GT>AG (complex)
		V600D1	1799,1800TG>AC (complex)
		V600D2	1799,1800TG>AT (complex)

40 patients  $\leq 60$  years-old. The tumor sites were as follows: 66 cases were right colon cancer (including ascending colon cancer and transverse colon cancer), and 116 cases were left colon cancer (including descending colon cancer, sigmoid colon cancer, rectal and anal cancer). The general tumor type was endophytic in 116 patients and exophytic in 66 patients. There were 136 cases of well to moderate differentiation

and 46 cases of poor to no differentiation. Additionally, 150 patients had a tumor diameter  $>3$  cm, and 32 patients had a tumor diameter  $\leq 3$  cm. Lymph node metastasis was found in 46 cases, and no lymph node metastasis was identified in 136 cases. In addition, 90 patients had distant metastasis, and 92 patients did not have distant metastasis. Neurovascular invasion was identified in 76 patients, and no neurovascular invasion was identified in 106 patients. The TNM stage distribution was as follows: 12 cases of Stage I and 170 cases of Stage II-IV disease.

**KRAS, NRAS, BRAF and PIK3CA gene mutations.** As shown in Table IV, the total KRAS/NRAS/BRAF/PIK3CA mutation rate in 182 patients with CRC was 57.1% (104/182), the single mutation rates of KRAS, NRAS, BRAF and PIK3CA were 40.7% (74/182), 4.4% (8/182), 4.4% (8/182), and 3.3% (6/182), respectively. The concomitant mutation rate of KRAS/PIK3CA was 2.7% (5/182) and that of KRAS/NRAS was 1.6% (3/182). Representative KRAS, NRAS, BRAF, and PIK3CA gene mutation amplification curves in patients with CRC were generated by ARMS (Fig. 1). There were no patients with 3 or more gene mutations. The KRAS exon 2 mutation rate was the highest (35.2%, 64/182), and KRAS exon 2 mutations mainly occurred at codons 12 and 13. The KRAS exon 4, PIK3CA exon 20, NRAS exon 2, BRAF exon 15, KRAS exon 3, NRAS exon 3 and NRAS exon 4 mutation rates were 6.6% (12/182), 6.0% (11/182), 4.4% (8/182), 4.4% (8/182), 3.3% (6/182), 1.1% (2/182) and 0.5% (1/182), respectively.

Among the 104 patients with gene mutations, the single KRAS exon 2 mutation frequency was the highest (55.8%, 58/104), followed by the KRAS exon 4 mutation frequency was the second highest (11.5%, 12/104), and the frequency of concomitant mutations was 7.7% (8/104). The distribution of single and concomitant KRAS, NRAS, BRAF and PIK3CA gene mutations is shown in a Venn diagram (Fig. 2A). All other mutation types are shown in Fig. 2B.

**Associations of KRAS, NRAS, BRAF and PIK3CA gene mutations with CRC patient clinical characteristics.** There were no statistically significant differences in the incidences of analyzed mutations between patients with different clinical characteristics, including sex, age, tumor differentiation, tumor size, distant metastasis, neurovascular invasion, postoperative recurrence, TNM stage and gene mutations ( $P>0.05$ ). However, the BRAF gene mutation rate in patients with poorly differentiated cancer was significantly higher than that in patients with well to moderately differentiated cancer ( $P=0.0037$ , Table III), and no significant differences in the mutation rates of other genes were found between patients with different degrees of tumor differentiation ( $P>0.05$ ). The PIK3CA mutation rate in the right colon subgroup was significantly higher than that in the left colon subgroup ( $P=0.0243$ ); however, no significant differences in other gene mutations were identified between patients with different primary tumor sites ( $P>0.05$ ). The gene mutation rate of patients with lymph node metastasis (76.1%, 35/46) was higher than that of patients without lymph node metastasis (50.8%, 69/136), and the difference was statistically significant ( $P=0.0027$ ). However, no significant difference in the rate of single-gene mutations was identified between patients with and without lymph node metastasis ( $P>0.05$ ).



Table III. Relationship between KRAS, NRAS, BRAF, and PIK3CA gene mutations and clinicopathological features in 182 patients with CRC [n (%)].

Clinicopathological features	n	KRAS (n=74)	P-value	NRAS (n=8)	P-value	BRAF (n=8)	P-value	PIK3CA (n=6)	P-value	≥2 mutations (n=8)	P-value	Total mutations (n=104)	P-value
Sex			0.1047										
Male	130	48 (36.9)		5 (3.9)		6 (4.6)		5 (3.9)		5 (3.9)	0.6904	69 (53.1)	0.0977
Female	52	26 (50.0)		3 (5.8)		2 (3.9)		1 (1.9)		3 (5.8)		35 (67.3)	
Age, years			0.7173								0.6079		0.2556
>60	142	59 (41.6)		5 (3.5)		4 (2.8)		4 (2.8)		6 (4.2)		78 (54.9)	
≤60	40	15 (37.5)		3 (7.5)		4 (10.0)		2 (5.3)		2 (5.0)		26 (65.0)	
Localization			0.3735								0.0243		0.9291
Right	66	24 (36.4)		2 (4.3)		3 (4.6)		5 (7.6)		4 (6.1)		38 (48.1)	
Left	116	50 (43.1)		6 (4.6)		5 (4.3)		1 (0.9)		4 (3.5)		66 (61.9)	
Configuration			0.3488								>0.9999		0.1818
Endophytic	116	44 (37.9)		5 (5.2)		4 (3.6)		4 (3.5)		5 (4.3)		62 (53.5)	
Exophytic	66	30 (45.5)		3 (3.0)		4 (6.1)		2 (3.1)		3 (4.6)		42 (63.6)	
Differentiation			0.1196								0.1705		0.2005
Well to moderate	136	60 (44.1)		5 (3.7)		2 (1.5)		3 (2.2)		4 (2.9)		74 (54.4)	
Poor to undifferentiated	46	14 (30.4)		3 (6.5)		6 (13.0)		3 (6.5)		4 (8.7)		30 (65.2)	
Size			0.3217								0.2846		0.8456
>3 cm	150	64 (42.7)		6 (4.0)		5 (3.3)		4 (2.7)		6 (4.0)		85 (56.7)	
≤3 cm	32	10 (31.3)		2 (8.3)		3 (9.4)		2 (6.3)		2 (8.3)		19 (59.4)	
Lymph node metastasis			0.0826								0.1705		0.0027
Positive	46	24 (52.2)		3 (6.5)		2 (4.4)		3 (6.5)		3 (6.5)		35 (76.1)	
Negative	136	50 (35.8)		5 (3.7)		6 (4.4)		3 (2.2)		5 (3.7)		69 (50.8)	
Distant metastasis			0.3028								0.6824		0.1708
Positive	90	40 (44.4)		3 (3.3)		6 (6.7)		2 (2.2)		5 (5.6)		56 (62.2)	
Negative	92	34 (37.0)		5 (5.4)		2 (2.2)		4 (4.4)		3 (3.3)		48 (52.2)	
Neurovascular invasion			0.7828								0.2399		0.5392
Positive	76	30 (39.5)		4 (5.3)		5 (6.6)		4 (5.3)		3 (4.0)		46 (44.2)	
Negative	106	44 (41.5)		4 (3.8)		3 (2.8)		2 (1.9)		5 (4.7)		58 (48.3)	
TNM stage			0.7641								0.3398		0.0727
I	12	4 (33.3)		1 (8.3)		2 (16.7)		1 (8.3)		2 (16.7)		10 (83.3)	
II + III + IV	170	70 (41.2)		7 (4.1)		6 (3.5)		5 (2.9)		6 (3.5)		94 (55.3)	

Table IV. Mutations in the KRAS, NRAS, BRAF, and PIK3CA genes in 182 patients with CRC.

Test location	Mutant name	Number	Mutation rate (%)
KRAS Exon2	G12S, G12D	35	19.8
KRAS Exon2	G12C, G12R, G12V, G12A, G13C	17	9.3
KRAS Exon2	G13D	12	6.6
KRAS Exon3	Q61L, Q61R, Q61H(183A>C), Q61H(183A>T)	6	3.3
KRAS Exon4	K117N(351A>C), K117N(351A>T), A146T, A146V, A146P	12	6.6
NRAS Exon2	G12D, G12S	5	2.7
NRAS Exon2	G13R, G12C, G12V, G12A, G13V	3	1.6
NRAS Exon3	Q61R, Q61K, Q61L, Q61H	2	1.1
NRAS Exon4	A146T	1	0.5
PIK3CA Exon20	H1047R, H1047L	11	6.0
BRAF Exon15	V600E1, V600K, V600E2, V600R, V600D1, V600D2	8	4.4

Three patients had simultaneous mutations in KRAS exon 2 (G12S, G12D) and PIK3CA exon 20. Three patients had simultaneous mutations in KRAS exon 2 (G12C, G12R, G12V, G12A, G13C) and NRAS exon 2 (G12D, G12S). Two patients had simultaneous mutations in KRAS exon 3 and PIK3CA exon 20.

*Prognostic significance of KRAS, NRAS, BRAF and PIK3CA mutations in the entire cohort.* The average length of follow-up for all 182 CRC patients was 41.4 months (range, 14-79 months; 95% Confidence interval, 38.04-44.79 months), and 72.5% (132/182) of patients were alive when the present study was completed. In the KRAS/NRAS/BRAF/PIK3CA mutation group, there were 25 deaths; 40 patients were alive with liver, lung, or bone metastasis, and 39 patients were alive without evidence of tumor metastasis. In the KRAS wild-type group, 18 patients succumbed, and one of these patients had liver or brain metastasis. A total of 34 patients were alive with liver, lung, bone, or brain metastasis or tubular adenoma. The remaining 36 patients were alive and without evidence of tumor metastasis or recurrence. The majority of the KRAS wild-type patients (71.6%, 53/74) received targeted anti-EGFR therapy (cetuximab and panitumumab). However, no KRAS-mutant patients received anti-EGFR therapy. OS was defined from the date of pathological diagnosis of carcinoma to the date of death or the date of examination of surviving patients in September 2022. Disease-free survival (DFS) was calculated from the date of primary colorectal carcinoma resection to the date of recurrence or metastasis or the date of screening in September 2022. The KRAS/NRAS/BRAF/PIK3CA mutation group had a significantly shorter OS and DFS than the KRAS/NRAS/BRAF/PIK3CA wild-type group, as demonstrated in Fig. 3.

*IHC results.* Among 182 CRC patients, the rate of positive KRAS protein staining by IHC was 69.8% (127/182). The rate of positive KRAS protein staining in patients with mutated KRAS was 65.9% (54/82), while that in patients without KRAS mutation was 73.0% (73/100). No significant difference was found between KRAS-mutant patients and nonmutated patients ( $P=0.2692$ ). The rate of positive NRAS protein staining by IHC was 83.0% (151/182), and the rate of positive NRAS protein staining in NRAS-mutant patients was 72.7% (8/11). The rate of positive NRAS staining in non-mutated patients was 83.6% (143/171), and

no significant difference was identified between NRAS-mutated and nonmutated patients ( $P=0.4024$ ). The rate of positive BRAF protein staining by IHC was 85.7% (156/182). The rate of positive BRAF protein staining in BRAF-mutated patients was 62.5% (5/8), and that in patients without BRAF mutation was 87.3% (151/173). No significant difference was found between BRAF-mutated and nonmutated patients ( $P=0.0817$ ). The rate of positive PIK3CA protein staining by IHC was 84.1% (153/182). The rate of positive PIK3CA protein staining in PIK3CA-mutated patients was 81.8% (9/11), and that in patients without PIK3CA mutation was 84.8% (145/173). No significant difference was identified between PIK3CA-mutated and non-mutated patients ( $P=0.6785$ ). The total rate of positive of KRAS/NRAS/BRAF/PIK3CA protein staining by IHC was not significantly different between KRAS/NRAS/BRAF/PIK3CA-mutated and nonmutated patients ( $P=0.5882$ ), as shown in Table V. A typical sample with KRAS, NRAS, BRAF and PIK3CA protein IHC staining is shown in Fig. 4A-H.

*Association between gene mutation and RNF215 expression.* According to the results obtained from TIMER 2.0 database (Fig. 5), RNF215 expression was significantly higher in the mutated BRAF group than in the wild-type BRAF group in CRC ( $P<0.05$ ). However, no significant differences were identified between the mutated KRAS, NRAS and PIK3CA groups and their corresponding wild-type groups ( $P>0.05$ ) in patients with CRC. To further validate the expression level of RNF215 in CRC samples with KRAS, NRAS, BRAF and PIK3CA mutations, RNF215 immunoassays with 182 CRC samples were performed. Interestingly, the IHC results revealed no significant differences between the mutated and corresponding wild-type groups (all  $P>0.05$ ) (Fig. 6).

## Discussion

CRC is one of the most prevalent malignant tumors worldwide (3-5). In the past few decades, great advances have been

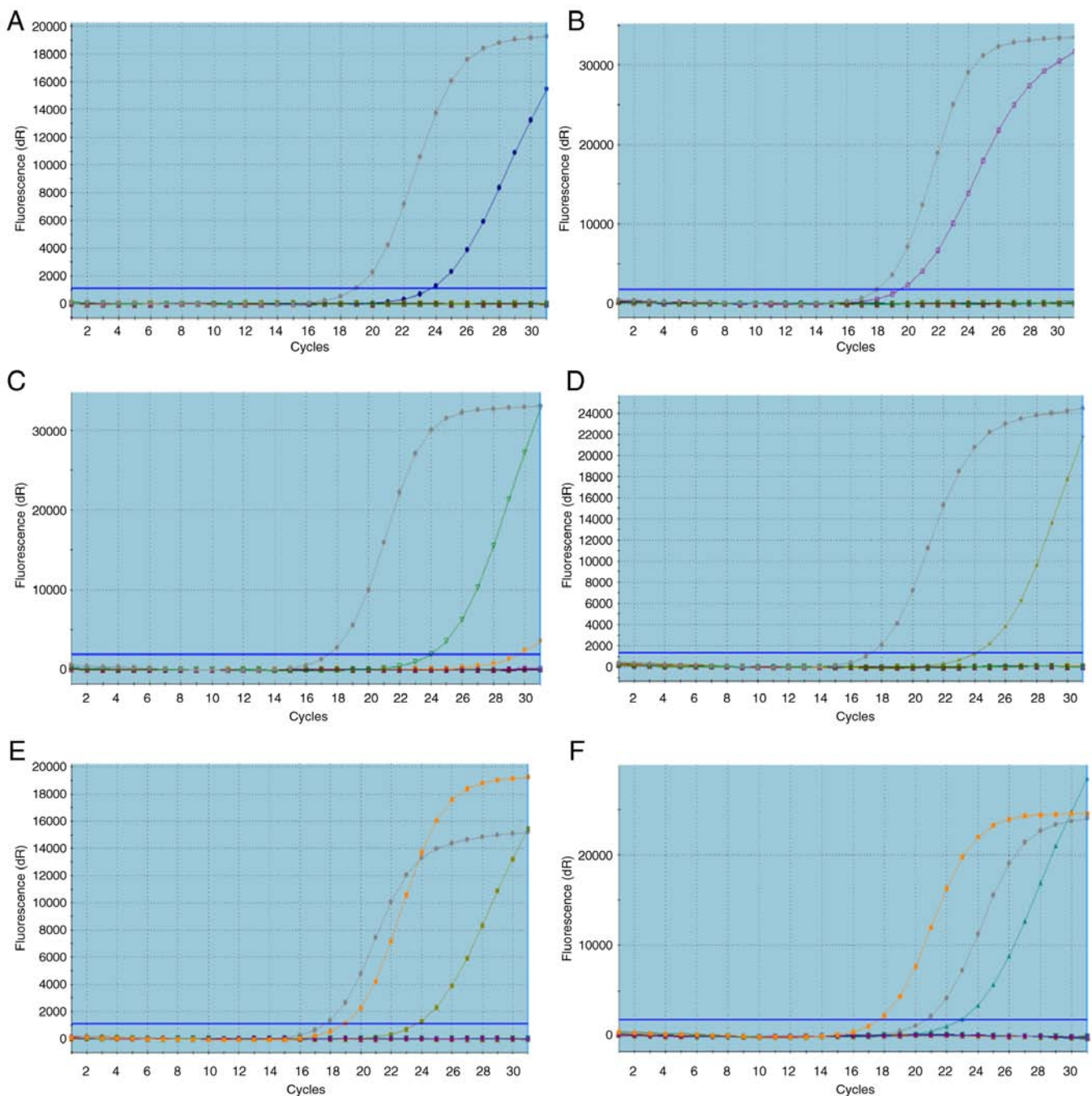


Figure 1. PCR mutation curves for the KRAS, NRAS, BRAF and PIK3CA genes in patients with colorectal cancer. (A) KRAS-EXON2-G13D mutation. (B) NRAS-EXON2-G12 mutation. (C) BRAF-EXON15 mutation. (D) PIK3CA-EXON10 mutation. (E) Concomitant KRAS-EXON2 and NRAS-EXON2 mutations. (F) Concomitant KRAS-EXON3 and PIK3CA-EXON10 mutations.

made in the clinical treatment of CRC through improvements in the understanding of its pathophysiology and molecular mechanisms. However, CRC is a heterogeneous disease with different treatment responses and prognoses (24). Therefore, it is necessary to identify molecular markers with predictive or prognostic value. EGFR has been suggested as a target for the treatment of CRC, and KRAS gene mutations play a dominant role in resistance to EGFR inhibitors. However, there are other possible mechanisms underlying this resistance; these mechanisms include ligand expression, increased EGFR copy number, BRAF gene mutations and activation of other signaling pathways (25). Therefore, gene mutations in exons 2,

3 and 4 of KRAS and NRAS, exon 15 of BRAF, and exon 20 of PIK3CA have been recognized for their predictive value in anti-EGFR-targeted therapy (26). Thus, in the present study, these exons of the aforementioned genes were analyzed in 182 patients with CRC, aiming to provide reference data for clinical treatment.

In CRC, the RAS family is the most frequently researched malignant gene family. In this family, the KRAS gene is the most commonly researched family member. KRAS can activate the downstream PI3K pathway and affect cell proliferation and differentiation. Reportedly, the KRAS mutation rate in patients with CRC is 30-50% (27,28). KRAS mutations most

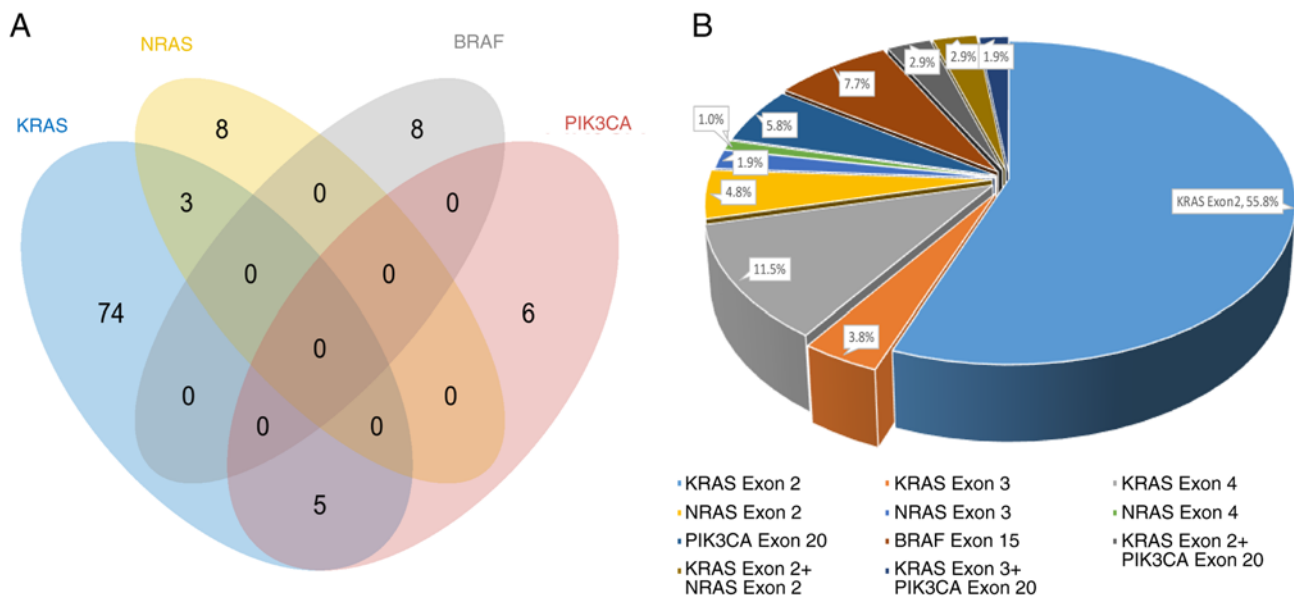


Figure 2. Analysis of concomitant mutations in KRAS, NRAS, BRAF and PIK3CA. (A) Venn diagram showing the distribution of single and concomitant mutations in the KRAS, NRAS, BRAF and PIK3CA genes. (B) Frequency of mutations in KRAS, NRAS, BRAF and PIK3CA exons in patients with CRC. The mutations in all exons are listed in the figure. Among them, the frequency of KRAS exon 2 mutations was the highest (55.8%), and concomitant mutations accounted for 7.7% (8/104) of mutations.

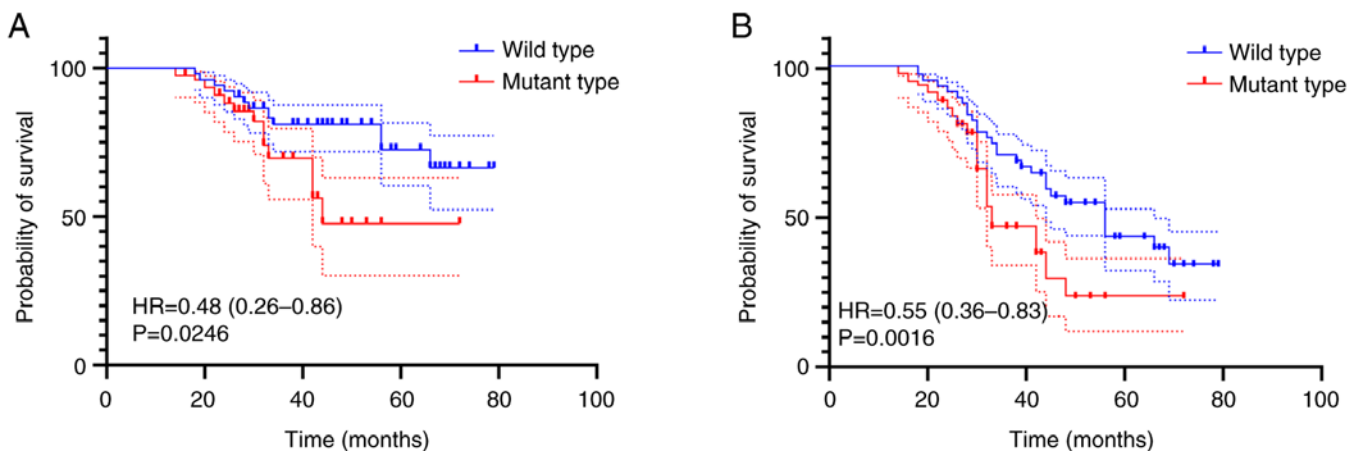


Figure 3. Kaplan-Meier curves for patients with colorectal cancer with and without KRAS mutations. (A) Comparison of overall survival, showing a significant difference ( $P=0.0246$ ). (B) Comparison of disease-free survival, showing a significant difference ( $P=0.0016$ ). HR, hazard ratio.

commonly occur in codons 12 and 13 of exon 2 and codons 59 and 61 of exon 3 (21). In the present study, the mutation rate of the KRAS gene alone was 40.7% (74/182), and the concomitant mutation rate of KRAS and other genes was 4.4% (8/182). These mutations mainly occurred in the 12 and 13th codons of exon 2, which demonstrated the highest mutation rate (61.5%, 64/104). The mutation rate of the Q61 codon of exon 3 was 3.3% (6/182). The mutation rate of the K117 and A146 codons in exon 4 was 6.6% (12/182), which was consistent with the results in related studies (29). Numerous studies have claimed that KRAS gene mutations are more likely to occur in women and right-sided colon cancer patients (27,30). Chang *et al* (31) suggested that KRAS gene mutations are associated with pathological differentiation and the number of metastatic lymph nodes. A previous study also suggested correlations among patient age, tumor site and KRAS mutations (32). However,

no significant association between KRAS gene mutations and the clinicopathological characteristics of patients was found in the present study ( $P>0.05$ ), which may be related to ethnic and regional differences and the sample size. Therefore, this finding requires further validation with a larger sample size.

NRAS is a common oncogene in human tumors and an important member of the RAS gene family. The NRAS mutation rate in CRC has been reported to be 2.2–7% (33,34). In the present study, the total NRAS mutation rate was 6.0% (11/182). The rate of mutation in codons 12 and 13 in exon 2 was 4.4% (8/182), that in codon Q61 of exon 3 was 1.1% (2/182), and that in codon A146T of exon 4 was 0.5% (1/182), which was consistent with the findings of a previous study (33). A previous study by Russo *et al* (35) revealed that NRAS gene mutations were more likely to occur in older adults. However, no significant association was found between the clinicopathological



Table V. Immunohistochemical results of the KRAS, NRAS, BRAF, and PIK3CA genes in 182 patients with CRC.

	Positive (n)	Negative (n)	P-value
KRAS			0.2692
Mutated	54	28	
Unmutated	73	27	
NRAS			0.4024
Mutated	8	3	
Unmutated	143	28	
BRAF			0.0817
Mutated	5	3	
Unmutated	151	22	
PIK3CA			0.6785
Mutated	9	2	
Unmutated	145	26	
Total (KRAS + NRAS + BRAF + PIK3CA)			0.5882
Mutated	94	10	
Unmutated	73	5	

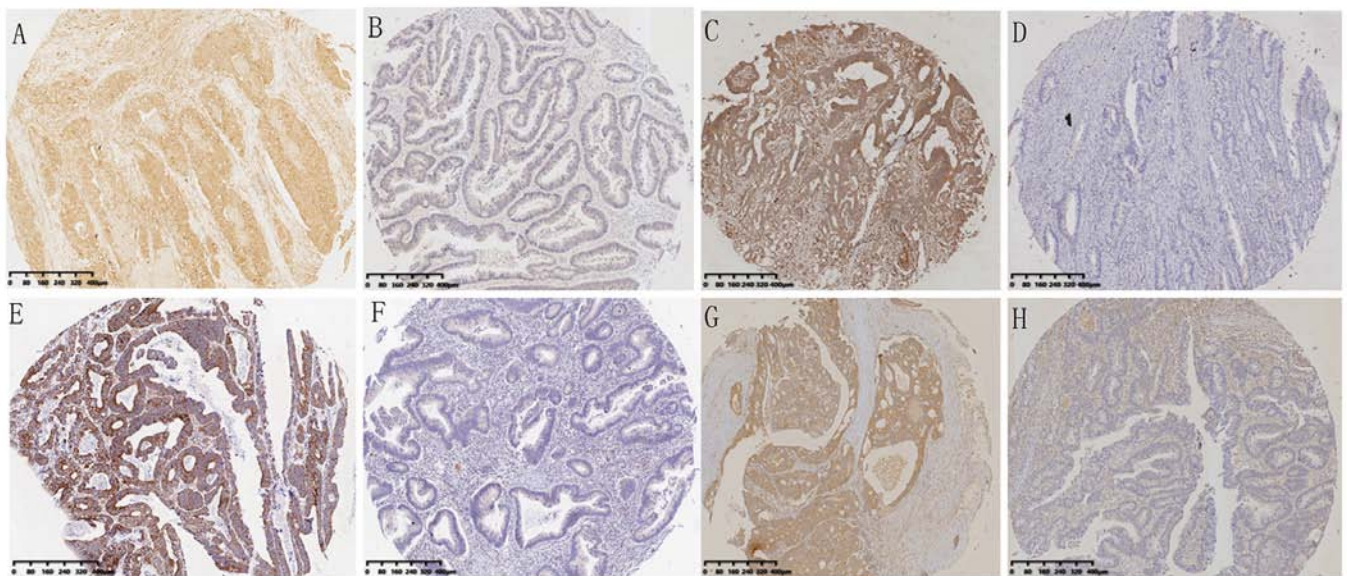


Figure 4. Typical IHC images of KRAS, NRAS, BRAF and PIK3CA staining in samples from patients with colorectal cancer observed under a light microscope. The brown-yellow particles of the cytoplasm indicate positive staining. (A) Positive expression of KRAS on IHC. (B) Negative expression of KRAS on IHC. (C) Positive expression of NRAS on IHC. (D) Negative expression of NRAS on IHC. (E) Positive expression of BRAF on IHC. (F) Negative expression of BRAF on IHC. (G) Positive expression of PIK3CA on IHC. (H) Negative expression of PIK3CA on IHC. IHC, immunohistochemistry.

features of CRC and NRAS mutations in the present study ( $P>0.05$ ). The differences may be associated with sample size, detection methods, race and geographical scope.

The BRAF gene is one of the most important proto-oncogenes in humans. Reportedly, the BRAF mutation rate in CRC is 1.8-20% (36,37). The mutation rate in the present study was 4.4% (8/182), consistent with previous studies (38,39). Numerous studies have suggested that BRAF gene mutations are associated with certain clinicopathological features, including right-sided tumor location, poor differentiation and peritoneal metastasis (40). A study by Siena *et al* (41) suggested that the BRAF mutation rate in female CRC patients was significantly

higher than that in male patients. The present study found that BRAF gene mutations were more likely to occur in poorly differentiated CRC patients ( $P<0.05$ ). This may suggest that BRAF-targeted inhibitors (including vemurafenib, dabrafenib and encorafenib) may be beneficial in poorly differentiated CRC patients. However, the number of BRAF-mutant cases in the present study was too small to allow any meaningful statistical analysis, and more cases are needed for further confirmation. In addition, according to Siena *et al* (41), BRAF mutations and KRAS and NRAS mutations are mutually exclusive. In the present study, there were no KRAS or NRAS mutations in the 8 patients with BRAF gene mutations, which



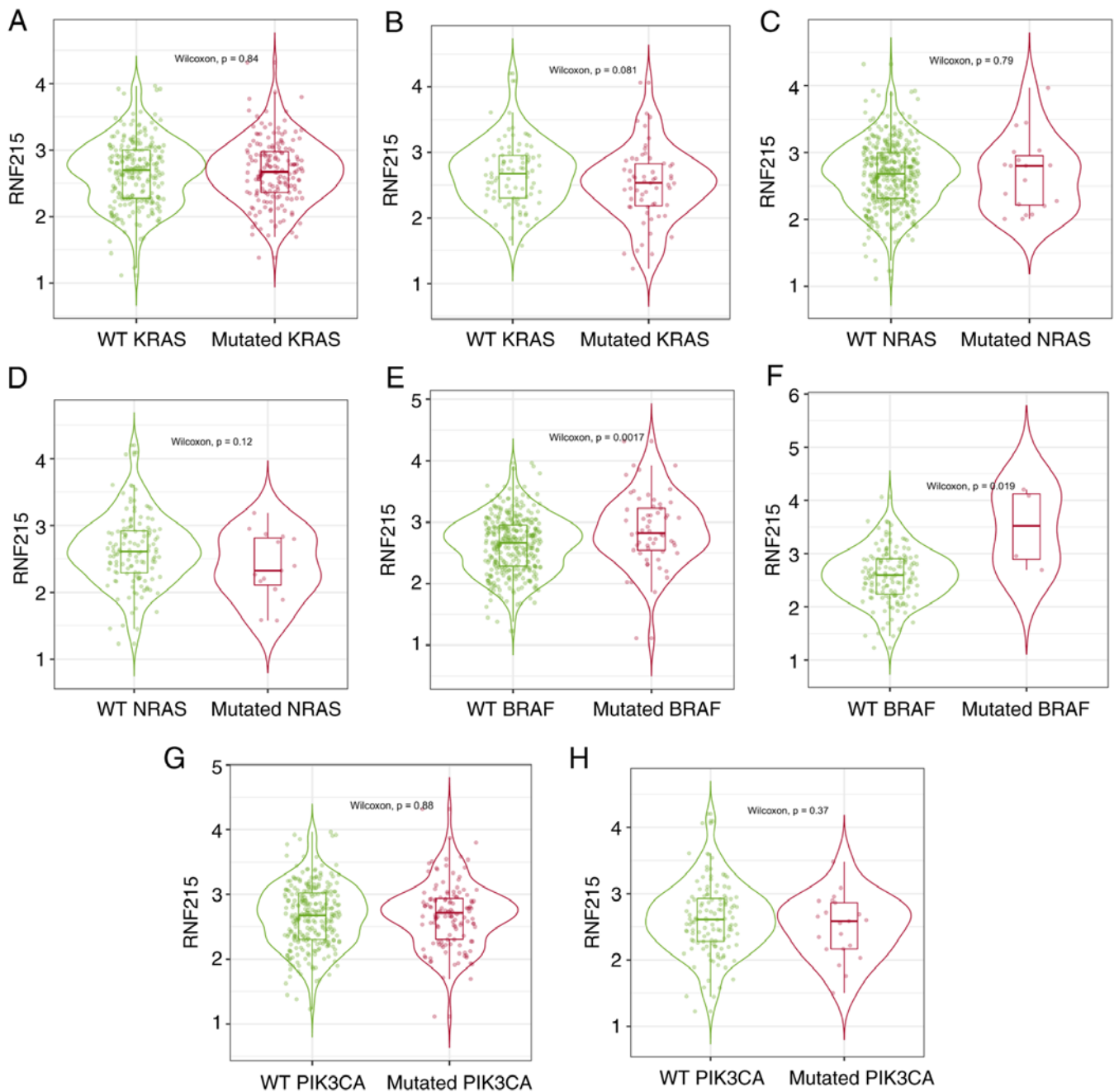


Figure 5. Comparison of RNF215 expression in the KRAS, NRAS, BRAF and PIK3CA mutant vs. WT colorectal cancer groups via the TIMER 2.0 database. (A) Expression of RNF215 in KRAS mutant vs. WT colon cancer ( $P=0.84$ ). (B) Expression of RNF215 in KRAS mutant vs. WT rectal cancer ( $P=0.081$ ). (C) Expression of RNF215 in NRAS mutant vs. WT colon cancer ( $P=0.79$ ). (D) Expression of RNF215 in NRAS mutant vs. WT rectal cancer ( $P=0.12$ ). (E) Expression of RNF215 in BRAF mutant vs. WT colon cancer ( $P=0.0017$ ). (F) Expression of RNF215 in BRAF mutant vs. WT rectal cancer ( $P=0.019$ ). (G) Expression of RNF215 in PIK3CA mutant vs. WT colon cancer ( $P=0.88$ ). (H) Expression of RNF215 in PIK3CA mutant vs. WT rectal cancer ( $P=0.37$ ). RNF215, ring finger protein 215; WT, wild-type.

was consistent with the study by Siena *et al* (41). Guo *et al* (42) suggested that the mutation rate of the BRAF gene was higher in CRC patients with lymph node metastasis. EGFR-targeted therapy is recommended for patients without driver mutations in KRAS/NRAS. Lymph node metastasis is not a criterion for targeted therapy. However, interestingly, the present study did not find that a single BRAF gene mutation was associated with the presence of lymph node metastasis. However, the total mutation rate of KRAS/NRAS/BRAF/PIK3CA in patients with lymph node metastasis was 76.1% (35/46), which was significantly higher than that in patients without lymph

node metastasis (50.8%, 69/136) ( $P<0.05$ ). EGFR selectively binds to EGFR and triggers the receptor to form a dimer that activates RAS which can transmit signals from the activated transmembrane receptor EGFR to effectors in the MAPK and PI3K/AKT signaling pathways in the cytoplasm, regulating cell survival and proliferation (43). This finding suggests that EGFR-targeted therapy may be beneficial for patients with CRC and lymph node metastasis, providing a meaningful reference index for the clinical identification of these patients, although this finding does need to be confirmed in a larger sample size.

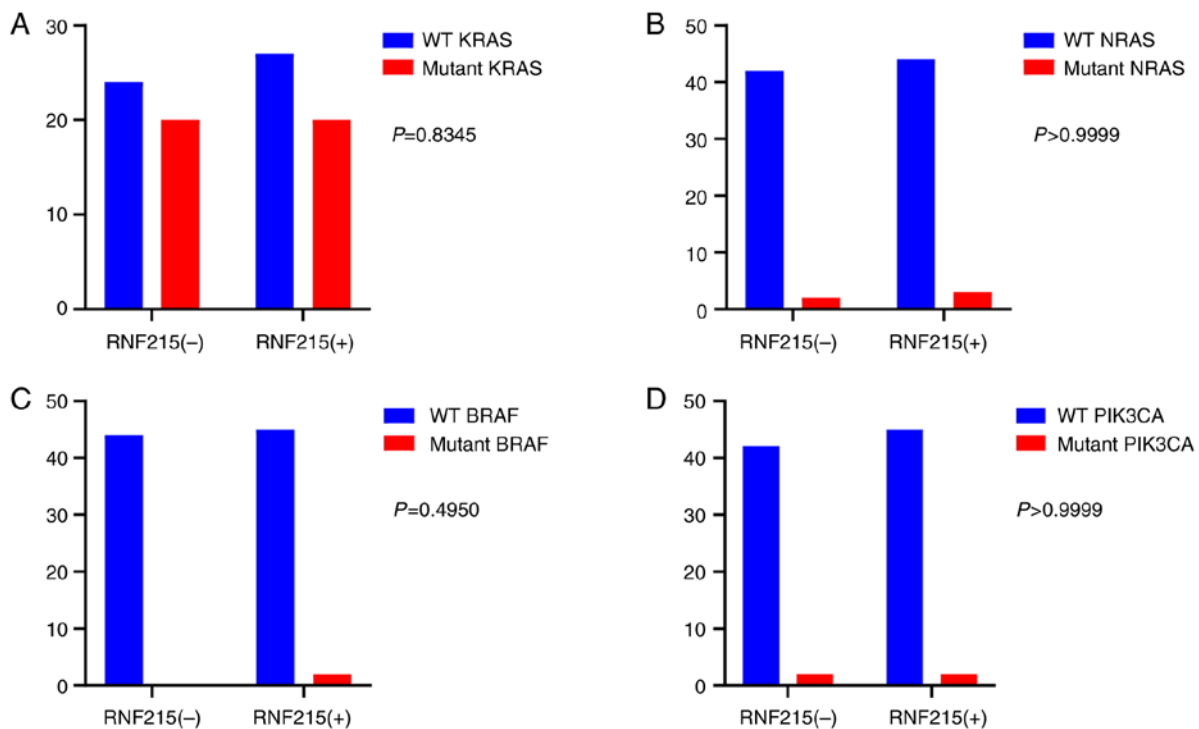


Figure 6. Comparison of RNF215 expression in the KRAS, NRAS, BRAF and PIK3CA mutant vs. WT CRC groups in 182 CRC patients. (A) Expression of RNF215 in KRAS mutant vs. WT CRC patients ( $P=0.8345$ ). (B) Expression of RNF215 in NRAS mutant vs. WT CRC patients ( $P>0.9999$ ). (C) Expression of RNF215 in BRAF mutant vs. WT CRC patients ( $P=0.4950$ ). (D) Expression of RNF215 in PIK3CA mutant vs. WT CRC patients ( $P>0.9999$ ). RNF215, ring finger protein 215; CRC, colorectal cancer; WT, wild-type.

As one of the common proto-oncogenes in CRC, PIK3CA participates in regulating cell proliferation and differentiation, apoptosis, and other functions by activating the PI3K-AKT-mTOR pathway. When PIK3CA is mutated, it can cause continuous abnormal activation of the aforementioned pathway, resulting in the development of CRC. Chen *et al* (44) confirmed that aspirin enhances the cytotoxic effect of RSL3 in PIK3CA-mutated CRC, and the combination of aspirin and a ferroptosis inducer showed promising therapeutic effects in CRC treatment. In recent years, it has been shown that in addition to KRAS mutations, PIK3CA gene mutations are common in patients with CRC, and the potential clinical value of PIK3CA mutation as a tumor marker and molecular target has been studied. Studies have shown that PIK3CA has a high mutation rate of 14-32% in Western CRC patients (45,46). In the present study, 11 mutations of the PIK3CA gene were confirmed, a mutation rate of 6.0% (11/182). A total of five of the samples with PIK3CA mutations also had mutations in the KRAS gene. The low mutation rate of PIK3CA in the present study may be related to the low mutation rate of PIK3CA in Chinese CRC patients and the fact that the current analysis only assessed exon 20 (a high mutation frequency region) of the PIK3CA gene. Studies have suggested that mutations in PIK3CA are often accompanied by mutations in other genes (8), especially KRAS, and the present study found similar results. Ye *et al* (27) found that the most common mutation was in exon 9 in the PIK3CA gene, and the exon 9 mutation of PIK3CA depended on the RAS-GTP mode. The mutation of exon 20 did not involve Ras. The study by Jang *et al* (28) revealed an association between certain KRAS and PIK3CA variants and aggressive clinicopathological characteristics.

The present study also found that PIK3CA gene mutations were related to the site of tumor occurrence, but because of the small sample size, it was not possible to perform additional analyses. Moreover, Mao *et al* (47) suggested that mutations in exon 20 of the PIK3CA gene are a biomarker of anti-EGFR monoclonal antibody resistance in patients with KRAS wild-type metastatic CRC, and failure of anti-EGFR therapy in KRAS wild-type patients may be caused by PIK3CA gene mutations.

In patients with CRC, EGFR-mediated signaling pathway activation via ligand binding can lead to the activation of the two major downstream signaling pathways (RAS/RAF/MAPK and PI3K/AKT/mTOR). PIK3CA gene mutations are often accompanied by KRAS mutations (the concomitant mutation rate in the present study was 45.4%), and studies have shown that EGFR-targeted therapy is beneficial for wild-type CRC patients without KRAS and PIK3CA mutations. In addition, KRAS wild-type patients with PIK3CA mutations are more likely to develop resistance to EGFR-targeted therapy, which may be due to EGFR-targeted drugs blocking the RAS/RAF/MAPK pathway. However, cell proliferation can still be caused by the activation of PI3K/AKT/mTOR. Hence, it is considered that the failure of EGFR-targeted treatment in KRAS wild-type patients may be associated with mutations in the PIK3CA gene, which may represent a new mechanism underlying EGFR-targeted treatment failure (10). Therefore, a combined assessment of multiple genes is crucial for the selection of treatment options, and PIK3CA gene mutations may become a new target for the treatment of CRC. When choosing a molecularly targeted therapy plan for patients with CRC, multiple genes involved in the signaling pathways being

targeted should be assessed before supporting the development of a more precise treatment plan (48).

Although the present study showed that the KRAS/NRAS/BRAF/PIK3CA gene mutation in patients with lymph node metastasis was significantly higher than that in patients without lymph node metastasis, the present study also suggested that KRAS, NRAS, BRAF and PIK3CA mutations were not significantly associated with IHC protein expression. It is considered that this may be attributed to the fact that the primary anti-KRAS antibody used in the present study was a polyclonal antibody, and although the antibodies used to detect NRAS, BRAF and PIK3CA were monoclonal antibodies, only common hotspot mutation sites for gene mutations were detected, and these hotspot mutation sites were not detected in the IHC analysis (NRAS, clone sp174; BRAF, clone VE1; PIK3CA, clone sp139). In addition, it is also considered that the sensitivity and specificity of the IHC antibodies may also need to be further improved. Another explanation may be the fact that only 182 Chinese CRC cases were analysed. Therefore, although IHC is more convenient and inexpensive, the results of the present study, suggested that KRAS, NRAS, BRAF and PIK3CA gene mutations in CRC patients cannot be detected by IHC. DNA sequencing and ARMS are still the most effective means of detecting genetic mutations.

In addition, 39 patients were alive without evidence of tumor metastasis in the present cohort study. This could be caused by the following reasons: First, some patients had a short follow-up time, and when the follow-up time is extended, distant metastasis may occur in these patients. Second, some patients were early-stage patients, therefore, they may receive timely treatment and did not develop metastasis. Third, the number of specimens in the present study was relatively small, with only 182 patients, and if the number of specimens is sufficiently large, metastasis might occur in more patients. Finally, this might be related to race, as all the patients selected for the present study, were Chinese patients with CRC, and it is possible that more patients with distant metastases would have developed if more racial patients from multiple centers had been included.

In recent years, it has been postulated that genetic mutations can be used for the early diagnosis of CRC. He *et al* (49) suggested that fecal TP53 and KRAS could be used as specific genes for CRC screening, diagnosis, prognosis prediction and recurrence monitoring (49). Similar results were obtained by Lin *et al* (50), who found that the combined detection of fecal KRAS/BRAF/APC mutations and SFRP2/SDC2 methylation had potential application value for the auxiliary diagnosis of CRC. It has been also hypothesized that independent clones with pathogenic KRAS and TP53 mutations are common in individuals with CRC (51). Alizadeh-Sedigh *et al* (52) found that a panel identifying PIK3CA, KRAS and BRAF mutations had favorable performance in detecting CRC DNA in plasma circulating-free DNA. According to the results of the gene mutation analysis of the present study and literature review, it was hypothesized that KRAS/NRAS/BRAF/PIK3CA gene mutations could be biomarkers of carcinogenesis in colorectal adenoma patients. However, this needs to be further verified with further research.

Increasing attention has been given to personalized targeted therapy for CRC. Some targeted drugs, such as cetuximab,

panitumumab and bevacizumab, have been shown to have a positive effect in the treatment of CRC patients. KRAS mutations are a poor prognostic factor in CRC patients (53), and mutations in codons 12 and 13 specifically are potentially associated with reduced efficacy of anti-EGFR monoclonal antibodies (54,55). The present study also suggested that KRAS wild-type CRC patients had longer OS and DFS than KRAS-mutated CRC patients. This may be related to the fact that the majority of the KRAS wild-type patients received targeted therapy (cetuximab and panitumumab), and numerous patients benefit greatly from these targeted drugs in the clinic. However, some researchers have shown that KRAS G12C can be targeted in KRAS-mutated patients by a covalent compound that locks the mutant protein in its inactive GDP-bound state (56). Furthermore, NRAS, BRAF and PIK3CA mutations may negatively affect the response to EGFR inhibitors. Patients with BRAF (4.7%), PIK3CA exon 20 (3%), and NRAS mutations (2%) had a lower response rate to cetuximab plus chemotherapy (57). Cathomas (55) suggested that PIK3CA mutations were associated with poorer clinical outcomes and poor response to targeted therapy with anti-EGFR monoclonal antibodies in CRC patients with wild-type RAS. Moreover, it may be possible to design vaccines for RAS, BRAF, or PIK3CA mutant peptides or immunotherapies using polyclonal T cells to target these gene mutations in the future.

The present study assessed the relationship between KRAS, NRAS, BRAF and PIK3A mutations and RNF215 expression for the first time. No significant differences in RNF215 expression were found between the mutated KRAS, NRAS and PIK3CA groups and their corresponding wild-type groups ( $P>0.05$ ) in CRC patients. RNF215 expression was significantly higher in the mutated BRAF group than in the wild-type BRAF group according to the TIMER 2.0 database, indicating that BRAF mutations may be associated with RNF215 expression. However, interestingly, the immunostaining results of the present study did not show a significant difference. This may be caused by the small number of patients in the present study, with only three patients with BRAF mutations. Therefore, the association between KRAS, NRAS, BRAF, and PIK3CA mutations and RNF215 expression needs to be further investigated in more patients in the future. According to a previous study by the authors (20), RNF215 was associated with several important pathways involved in CRC occurrence, including MAPK signaling pathway and the RAS signaling pathway. Therefore, it was considered that BRAF may regulate the RNF215 expression via the MAPK signaling pathway. However, the detailed mechanism by which BRAF regulates RNF215 expression needs to be further confirmed because of the lack of relevant studies.

In addition, apart from CRC, through a search in PubMed, three types of tumors that may be associated with the KRAS/NRAS/BRAF/PIK3CA axis were identified, including lung cancer, ameloblastoma and ovarian cancer. Seo *et al* (58) identified driver somatic mutations in EGFR, KRAS, NRAS, BRAF and PIK3CA in lung adenocarcinoma. Nguyen *et al* (59) revealed driver mutations in FGFR2, KRAS, NRAS, PIK3CA and SMO in ameloblastoma. Somatic mutations were identified in KRAS, NRAS, BRAF, PIK3CA, EGFR and PTEN in ovarian cancer patients by

Despierre *et al* (60). Rachiglio *et al* (61) investigated the presence of hotspot mutations in genes, including KRAS, NRAS, BRAF, ERBB2, PIK3CA and MET, in patients with non-small cell lung cancer.

It appears that the collection of samples in the present study is quite biased to KRAS mutation and there are numerous KRAS mutations. However, in fact, CRC cases were not intentionally selected, and this result is a true reflection of the KRAS, NRAS, BRAF and PIK3CA mutation status of these cases, with similar findings to some recent studies (62,63). The KRAS mutation rate was high, and it is considered that this may be due to the limitations of the present study's analysis. First, the present study was performed on a relatively small number of patients at a single center, with only 182 patients, and the limited sample size prevented the authors from drawing firm conclusions. Second, ARMS was utilized to detect KRAS/NRAS/BRAF/PIK3CA gene mutations and selected coding regions of the four genes were only analyzed, and the KRAS hotspot (up to 17) mutation was the highest, as shown in Table II. Therefore, all mutation sites were not assessed with NGS or other sequencing analyses. Other limitations in the present study include the retrospective nature of the analysis, the small number of genes analyzed, the limited number of exons tested for each gene, ethnic and regional differences. In addition, some of the statistically significant associations between the mutated genes and clinicopathological features may be random effects based on the limited number of cases. In summary, there are few overall data, and more research is needed. Furthermore, there is very limited information regarding the prognostic/predictive value of simultaneous detection of these genetic alterations in the same tumor. Therefore, in the future studies, the authors will collaborate with multiple research centers to include CRC patients from more geographically diverse populations to further validate the present findings.

In summary, in the present study, more than 50% of the patients with CRC had one or more gene mutations. KRAS mutations were the most common mutations, mainly in codons 12 and 13 of exon 2, while mutations in NRAS, BRAF and PIK3CA were relatively rare. KRAS wild-type CRC patients could benefit from EGFR-targeted drugs, and KRAS mutations may be a poor prognostic factor in CRC patients. The simultaneous detection of KRAS/NRAS/BRAF/PIK3CA gene mutations is conducive to the development of the most suitable treatment regimen for CRC patients and will help provide new targets for the development of new therapeutic drugs for CRC. It was found that KRAS/NRAS/BRAF/PIK3CA gene mutations were associated with certain clinicopathological characteristics of CRC patients. The present study also showed that PIK3CA mutations may often be accompanied by KRAS mutations. Therefore, PIK3CA gene mutations may prevent patients with wild-type KRAS from benefiting from EGFR-targeted therapy. In addition, the present study also demonstrated that BRAF mutations may be associated with RNF215 expression. However, these findings need to be further verified in larger cohorts. In addition, the results of the present study indicated that gene mutations in CRC patients are best detected by DNA sequencing or ARMS.

## Acknowledgements

Not applicable.

## Funding

The present study was supported by the High-level Professional Physician Training Program of Minhang, Shanghai (grand no. 2020MZYS10) and the Natural Science Research Project of the Science and Technology Commission in Minhang, Shanghai (grand no. 2022MHZ043).

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

JBW conceptualized the study, developed methodology and wrote the original draft. HL and YJL conducted the research and provided experimental suggestions. XJL performed data curation and analysis. XPL designed the study and performed the final revision of the manuscript. All authors have read and approved the final version of the manuscript. JBW and XPL confirm the authenticity of all the raw data.

## Ethics approval and consent to participate

The studies were reviewed and approved by the Ethical Committee of Shanghai Fifth People's Hospital, Fudan University (approval no. 2021071; Shanghai, China). Written informed consent was provided by all patients/participants to participate in the present study.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

## References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A and Bray F: Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA Cancer J Clin* 71: 209-249, 2021.
2. Li N, Lu B, Luo C, Cai J, Lu M, Zhang Y, Chen H and Dai M: Incidence, mortality, survival, risk factor and screening of colorectal cancer: A comparison among China, Europe, and northern America. *Cancer Lett* 522: 255-268, 2021.
3. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* 68: 394-424, 2018.
4. Zheng ZX, Zheng RS, Zhang SW and Chen WQ: Colorectal cancer incidence and mortality in China, 2010. *Asian Pac J Cancer Prev* 15: 8455-8460, 2014.
5. Zhang L, Cao F, Zhang G, Shi L, Chen S, Zhang Z, Zhi W and Ma T: Trends in and predictions of colorectal cancer incidence and mortality in China from 1990 to 2025. *Front Oncol* 9: 98, 2019.

6. Reggiani Bonetti L, Barresi V, Bettelli S, Caprera C, Manfredini S and Maiorana A: Analysis of KRAS, NRAS, PIK3CA, and BRAF mutational profile in poorly differentiated clusters of KRAS-mutated colon cancer. *Hum Pathol* 62: 91-98, 2017.
7. Barresi V, Reggiani Bonetti L, Vitarelli E, Di Gregorio C, Ponz de Leon M and Barresi G: Immunohistochemical assessment of lymphovascular invasion in stage I colorectal carcinoma: Prognostic relevance and correlation with nodal micrometastases. *Am J Surg Pathol* 36: 66-72, 2012.
8. Hossain MS, Karuniawati H, Jairoun AA, Urbi Z, Ooi J, John A, Lim YC, Kibria KMK, Mohiuddin AKM, Ming LC, *et al*: Colorectal cancer: A review of carcinogenesis, global epidemiology, current challenges, risk factors, preventive and treatment strategies. *Cancers (Basel)* 14: 1732, 2022.
9. Liu J, Hu J, Cheng L, Ren W, Yang M, Liu B, Xie L and Qian X: Biomarkers predicting resistance to epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer with wild-type KRAS. *Oncotargets Ther* 9: 557-565, 2016.
10. Palomba G, Doneddu V, Cossu A, Paliogiannis P, Manca A, Casula M, Colombino M, Lanzillo A, Defraia E, Pazzola A, *et al*: Prognostic impact of KRAS, NRAS, BRAF, and PIK3CA mutations in primary colorectal carcinomas: A population-based study. *J Transl Med* 14: 292, 2016.
11. Li QH, Wang YZ, Tu J, Liu CW, Yuan YJ, Lin R, He WL, Cai SR, He YL and Ye JN: Anti-EGFR therapy in metastatic colorectal cancer: Mechanisms and potential regimens of drug resistance. *Gastroenterol Rep (Oxf)* 8: 179-191, 2020.
12. De Roock W, De Vriendt V, Normanno N, Ciardiello F and Tejpar S: KRAS, BRAF, PIK3CA, and PTEN mutations: Implications for targeted therapies in metastatic colorectal cancer. *Lancet Oncol* 12: 594-603, 2011.
13. Cremolini C, Benelli M, Fontana E, Pagani F, Rossini D, Fucà G, Busico A, Conca E, Di Donato S, Loupakakis F, *et al*: Benefit from anti-EGFRs in RAS and BRAF wild-type metastatic transverse colon cancer: A clinical and molecular proof of concept study. *ESMO Open* 4: e000489, 2019.
14. McCubrey JA, Steelman LS, Abrams SL, Lee JT, Chang F, Bertrand FE, Navolanic PM, Terrian DM, Franklin RA, D'Assoro AB, *et al*: Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. *Adv Enzyme Regul* 46: 249-279, 2006.
15. Liao X, Lochhead P, Nishihara R, Morikawa T, Kuchiba A, Yamauchi M, Imamura Y, Qian ZR, Baba Y, Shima K, *et al*: Aspirin use, tumor PIK3CA mutation, and colorectal-cancer survival. *N Engl J Med* 367: 1596-1606, 2012.
16. Wojas-Krawczyk K, Kalinka-Warzechka E, Reszka K, Nicos M, Szumilo J, Mańdziuk S, Szczepaniak K, Kupnicka D, Lewandowski R, Milanowski J and Krawczyk P: Analysis of KRAS, NRAS, BRAF, and PIK3CA mutations could predict metastases in colorectal cancer: A preliminary study. *Adv Clin Exp Med* 28: 67-73, 2019.
17. Wu Y, Chen D, Hu Y, Zhang S, Dong X, Liang H, Liang M, Zhu Y, Tan C, An S, *et al*: Ring finger protein 215 negatively regulates type I IFN production via blocking NF- $\kappa$ B p65 activation. *J Immunol* 209: 2012-2021, 2022.
18. Ma J, Li R and Wang J: Characterization of a prognostic fourgene methylation signature associated with radiotherapy for head and neck squamous cell carcinoma. *Mol Med Rep* 20: 622-632, 2019.
19. McIntosh LA, Marion MC, Sudman M, Comeau ME, Becker ML, Bohnsack JF, Fingerlin TE, Griffin TA, Haas JP, Lovell DJ, *et al*: Genome-Wide association meta-analysis reveals novel juvenile idiopathic arthritis Susceptibility Loci. *Arthritis Rheumatol* 69: 2222-2232, 2017.
20. Wu JB, Li XJ, Liu H and Liu XP: Ring finger protein 215 is a potential prognostic biomarker involved in immune infiltration and angiogenesis in colorectal cancer. *Biomedical Reports* 19: 50, 2023.
21. Lindner AU, Carberry S, Monsefi N, Barat A, Salvucci M, O'Byrne R, Zanella ER, Cremona M, Hennessy BT, Bertotti A, *et al*: Systems analysis of protein signatures predicting cetuximab responses in KRAS, NRAS, BRAF and PIK3CA wild-type patient-derived xenograft models of metastatic colorectal cancer. *Int J Cancer* 147: 2891-2901, 2020.
22. Kononen J, Bubendorf L, Kallioniemi A, Bärklund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G and Kallioniemi OP: Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4: 844-847, 1998.
23. Li T, Fu J, Zeng Z, Cohen D, Li J, Chen Q, Li B and Liu XS: TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res* 48: W509-W514, 2020.
24. Peng J, Huang D, Poston G, Ma X, Wang R, Sheng W, Zhou X, Zhu X and Cai S: The molecular heterogeneity of sporadic colorectal cancer with different tumor sites in Chinese patients. *Oncotarget* 8: 49076-49083, 2017.
25. Yao S, Wang X, Li C, Zhao T, Jin H and Fang W: Kaempferol inhibits cell proliferation and glycolysis in esophagus squamous cell carcinoma via targeting EGFR signaling pathway. *Tumour Biol* 37: 10247-10256, 2016.
26. Wang B, Wu S, Huang F, Shen M, Jiang H, Yu Y, Yu Q, Yang Y, Zhao Y, Zhou Y, *et al*: Analytical and clinical validation of a novel amplicon-based NGS assay for the evaluation of circulating tumor DNA in metastatic colorectal cancer patients. *Clin Chem Lab Med* 57: 1501-1510, 2019.
27. Ye ZL, Qiu MZ, Tang T, Wang F, Zhou YX, Lei MJ, Guan WL and He CY: Gene mutation profiling in Chinese colorectal cancer patients and its association with clinicopathological characteristics and prognosis. *Cancer Med* 9: 745-756, 2020.
28. Jang S, Hong M, Shin MK, Kim BC, Shin HS, Yu E, Hong SM, Kim J, Chun SM, Kim TI, *et al*: KRAS and PIK3CA mutations in colorectal adenocarcinomas correlate with aggressive histological features and behavior. *Hum Pathol* 65: 21-30, 2017.
29. Li ZZ, Wang F, Zhang ZC, Wang F, Zhao Q, Zhang DS, Wang FH, Wang ZQ, Luo HY, He MM, *et al*: Mutation profiling in Chinese patients with metastatic colorectal cancer and its correlation with clinicopathological features and anti-EGFR treatment response. *Oncotarget* 7: 28356-28368, 2016.
30. Fan JZ, Wang GF, Cheng XB, Dong ZH, Chen X, Deng YJ and Song X: Relationship between mismatch repair protein, RAS, BRAF, PIK3CA gene expression and clinicopathological characteristics in elderly colorectal cancer patients. *World J Clin Cases* 9: 2458-2468, 2021.
31. Chang XN, Shang FM, Jiang HY, Chen C, Zhao ZY, Deng SH, Fan J, Dong XC, Yang M, Li Y, *et al*: Clinicopathological features and prognostic value of KRAS/NRAS/BRAF mutations in colorectal cancer patients of central China. *Curr Med Sci* 41: 118-126, 2021.
32. Chang YY, Lin PC, Lin HH, Lin JK, Chen WS, Jiang JK, Yang SH, Liang WY and Chang SC: Mutation spectra of RAS gene family in colorectal cancer. *Am J Surg* 212: 537-544.e3, 2016.
33. Zheng G, Tseng LH, Haley L, Ibrahim J, Bynum J, Xian R, Gocke CD, Eshleman JR and Lin MT: Clinical validation of coexisting driver mutations in colorectal cancers. *Hum Pathol* 86: 12-20, 2019.
34. Bokemeyer C, Kohne CH, Ciardiello F, Lenz HJ, Heinemann V, Klinkhardt U, Beier F, Duecker K, van Krieken JH and Tejpar S: FOLFOX4 plus cetuximab treatment and RAS mutations in colorectal cancer. *Eur J Cancer* 51: 1243-1252, 2015.
35. Russo AL, Borger DR, Szymonifka J, Ryan DP, Wo JY, Blaszkowsky LS, Kwak EL, Allen JN, Wadlow RC, Zhu AX, *et al*: Mutational analysis and clinical correlation of metastatic colorectal cancer. *Cancer* 120: 1482-1490, 2014.
36. Knickelbein K and Zhang L: Mutant KRAS as a critical determinant of the therapeutic response of colorectal cancer. *Genes Dis* 2: 4-12, 2015.
37. Jass JR: Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 50: 113-130, 2007.
38. Zeng C, Wang M, Xie S, Wang N, Wang Z, Yi D, Kong F and Chen L: Clinical research progress on BRAF V600E-mutant advanced colorectal cancer. *J Cancer Res Clin Oncol*: Aug 28, 2023 doi: 10.1007/s00432-023-05301-0 (Epub ahead of print).
39. Zeng J, Fan W, Li J, Wu G and Wu H: KRAS/NRAS mutations associated with distant metastasis and BRAF/PIK3CA mutations associated with poor tumor differentiation in colorectal cancer. *Int J Gen Med* 16: 4109-4120, 2023.
40. Chen D, Huang JF, Liu K, Zhang LQ, Yang Z, Chuai ZR, Wang YX, Shi DC, Huang Q and Fu WL: BRAFV600E mutation and its association with clinicopathological features of colorectal cancer: A systematic review and meta-analysis. *PLoS One* 9: e90607, 2014.
41. Siena S, Sartore-Bianchi A, Di Nicolantonio F, Balfour J and Bardelli A: Biomarkers predicting clinical outcome of epidermal growth factor receptor-targeted therapy in metastatic colorectal cancer. *J Natl Cancer Inst* 101: 1308-1324, 2009.
42. Guo F, Gong H, Zhao H, Chen J, Zhang Y, Zhang L, Shi X, Zhang A, Jin H, Zhang J and He Y: Mutation status and prognostic values of KRAS, NRAS, BRAF and PIK3CA in 353 Chinese colorectal cancer patients. *Sci Rep* 8: 6076, 2018.



43. Li ZN, Zhao L, Yu LF and Wei MJ: BRAF and KRAS mutations in metastatic colorectal cancer: Future perspectives for personalized therapy. *Gastroenterol Rep (Oxf)* 8: 192-205, 2020.
44. Chen H, Qi Q, Wu N, Wang Y, Feng Q, Jin R and Jiang L: Aspirin promotes RSL3-induced ferroptosis by suppressing mTOR/SREBP-1/SCD1-mediated lipogenesis in PIK3CA-mutated colorectal cancer. *Redox Biol* 55: 102426, 2022.
45. Liao X, Morikawa T, Lochhead P, Imamura Y, Kuchiba A, Yamauchi M, Nosho K, Qian ZR, Nishihara R, Meyerhardt JA, *et al*: Prognostic role of PIK3CA mutation in colorectal cancer: cohort study and literature review. *Clin Cancer Res* 18: 2257-2268, 2012.
46. Baldus SE, Schaefer KL, Engers R, Hartleb D, Stoecklein NH and Gabbert HE: Prevalence and heterogeneity of KRAS, BRAF, and PIK3CA mutations in primary colorectal adenocarcinomas and their corresponding metastases. *Clin Cancer Res* 16: 790-799, 2010.
47. Mao C, Yang ZY, Hu XF, Chen Q and Tang JL: PIK3CA exon 20 mutations as a potential biomarker for resistance to anti-EGFR monoclonal antibodies in KRAS wild-type metastatic colorectal cancer: A systematic review and meta-analysis. *Ann Oncol* 23: 1518-1525, 2012.
48. Li W, Li H, Liu R, Yang X, Gao Y, Niu Y, Geng J, Xue Y, Jin X, You Q, *et al*: Comprehensive analysis of the relationship between RAS and RAF mutations and MSI status of colorectal cancer in Northeastern China. *Cell Physiol Biochem* 50: 1496-1509, 2018.
49. He SY, Li YC, Wang Y, Peng HL, Zhou CL, Zhang CM, Chen SL, Yin JF and Lin M: Fecal gene detection based on next generation sequencing for colorectal cancer diagnosis. *World J Gastroenterol* 28: 2920-2936, 2022.
50. Lin J, Zhang L, Chen M, Chen J, Wu Y, Wang T, Lu Y, Ba Z, Cheng X, Xu R, *et al*: Evaluation of combined detection of multi-gene mutation and SDC2/SFRP2 methylation in stool specimens for colorectal cancer early diagnosis. *Int J Colorectal Dis* 37: 1231-1238, 2022.
51. Matas J, Kohn B, Fredrickson J, Carter K, Yu M, Wang T, Gui X, Soussi T, Moreno V, Grady WM, *et al*: Colorectal cancer is associated with the presence of cancer driver mutations in normal colon. *Cancer Res* 82: 1492-1502, 2022.
52. Alizadeh-Sedigh M, Mahmoodzadeh H, Fazeli MS, Haddadi-Aghdam M and Teimoori-Toolabi L: The potential of PIK3CA, KRAS, BRAF, and APC hotspot mutations as a non-invasive detection method for colorectal cancer. *Mol Cell Probes* 63: 101807, 2022.
53. Ma BB, Mo F, Tong JH, Wong A, Wong SC, Ho WM, Wu C, Lam PW, Chan KF, Chan TS, *et al*: Elucidating the prognostic significance of KRAS, NRAS, BRAF and PIK3CA mutations in Chinese patients with metastatic colorectal cancer. *Asia Pac J Clin Oncol* 11: 160-169, 2015.
54. Cremolini C, Rossini D, Dell'Aquila E, Lonardi S, Conca E, Del Re M, Busico A, Pietrantonio F, Danesi R, Aprile G, *et al*: Rechallenge for patients with RAS and BRAF Wild-type metastatic colorectal cancer with acquired resistance to First-line cetuximab and irinotecan: A phase 2 Single-arm clinical trial. *JAMA Oncol* 5: 343-350, 2019.
55. Segelov E, Thavaneswaran S, Waring PM, Desai J, Robledo KP, Gebbski VJ, Elez E, Nott LM, Karapetis CS, Lunke S, *et al*: Response to cetuximab with or without irinotecan in patients with refractory metastatic colorectal cancer harboring the KRAS G13D mutation: Australasian Gastro-intestinal trials group ICECREAM study. *J Clin Oncol* 34: 2258-2264, 2016.
56. Ostrem JM, Peters U, Sos ML, Wells JA and Shokat KM: K-Ras(G12C) inhibitors allosterically control GTP affinity and effector interactions. *Nature* 503: 548-551, 2013.
57. De Roock W, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, *et al*: Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: A retrospective consortium analysis. *Lancet Oncol* 11: 753-762, 2010.
58. Seo JS, Ju YS, Lee WC, Shin JY, Lee JK, Bleazard T, Lee J, Jung YJ, Kim JO, Shin JY, *et al*: The transcriptional landscape and mutational profile of lung adenocarcinoma. *Genome Res* 22: 2109-2119, 2012.
59. Nguyen J, Saffari PS, Pollack AS, Vennam S, Gong X, West RB and Pollack JR: New amelanoma cell lines enable preclinical study of targeted therapies. *J Dent Res* 101: 1517-1525, 2022.
60. Despiere E, Vergote I, Anderson R, Coens C, Katsaros D, Hirsch FR, Boeckx B, Varela-Garcia M, Ferrero A, Ray-Coquard I, *et al*: Epidermal growth factor receptor (EGFR) pathway biomarkers in the randomized phase III trial of erlotinib versus observation in ovarian cancer patients with No evidence of disease progression after First-line platinum-based chemotherapy. *Target Oncol* 10: 583-596, 2015.
61. Rachiglio AM, Fenizia F, Piccirillo MC, Galetta D, Crinò L, Vincenzi B, Barletta E, Pinto C, Ferrà F, Lambiase M, *et al*: The presence of concomitant mutations affects the activity of EGFR tyrosine kinase inhibitors in EGFR-mutant non-small cell lung cancer (NSCLC) patients. *Cancers (Basel)* 11: 341, 2019.
62. Sclafani F, Wilson SH, Cunningham D, Gonzalez De Castro D, Kalaitzaki E, Begum R, Wotherspoon A, Capdevila J, Glimelius B, Roselló S, *et al*: Analysis of KRAS, NRAS, BRAF, PIK3CA and TP53 mutations in a large prospective series of locally advanced rectal cancer patients. *Int J Cancer* 146: 94-102, 2020.
63. Mahdi Y, Khmou M, Souadka A, Agouri HE, Ech-Charif S, Mounjid C and Khannoussi BE: Correlation between KRAS and NRAS mutational status and clinicopathological features in 414 cases of metastatic colorectal cancer in Morocco: The largest North African case series. *BMC Gastroenterol* 23: 193, 2023.



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