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ORIGINAL RESEARCH

KRAS/NRAS Mutations Associated with Distant Metastasis and *BRAF/PIK3CA* Mutations Associated with Poor Tumor Differentiation in Colorectal Cancer

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Background: The occurrence, progression, and prognosis of colorectal cancer (CRC) are regulated by EGFR-mediated signaling pathways. However, the relationship between the core genes (*KRAS*/*NRAS*/*BRAF*/*PIK3CA*) status in the signaling pathways and clinicopathological characteristics of CRC patients in Hakka population remains controversial.

Methods: Patients were genotyped for *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12, 61, 117, and 146), *BRAF* (codons 600), and *PIK3CA* (codons 542, 545 and 1047) mutations. Clinical records were collected, and clinicopathological characteristic associations were analyzed together with mutations of studied genes.

Results: Four hundred and eight patients (256 men and 152 women) were included in the analysis. At least one mutation in the four genes was detected in 216 (52.9%) patients, while none was detected in 192 (47.1%) patients. *KRAS, NRAS, BRAF*, and *PIK3CA* mutation status were detected in 190 (46.6%), 11 (2.7%), 10 (2.5%), 34 (8.3%) samples, respectively. *KRAS* exon 2 had the highest proportion (62.5%). Age, tumor site, tumor size, lymphovascular invasion, and perineural invasion were not associated with gene mutations. *KRAS* mutations (adjusted OR 1.675, 95% CI 1.017–2.760, *P*=0.043) and *NRAS* mutations (adjusted OR 5.183, 95% CI 1.239–21.687, *P*=0.024) appeared more frequently in patients with distant metastasis. *BRAF* mutations (adjusted OR 7.224, 95% CI 1.356–38.488, *P*=0.021) and *PIK3CA* mutations (adjusted OR 3.811, 95% CI 1.268–11.455, *P*=0.017) associated with poorly differentiated tumor.

Conclusion: *KRAS/NRAS* mutations are associated with distant metastasis and *BRAF/PIK3CA* mutations are associated with poor tumor differentiation in CRC. And the results provided a better understanding between clinicopathological characteristics and gene mutations in CRC patients.

Keywords: colorectal cancer, *KRAS*, *NRAS*, *BRAF*, *PIK3CA*, clinicopathological feature

Introduction

Colorectal cancer (CRC) is a common malignancy of the gastrointestinal tract. According to the latest estimates of global cancer incidence and mortality, CRC (10.0%) ranks third in incidence after breast cancer and lung cancer (11.7% and 11.4%, respectively), and ranks second in mortality (9.4%) after lung cancer (18%).[1] In China, from 2015 to 2020, the incidence of CRC increased rapidly, while gastrointestinal cancer (stomach, colorectal, liver and esophageal cancer) had the highest mortality rate, and CRC ranked fifth in mortality after lung, liver, stomach and esophageal cancer.[2] With the clinical application of anti-epidermal growth factor receptor (EGFR) drug cetuximab and anti-angiogenic drug bevacizumab, targeted therapy has become the first-line treatment for CRC.[3]

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The occurrence, progression, and prognosis of CRC are regulated by some molecular signal transduction pathways, such as EGFR-mediated signaling pathways. As a transmembrane tyrosine kinase receptor, EGFR can activate Ras/RAF/MAPK and PI3K/AKT/mTOR pathways after binding to corresponding ligands, thereby inducing tumor cell proliferation, invasion, metastasis and angiogenesis.[4] Target drug tyrosine kinase inhibitors (TKI) targeting the EGFR have a significant therapeutic effect in cancer patients with *EGFR* gene mutations.[5] In addition, mutations in some genes downstream of EGFR signaling pathway, such as KRAS proto-oncogene (*KRAS*), NRAS proto-oncogene (*NRAS*), B-Raf proto-oncogene (*BRAF*) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), will affect the targeted therapeutic effect of anti-EGFR monoclonal antibody drugs.[6] Mutations in these genes result in the EGFR signaling pathway always in an active status, making EGFR-targeted drug therapy ineffective.[7,8] In recent years, *KRAS/NRAS/BRAF/PIK3CA* wild-type CRC patients have significantly benefited from anti-EGFR monoclonal antibodies.

The different populations, lifestyles, and the interaction between some environmental factors and genetic polymorphisms will affect the clinicopathological characteristics of CRC patients.[9] In addition, the clinical characteristics of CRC are also related to age, and although study has found that older pT4 patients were more likely to develop serious postoperative complications, the prognosis of older patients may vary depending on the stage, tumor site, and pre-existing comorbidities.[10] Study has shown that *KRAS, NRAS* or *BRAF* mutations were associated with CRC metastasis.[11,12] However, study has shown that distant metastasis was not related to *KRAS, NRAS, BRAF*, or *PIK3CA* mutations.[13] Study has shown that *BRAF* mutations were associated with poorer differentiation, but mutant *KRAS* was associated with greater differentiation,[14] and *PIK3CA* was not associated with differentiation.[15–18] Therefore, in different sample sizes, different populations and different studies, the results of the correlation between the studied gene status and clinicopathological features are inconsistent.

The association of the studied gene status with clinicopathological features will provide valuable information for clinicians to better assess the disease severity and disease progression, such as colorectal cancer brain metastases. The development of brain metastases is an important factor in overall cancer mortality in patients with advanced cancer, and the prognosis is poor.[19] Clinicians can monitor the progress of such patients with a combination of screening techniques to achieve early detection and treatment. Electroencephalogram (EEG) biomarkers differ significantly in patients with consciousness after severe acquired brain injury, which can be used as diagnosis and prediction for clinical evaluation of patients.[20] EEG alpha band indices can be used by clinicians to diagnose, select and evaluate treatment for main neuropsychiatric disorders of the brain.[21]

The Hakka is a Han ethnic group with a unique genetic background formed by the Hakka ancestors from the Han nationality in central China, who migrated southward for many times and fused with the ancient Yue residents in Guangdong, Fujian, and Jiangxi.[22] At present, there is no study on the relationship between the mutation status of *KRAS, NRAS, BRAF, PIK3CA* genes in EGFR signaling pathway and the clinicopathological features of CRC in Hakka population. Therefore, this study retrospectively analyzed mutations of these genes in CRC tissues, and explored their relationship with clinicopathological features, aiming to provide valuable data for precise treatment of CRC individuals.

Materials and Methods

Participants

After approval from the Ethics Committee of the Meizhou People's Hospital (Clearance No.: 2020-A-57), 408 CRC patients who received *KRAS*/*NRAS*/*BRAF*/*PIK3CA* molecular testing were retrospectively investigated between January 2019 and October 2020. The inclusion criteria for the study subjects were: (1) colorectal cancer was confirmed by hematoxylin and eosin (HE) staining and histological analysis; (2) at the time of enrolment, patients presenting in stage I to IV (according to the American Joint Committee on Cancer (AJCC) guidelines[23]) were enrolled; (3) there was complete medical records; (4) aged more than 18 years. The exclusion criteria for the study subjects as follows: (1) patients with other tumors disease other than colorectal cancer; (2) other circumstances inconsistent with the inclusion criteria mentioned above.

Detection of Mismatch Repair (MMR) Protein by Immunohistochemistry

All specimens were immobilized with 4% neutral methanol solution and embedded with paraffin. Max Vision two-step method was used for immunohistochemistry. Paraffin sections were dewaxed and hydrated, then washed with PBS. Peroxidase blocker was used to block endogenous peroxidase activity. Primary antibody was added and incubated at room temperature, biotin-labeled secondary antibody was added and streptomycin antibiotin peroxidase solution was added. After incubation, Hematoxylin-3,3'-Diaminobenzidine (DAB) was added for coloration, restained with hematoxylin, dehydration, transparent, and tablets were sealed. The expression of mismatch repair proteins (MutS homolog 2 (MSH2), MutS homolog 6 (MSH6), MutL homolog 1 (MLH1), and PMS1 homolog 2 (PMS2)) was observed under the microscope. When samples from patients were positive for all four of these mismatch repair proteins (>30%) were identified as mismatch repair-proficient (pMMR), and mismatch repair-deficient (dMMR) was identified when one or more proteins were missing.

Genotyping for *KRAS, NRAS, BRAF* and *PIK3CA* Genes

Formalin-fixed and paraffin-embedded (FFPE) tissue was cut into 5μm thick slices, and 10 slices were loaded into EP tubes for DNA extraction. DNA was extracted using the AmoyDx® Tissue DNA Kit (Spin Column) (Amoy Diagnostics, Xiamen, China) according to the manufacturers' instructions. NanoDrop 2000 (Thermo Scientific) was used to measure DNA concentration. The DNA samples should be diluted to 2 ng/μL, and the OD260/OD280 should be 1.8–2.0.

Genetic tests were performed using amplification refractory mutation system polymerase-chain reaction (ARMS-PCR) with corresponding mutation detection kits, respectively (Amoy Diagnostics Co. Ltd, Xiamen, China). The DNA of the sample to be tested, positive quality control DNA, and negative quality control DNA were added to the PCR reaction solution, respectively. PCR amplification was performed according to the following conditions: initial denaturation at 95°C for 5 minutes; 95°C 25 seconds, 64°C 20 seconds, 72°C 20 seconds run 15 cycles; 95°C 25 seconds, 60°C 35 seconds, 72°C 20 seconds run 31 cycles; Amplification was performed in a LightCycler 480 real-time PCR system (Roche Diagnostics, Germany). According to the amplification curve of the experimental results, the mutation cycle threshold (Ct) values of each reaction tube and external control of the sample were determined, and the sample to be tested was determined as mutation positive or negative according to the obtained Ct values. The genetic sites tested mainly included common mutations in exons 2, 3 and 4 of *KRAS* (codons 12, 13, 61, 117, and 146), exons 2, 3 and 4 of *NRAS* (codons 12, 61, 117, and 146), exons 9 and 20 of *PIK3CA* (codons 542, 545 and 1047), and exon 15 of *BRAF* (codons 600).

Data Collection and Statistical Analysis

Clinical records, including age, sex, tumor site, maximum diameter of tumor, histological type, tumor differentiation, lymphovascular invasion, perineural invasion, and TNM stage were collected. SPSS statistical software version 21.0 (IBM Inc., State of New York, USA) was used for data analysis. The patients' clinicopathological features were summarized with descriptive statistics. Categorical variables were compared using χ^2 test and Fisher's exact test. Gender, age, tumor site, maximum diameter of tumor, tumor differentiation, lymphovascular invasion, perineural invasion, and distant metastasis were selected as covariates in the multivariate logistic regression analysis for *KRAS, NRAS, BRAF, PIK3CA* mutations, based on estimating the odds ratios (OR) and their 95% confidence intervals (CIs). The significance test was two-sided, and a P value <0.05 was considered statistically significant.

Results

Characteristics of Subjects

Four hundred and eight patients were included in the analysis, including 256 (62.7%) men and 152 (37.3%) women. The age of all patients was ranging from 25 to 86 years old. There were 185 (45.3%) patients with <60 years old, and 223 (54.7%) patients with ≥60 years old. The number of colon, rectum and cecum tumors was 227 (55.6%), 175 (42.9%) and 6 (1.5%), respectively. There were 255 patients (62.5%) with tumor maximum diameter less than 5 cm, and 138 patients (33.8%) with tumor maximum diameter ≥5 cm. Well differentiation, moderate differentiation and poor differentiation

tumor detected in 4 (1.0%), 370 (90.7%) and 34 (8.3%) cases, respectively. There were 80 (19.6%) and 54 (13.2%) patients with lymphatic vascular space invasion and perineural invasion, respectively. And 317 (77.7%) cases, and 91 (22.3%) cases were classified as stage I-III, and stage IV, respectively. There were 15 (3.7%) cases with dMMR (Table 1).

Frequency and Composition Ratio of *KRAS/NRAS/BRAF/PIK3CA* Mutations

All patients were genotyped for *KRAS* (codons 12, 13, 61, 117, and 146), *NRAS* (codons 12, 61, 117, and 146), *BRAF* (codons 600), and *PIK3CA* (codons 542, 545 and 1047) mutations. At least one mutation in these genes was detected in 216 (52.9%) patients, while none was detected in 192 (47.1%) patients. Mutation in *KRAS, NRAS, BRAF*, and *PIK3CA* was detected in 190 (46.6%), 11 (2.7%), 10 (2.5%), 34 (8.3%) samples, respectively. Among them, mutations in *KRAS* and *NRAS, KRAS* and *BRAF, KRAS* and *PIK3CA* were detected simultaneously in 1 (0.2%), 1 (0.2%), and 27 (6.6%) samples, respectively.

Ranking the mutations in order of composition, mutations in *KRAS* exon 2 had the highest proportion (62.5%), followed by mutations in both *KRAS* exon 2 and *PIK3CA* exon 9 simultaneously (7.9%), *KRAS* exon 4 (6.9%), *KRAS* exon 3 (5.1%), *BRAF* exon 15 (4.2%), both *KRAS* exon 2 and *PIK3CA* exon 20 simultaneously (3.7%), *NRAS* exon 2 (3.2%), and *PIK3CA* exon 9 (2.8%) (Table 2).

Table 1 Baseline Characteristics of CRC Patients

	Colorectal Cancer (n=408)
Gender	
Male, n (%)	256(62.7%)
Female, n (%)	152(37.3%)
Age	
<60, n (%)	185(45.3%)
≥60, n (%)	223(54.7%)
Tumor site	
Colon	227(55.6%)
Rectum	175(42.9%)
Cecum	6(1.5%)
Maximum diameter of tumor	
<5cm, n (%)	255(62.5%)
≥5cm, n (%)	138(33.8%)
Unknown, n (%)	15(3.7%)
Tumor differentiation	
Well, n (%)	4(1.0%)
Moderate, n (%)	370(90.7%)
Poor, n (%)	34(8.3%)
Lymphovascular invasion	
Present, n (%)	80(19.6%)
Absent, n (%)	328(80.4%)
Perineural invasion	
Present, n (%)	54(13.2%)
Absent, n (%)	354(86.8%)
Disease stage at diagnosis	
I-III, n (%)	317(77.7%)
IV, n (%)	91(22.3%)
MMR	
pMMR, n (%)	380(93.1%)
dMMR, n (%)	15(3.7%)
Unknown, n (%)	13(3.2%)

Abbreviation: MMR, mismatch repair.

Table 2 Frequency and Composition Ratio of *KRAS/NRAS/BRAF/PIK3CA* Mutation in CRC Patients

Gene Name	Exon	Number and Frequency of Mutation (n,%)	Composition Ratio of Mutations
KRAS	Exon2	135(33.1%)	62.5%
	Exon3	11(2.7%)	5.1%
	Exon4	15(3.7%)	6.9%
NRAS	Exon2	7(1.7%)	3.2%
	Exon3	3(0.7%)	1.4%
BRAF	Exon15	9(2.2%)	4.2%
PIK3CA	Exon9	6(1.5%)	2.8%
	Exon20	1(0.2%)	0.5%
KRAS and *NRAS*	*KRAS* exon2 and *NRAS* exon2	1(0.2%)	0.5%
KRAS and *BRAF*	*KRAS* exon2 and *BRAF* exon15	1(0.2%)	0.5%
KRAS and *PIK3CA*	*KRAS* exon2 and *PIK3CA* exon9	17(4.2%)	7.9%
	KRAS exon2 and *PIK3CA* exon20	8(2.0%)	3.7%
	KRAS exon3 and *PIK3CA* exon9	1(0.2%)	0.5%
	KRAS exon3 and *PIK3CA* exon20	1(0.2%)	0.5%
Total		216(52.9%)	100.0%

Relationship of Clinicopathological Characteristics and *KRAS/NRAS/BRAF/PIK3CA* Gene Mutation Status

Fisher's exact test was used to compare the clinicopathological differences between the *KRAS/NRAS/BRAF/PIK3CA* mutant and wild-type patients. *KRAS, NRAS, BRAF*, and *PIK3CA* mutations were not related to gender, age, tumor site, tumor size (maximum diameter of tumor), perineural invasion, lymph node metastasis, and MMR status (all $P>0.05$). The percentage of mutations in *KRAS* gene in stage IV patients was significantly higher than that in stage I-III patients (56.0% (51/91) vs 43.8% (139/317), $P=0.043$), and that in patients with distant metastasis was higher than that in patients without distant metastasis (57.1% (52/91) vs 43.5% (138/317), $P=0.024$). The mutation percentage of *BRAF* gene was higher in the tumor with poor differentiation than that in the tumor with moderate and well differentiation (13.3% (4/30) vs 1.6% (6/364) and 0 (0/4), $P=0.012$), and it was also significantly higher in the patients with lymphovascular invasion than without it (6.3% (5/80) vs 1.5% (5/328), $P=0.029$) (Table 3).

The regression analysis was performed with *KRAS* mutations, *NRAS* mutations, *BRAF* mutations and *PIK3CA* mutations as the response variables, and gender (X1), age (X2), tumor site (X3), maximum diameter of tumor (X4), tumor differentiation (X5), lymphovascular invasion (X6), perineural invasion (X7), and distant metastasis (X8) as independent variables. And multicollinearity and model goodness-of-fit was checked. The multivariate logistic regression analysis demonstrated that *KRAS* mutations (adjusted OR 1.675, 95% CI 1.017–2.760, $P=0.043$) and *NRAS* mutations (adjusted OR 5.183, 95% CI 1.239–21.687, $P=0.024$) appeared more frequently in patients with distant metastasis. *BRAF* mutations (adjusted OR 7.224, 95% CI 1.356–38.488, $P=0.021$) and *PIK3CA* mutations (adjusted OR 3.811, 95% CI 1.268–11.455, $P=0.017$) appeared more frequently in tumor with poor differentiation. Moreover, a relationship still existed between *PIK3CA* mutations and female patients (male/female adjusted OR 0.443, 95% CI 0.210–0.934, $P=0.032$) (Table 4).

Discussion

CRC is a common malignant tumor in human digestive tracts.[24] There is evidence that the occurrence of tumors is related to inflammation, and blood inflammatory markers can be used as markers for risk prediction and prognosis assessment of CRC. In addition, the significance of microRNAs (miRNAs) in tumor diagnosis, prognosis and prediction is constantly being explored.[25] In the past decades, a large number of research data have emerged in the studies on the molecular basis,[26,27] drug investigation and usage,[28,29] and gene testing[30] of colorectal cancer, leading to the rapid development of gene testing and targeted therapy for CRC. The targeted therapy of CRC patients needs to be based on the genes' status,

Table 3 Clinicopathological Characteristics According to *KRAS*/*NRAS*/*BRAF*/*PIK3CA* Gene Mutation Status in CRC Patients

	KRAS (Exon2/3/4)			*NRAS* (exon2/3)			*BRAF* (Exon15)			*PIK3CA* (Exon9/20)		
	Normal (n=218)	Mutated (n=190)	*P* value	Normal (n=397)	Mutated (n=11)	*P* value	Normal (n=398)	Mutated (n=10)	*P* value	Normal (n=374)	Mutated (n=34)	*P* value
Gender												
Male	144	112	0.151	249	7	1.000	251	5	0.510	240	16	0.063
Female	74	78		148	4		147	5		134	18	
Age												
<60	97	88	0.765	179	6	0.556	180	5	0.761	173	12	0.281
≥60	121	102		218	5		218	5		201	22	
Tumor site												
Colon	117	110	0.682	223	4	0.340	219	8	0.309	208	19	1.000
Rectum	98	77		168	7		173	2		160	15	
Cecum	3	3		6	0		6	0		6	0	
Maximum diameter of tumor												
<5cm	135	120	0.916	248	7	1.000	251	4	0.288	232	23	0.851
≥5cm	72	66		135	3		133	5		127	11	
Tumor differentiation												
Well	1	3	0.388	3	1	0.112	4	0	**0.012**	4	0	0.121
Moderate	201	169		360	10		364	6		342	28	
Poor	16	18		34	0		30	4		28	6	
Lymphovascular invasion												
Present	43	37	1.000	80	0	0.132	75	5	**0.029**	73	7	0.824
Absent	175	153		317	11		323	5		301	27	
Perineural invasion												
Present	25	29	0.306	54	0	0.373	52	2	0.628	52	2	0.288
Absent	193	161		343	11		346	8		322	32	
Disease stage at diagnosis												
I-III	178	139	**0.043**	310	7	0.273	310	7	0.699	288	29	0.294
IV	40	51		87	4		88	3		86	5	
Lymph node metastasis												
No	104	73	0.071	175	2	0.124	174	3	0.524	164	13	0.590
Yes	114	117		222	9		224	7		210	21	
Distant metastasis												
No	179	138	**0.024**	310	7	0.273	310	7	0.699	288	29	0.294
Yes	39	52		87	4		88	3		86	5	
MMR												
pMMR	200	180	1.000	370	10	1.000	371	9	1.000	350	30	0.121
dMMR	8	7		15	0		15	0		12	3	

Note: Bold values for $P<0.05$.

Table 4 Multivariate Logistic Regression in CRC Patients Between Gene Mutations and Clinicopathological Characteristics

Characteristics	*KRAS*			*NRAS*			*BRAF*			*PIK3CA*		
	Adjusted Odds Ratio (95% CI)	**R^2**	***P* value**	**Adjusted Odds Ratio (95% CI)**	**R^2**	***P* value**	**Adjusted Odds Ratio (95% CI)**	**R^2**	***P* value**	**Adjusted Odds Ratio (95% CI)**	**R^2**	***P* value**
Gender (Male/Female)	0.691(0.450–1.061)	0.154	0.091	2.252(0.425–11.947)	0.164	0.340	0.555(0.120–2.562)	0.250	0.450	0.443(0.210–0.934)	0.181	**0.032**
Age (≥60/<60)	0.915(0.605–1.383)		0.673	0.956(0.241–3.799)		0.950	0.778(0.157–3.856)		0.759	1.723(0.798–3.720)		0.166
Tumor site (Colon/Other)	1.191(0.769–1.845)		0.434	0.298(0.065–1.362)		0.119	3.325(0.346–31.990)		0.298	1.093(0.496–2.407)		0.825
Maximum diameter of tumor (≥5cm/<5cm)	0.992(0.633–1.555)		0.971	1.325(0.294–5.969)		0.714	2.362(0.476–11.727)		0.293	0.896(0.394–2.035)		0.792
Tumor differentiation (Poor/Moderate+well)	1.646(0.729–3.717)		0.231	–		0.998	7.224(1.356–38.488)		**0.021**	3.811(1.268–11.455)		**0.017**
Lymphovascular invasion (Present/Absent)	0.745(0.423–1.314)		0.310	–		0.997	4.547(0.874–23.666)		0.072	0.968(0.365–2.567)		0.948
Perineural invasion (Present/Absent)	1.897(0.962–3.738)		0.064	–		0.997	0.553(0.054–5.640)		0.617	0.387(0.083–1.808)		0.227
Distant metastasis (Yes/No)	1.675(1.017–2.760)		**0.043**	5.183(1.239–21.687)		**0.024**	0.495(0.075–3.265)		0.465	0.512(0.184–1.428)		0.201

Notes: *P*< 0.05 was considered statistically significant. Bold values for *P*<0.05.
Abbreviation: CI, confidence interval.

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and molecular testing has been paid more and more attention.[30] Studies have shown that genes downstream of the EGFR signaling pathway can affect the efficacy of targeted therapy with monoclonal antibodies.[31]

KRAS is one of the most important oncogenic genes in human genome, *KRAS* gene activating mutations are found in >80% of pancreatic cancer, >30% of CRC, cholangiocarcinoma and lung adenocarcinoma.[32] The frequency of mutations may vary from region to region and population to population. Approximately 41.5% of Danish CRC patients have *KRAS* mutations,[33] 45.5% in Slovene patients,[34] 49.6% in Saudi Arabian patients,[35] 34.7% in sigmoid colon and 58.2% in cecum of patients in the United States,[36] 29% in Iranian patients,[37] 54.84% in Turks patients,[38] 42% in Japanese patients,[39] 41.0% in Vietnamese patients,[40] and 23% in Indian patients.[41] The frequency of *KRAS* mutations was 35.0%–50.0% in Chinese CRC patients.[42–46] The frequency of *NRAS* mutations was 4.2% in Danish CRC patients,[33] 7.3% in Tunisian patients,[47] 2.0% in Saudi Arabian patients,[35] 5% in Japanese patients,[39] 9.6% in Vietnamese patients,[40] 2.0% in Indian patients,[41] and 7% in Mexican patients.[48] The mutation percentage of *NRAS* was 1.2%–3.85% in Chinese CRC patients.[45,46,49] In this study, at least one mutation in *KRAS, NRAS, BRAF* and *PIK3CA* was detected in 219 (52.6%) CRC patients, while none was detected in 197 (47.4%) CRC patients. *KRAS* and *NRAS* mutation was detected in 190 (46.6%) and 11 (2.7%) samples, respectively. The mutation percentage of *KRAS* and *NRAS* gene of CRC patients in this study is basically the same as that in other populations.

The frequency of *BRAF* was 18.0% in Danish CRC patients,[33] 0.4% in Saudi Arabian patients,[35] 7% in Iranian patients,[37] 12.9% in Turks patients,[38] 7% in Japanese patients,[39] 8.3% in Vietnamese patients,[40] 17% in Indian patients,[41] and 12.44% in Greek and Romanian patients.[50] The frequency of *BRAF* mutations was 2.3%–8% in Chinese CRC patients.[45,49,51–53] The frequency of *PIK3CA* mutations was 18.8% in Danish CRC patients,[33] 13.3% in Arab patients,[54] and 8% in Italian patients.[55] The frequency of *PIK3CA* mutations was about 9.4%–18.9% in Chinese CRC patients.[42,51,56,57] In this study, *BRAF* and *PIK3CA* mutation status were detected in 10 (2.5%) and 34 (8.3%) samples, respectively. Compared with other populations, the mutation percentage of *BRAF* and *PIK3CA* genes of CRC patients in this study was relatively low.

In this study, *KRAS* or *NRAS* mutations appeared more frequently in CRC patients with distant metastasis. Studies have shown that *KRAS, NRAS* or *BRAF* mutations were associated with lung metastasis in CRC.[11,12] *KRAS/NRAS/BRAF* mutations may predict late distant metastasis.[58] *KRAS* mutations were related to poor tumour differentiation and liver metastasis.[59] Distant metastatic tumors had a higher mutation percentage of *NRAS* mutation but not *KRAS*.[15] *BRAF* mutations were more common in peritoneal metastasis patients.[16] However, another study has shown that distant metastasis was not related to *KRAS, NRAS, BRAF*, or *PIK3CA* mutations.[13] In addition, *BRAF* and *PIK3CA* mutations appeared more frequently in tumor with poor differentiation in this study. Study has shown that *BRAF* mutations were associated with poorer differentiation, but mutant *KRAS* was associated with greater differentiation.[14] *BRAF* mutations were more common in poorly differentiated tumors, but *PIK3CA* is not.[15–18] *PIK3CA* mutations showed null associations with tumor differentiation.[60] In summary, the correlation of *KRAS, NRAS, BRAF, PIK3CA* mutations with distant metastasis, tumor differentiation was inconsistent. Different sample sizes, different populations, and different detection methods for genetic mutations can partly explain the results. In addition, the prognosis of CRC is also associated with DNA mismatch repair, and CRC patients with microsatellite instability-low (MSI-L) may experience shorter survival; however, there are challenges in the availability of data for MSI testing.[61]

In terms of molecular mechanisms, mutation-activated KRAS in tumor cells reprograms macrophages with tumor-associated macrophage (TAM)-like phenotypes, which not only promotes tumor progression but also induces resistance of tumor cells to targeted therapy.[62] MiR-450b-5p can activate Wnt/β-catenin signaling to promote cell proliferation, tumor growth, and inhibit the apoptosis of CRC cells, while miR-450b-5p can be up-regulated by KRAS/AP-1 signaling.[63] The differential expression of some RNAs in exosomes of CRC cells with *BRAF* V600E mutation is closely related to proliferation, metabolism of tumor cells and tumor microenvironment changes.[64] B-Raf/MEK/ERK pathway was related to inhibition of tumor cell differentiation.[65] The possible mechanisms of the relationship between distant metastasis and *KRAS* and *NRAS* mutations, as well as the relationship between poor tumor differentiation and *BRAF* and *PIK3CA* mutations, are still not completely clear and need further study.

The results of this study suggest that for CRC patients with *KRAS* and *NRAS* mutations, more attention should be paid to the possibility of distant metastases, such as liver metastasis, lung metastasis, and brain metastasis. Some techniques can be applied to the diagnosis of liver metastases in CRC.[66] Tumor brain metastases are the most common tumors of the adult central nervous system,[67] and the rate of brain metastases in CRC is the highest among

gastrointestinal tumors. Brain metastases usually occur in the advanced stages of the disease and the prognosis is poor in most patients. The quality of life and prognosis of patients with colorectal cancer with neurological symptoms can be improved by early detection and treatment of metastatic lesions. CRC patients with *KRAS* and *NRAS* mutations with neurological symptoms, through the application of some new technology such as non-invasive brain stimulation techniques (NIBS),[68–71] can be used to identify the risk factors for brain metastases, so that early detection and treatment.

The study has some limitations that are worth noting. First of all, the number of research objects in this study is relatively small, which leads to some deviations in the results. Second, we only studied the common mutation sites of *KRAS/NRAS/BRAF/PIK3CA* genes; the status of the other mutation sites in these genes is unknown. Third, this study was limited to the correlation between gene mutations and clinicopathological features, and did not analyze the correlation between gene mutations and clinical outcomes and treatment responses in CRC patients receiving chemotherapy or radiotherapy. Therefore, the next step is to conduct a multi-center study with a larger sample size and a comprehensive analysis of *KRAS/NRAS/BRAF/PIK3CA* gene.

Conclusions

The relationship between clinicopathological features and KRAS, NRAS, BRAF, PIK3CA genes status in CRC patients were studied, and found that there was a significant relationship between distant metastasis and KRAS or NRAS mutations, while poor tumor differentiation and BRAF or PIK3CA mutations. Importantly, more attention should be paid to the possibility of distant metastasis in colorectal cancer patients with KRAS and NRAS mutations. Monitoring these patients for early diagnosis and treatment of distant metastases will have the potential to improve their survival and prognosis.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics Approval and Consent to Participate

The study was approved by the Ethics Committee of Medicine, Meizhou People's Hospital. All participants signed informed consent in accordance with the Declaration of Helsinki.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no competing interests in this work.

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