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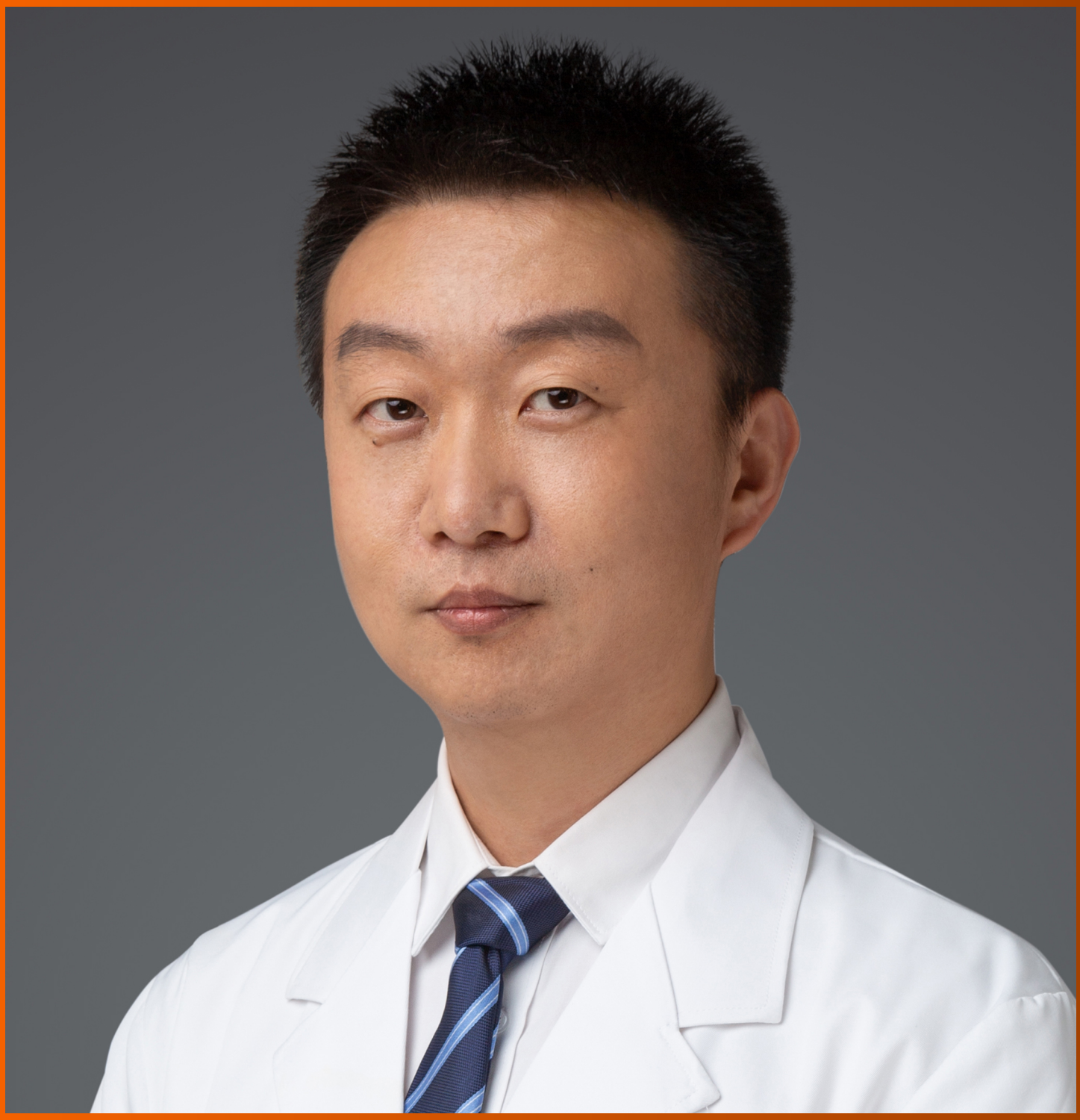

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ORIGINAL ARTICLE

Retrospective Study

Relationship between mismatch repair protein, *RAS*, *BRAF*, *PIK3CA* gene expression and clinicopathological characteristics in elderly colorectal cancer patients

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Abstract

BACKGROUND

Colorectal cancer (CRC) is common in elderly patients. Mismatch repair (MMR) protein deletion is one of the causes of CRC. The *RAS* (*KRAS/NRAS*), *BRAF*, and *PIK3CA* genes are important gene targets in CRC treatment and are closely related to the prognosis and survival of patients. However, little is known regarding the relationship between the expression of MMR, *RAS, BRAF, PIK3CA* and the clinicopathological features in CRC patients.

AIM

To analyze the relationship between the expression of MMR, *RAS, BRAF, PIK3CA* and the clinicopathological features in CRC.

METHODS

A total of 327 elderly patients with CRC were enrolled, and immunohistochemistry was used to detect the MMR protein. Real-time quantitative polymerase chain reaction was used to detect the *RAS* (*KRAS/NRAS*), *BRAF*, and *PIK3CA* genes. The clinicopathological data of the patients were recorded and analyzed by SPSS 19.0 statistical software.

RESULTS

In 327 elderly patients with CRC, the rate of MMR protein loss was 9.79% (32/327), and the deletion rate of four MMR proteins (MSH2, MSH6, MLH1, PMS2) was 1.83% (6/327), 3.06% (10/327), 7.65% (25/327), and 7.65% (25/327), respectively. There were no significant differences between MMR protein deletion

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and sex, pathological type, tumor morphology, differentiation degree or lymph node metastasis ($P > 0.05$), but there was a significant difference between MMR protein deletion and tumor diameter and tumor location ($P = 0.048$/$P = 0.000$). The mutation rates of the *KRAS, NRAS, BRAF* and *PIK3CA* genes in elderly CRC patients were 44.95% (147/327), 2.45% (8/327), 3.36% (11/327) and 2.75% (9/327), respectively; the *KRAS* gene mutation was closely related to tumor morphology ($P = 0.002$) but not to other clinicopathological features ($P > 0.05$), and there were no significant differences between *NRAS* gene mutation and clinicopathological features ($P > 0.05$). The *BRAF* gene mutation showed a significant difference in pathological type, tumor location, differentiation degree and lymph node metastasis ($P < 0.05$), but was not correlated with sex, tumor size and tumor morphology ($P > 0.05$). The *PIK3CA* gene mutation showed no significant differences in the above clinicopathological characteristics ($P > 0.05$). Significant differences were observed between MMR protein deletion and *KRAS*, *BRAF*, and *PIK3CA* gene mutations in elderly CRC patients ($P = 0.044$, $P = 0.000$, $P = 0.003$, respectively), but there was no significant difference between MMR protein deletion and *NRAS* mutation ($P > 0.05$).

CONCLUSION
In elderly CRC patients, the tumor is mainly located in the right colon, and the deletion rate of MMR protein is higher when the tumor diameter is greater than or equal to 5 cm; the deletion rate of MLH1 and PMS2 is more common; the mutation rate of *KRAS* gene is higher than that of the *NRAS*, *BRAF* and *PIK3CA* genes, the *BRAF* gene mutation has different degrees of correlation with clinicopathological characteristics; when the MMR protein is deleted, the *BRAF* and *PIK3CA* gene mutations are often present, and the *KRAS* gene mutation rate is low.

Key Words: Elderly patients; Colorectal cancer; Mismatch repair protein; Gene mutation; Expression; Diagnosis

Core Tip: The mismatch repair (MMR) protein deletion is one of the causes of colorectal cancer (CRC). The *RAS* (*KRAS/NRAS*), *BRAF*, and *PIK3CA* genes are important gene targets in CRC treatment and are closely related to the prognosis and survival of patients. However, little is known regarding the relationship between the expression of MMR, *RAS, BRAF, PIK3CA* and the clinicopathological features in CRC patients. In this study, we analyzed four target genes to provide a further theoretical basis for clinicians in relation to the diagnosis, treatment and prognosis of CRC.

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INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignant tumors in the digestive tract. Its incidence and mortality rates show an increasing trend worldwide. At present, the elderly are still the main group of patients with CRC[1]. Mismatch repair (MMR) protein deletion is one of the causes of CRC[2,3]. The *RAS* (*KRAS/NRAS*), *BRAF*, and *PIK3CA* genes are important gene targets in CRC treatment and are closely related to the prognosis and survival of patients[4-7]. This study analyzed pathological samples and clinical data from 327 elderly CRC patients to determine the relationship between MMR, *RAS* (*KRAS/NRAS*), *BRAF*, *PIK3CA* and clinicopathological characteristics, and the relationship between MMR and the four target genes to provide a further

theoretical basis for clinicians in the diagnosis, treatment and prognosis of CRC.

MATERIALS AND METHODS

Clinical data

Surgical resection specimens from 327 elderly patients with CRC were collected from April 2019 to January 2020 in the Department of Pathology, First Medical Center, People's Liberation Army General Hospital. The patients included 196 males and 131 females, aged 60-91 years with an average age of 70 years. There were 281 cases of adenocarcinoma, 43 cases of mucinous adenocarcinoma, and 3 cases of signet ring cell carcinoma. The tumor was located in the right colon in 78 cases, the left colon in 90 cases, and the rectum in 159 cases. There were 181 cases of ulcer type, 77 cases of protruding type, 45 cases of protruding type of ulcer, and 24 cases of infiltrating type and flat type. There were 112 cases with tumor diameter ≥ 5 cm, and 215 cases with tumor diameter < 5 cm. Four cases were well differentiated, 15 cases were well-moderately differentiated, 241 cases were moderately differentiated, 59 cases were moderately-poorly differentiated, and 8 cases were poorly differentiated. There were 130 cases with lymph node metastasis and 197 cases without lymph node metastasis.

Detection of MMR protein by immunohistochemistry

Cationic anti-stripping gel slides were used to cut the paraffin-embedded tissue sections after dehydration with neutral formalin to a thickness of 3-4 μm, which were then dried, placed in a 75°C baking table and baked for 20 min. After 20 min of xylene dewaxing, anhydrous ethanol X2, 95% ethanol X2, 85% ethanol X2 treatment, deionized water cleaning and high-pressure restoration of the antigen, the computer detected MSH2, MSH6, MLH1, and PMS2 MMR protein, antibody clone numbers were: RED2, EP49, ES05, EP51. The expression of MSH2, MSH6, MLH1, and PMS2 was observed under the microscope. When all four proteins were positive (> 30%), this was judged to be pMMR, and when at least one protein was missing, this was judged to be dMMR.

Real-time polymerase chain reaction detection of KRAS, NRAS, BRAF, and PIK3CA genes

The paraffin embedded tissues were cut into 3-4 pieces 4 μm thick and placed in a clean Eppendorf test tube, and the sample DNA was extracted using the FFPE-DNA sample nucleic acid extraction kit (Xiamen Aide Biomedicine Company), and an ultraviolet spectrophotometer was used to determine the concentration and purity of the sample for quality control. The *KRAS, NRAS, BRAF, PIK3CA* four gene joint detection kits (Xiamen Aide Biopharmaceutical Company) were used to carry out the polymerase chain reaction (PCR), and after PCR was complete, the results were recorded. The PCR steps are shown in Table 1.

Statistical analysis

SPSS 19.0 statistical software was used for data analysis, and the χ^2 test or Fisher's exact probability method were used to compare differences. A P value < 0.05 was considered statistically significant.

RESULTS

Relationship between the expression of MMR protein and clinicopathological characteristics in elderly patients with CRC

Of the 327 elderly patients with CRC, the loss rate of MMR protein expression was 9.79% (32/327), and the loss rate of the four MMR proteins (MSH2, MSH6, MLH1, PMS2) was 1.83% (6/327), 3.06% (10/327), 7.65% (25/327), and 7.65% (25/327), respectively, and the loss rate of MLH1 and PMS2 was significantly higher than that of MSH2 and MSH6 (P < 0.001). MMR protein loss was not statistically different in terms of patient's gender, pathological type, tumor morphology, degree of differentiation, lymph node metastasis, *etc.* (P > 0.05); however, a statistical difference was observed between MMR protein loss and both tumor diameter and tumor location (P = 0.048/P = 0.000). Patients with tumors ≥ 5 cm in diameter were more likely to have MMR protein loss or dMMR. The right colon was more likely to develop dMMR than the left

Table 1 Polymerase chain reaction steps

Polymerase chain reaction steps		
First stage	95°C, 5 min	1 cycle
Second stage	95°C, 25 s	15 cycles
	64°C, 20 s	
	72°C, 20 s	
Third stage	93°C, 25 s	31 cycles
	60°C, 35 s	
	72°C, 20 s	

colon and rectum (Table 2). Figure 1 shows the microscopic view of the immunohistochemical expression of the four proteins, MSH2, MSH6, MLH1, and PMS2.

Relationship between KRAS, NRAS, BRAF, PIK3CA gene expression and clinicopathological characteristics in elderly patients with CRC

KRAS, NRAS, BRAF, PIK3CA gene mutation rates in elderly patients with CRC were 44.95% (147/327), 2.45% (8/327), 3.36% (11/327), and 2.75% (9/327), respectively. The mutation rate of *KRAS* gene was significantly higher than that of the other three genes ($P < 0.001$); the mutation rate of *KRAS* gene was closely related to tumor morphology, and the mutation rate of elevated CRC was significantly lower than that of ulcerative and other types ($P = 0.002$). Pathological features were not related ($P > 0.05$). There was no significant difference between *NRAS* gene mutation and various clinicopathological characteristics ($P > 0.05$), *BRAF* gene mutation showed significant differences in relation to pathological type, tumor location, degree of differentiation, and lymph node metastasis ($P < 0.05$). *BRAF* gene was more likely to occur in the right colon, in poorly differentiated mucinous adenocarcinoma with lymph node metastasis, which was not related to gender, tumor size, or tumor morphology ($P > 0.05$), and *PIK3CA* gene mutation showed no significant differences in relation to the above-mentioned clinicopathological characteristics ($P > 0.05$, Table 3). The PCR mutation curves of the four genes are shown in Figure 2.

Relationship between MMR protein and KRAS, NRAS, BRAF, and PIK3CA genes in elderly patients with CRC

A statistically significant difference was observed between MMR protein deletion and *KRAS, BRAF, PIK3CA* gene mutations in elderly patients with CRC. When MMR protein deletion or dMMR was present, the *KRAS* gene mutation rate was significantly lower than that when pMMR was present ($P = 0.044$). When dMMR occurred, the mutation rate of *BRAF* and *PIK3CA* genes was significantly higher than that when pMMR occurred ($P = 0.000$/$P = 0.003$), and no significant difference was with *NRAS* mutation ($P > 0.05$, Table 4).

DISCUSSION

Many previous studies have shown that mutations or methylation inactivation of DNA MMR genes is not only the main cause of Lynch syndrome but also one of the causes of CRC[2,3]. The elderly are still the main patient group with CRC. The role of the MMR gene is mainly to repair base mismatches during DNA replication and recombination to ensure the stability of gene structure. When the MMR gene is mutated or methylated inactivated, it is prone to mutations of related oncogenes, leading to tumor formation. The most important protein families encoded by MMR genes are MSH2, MSH6, MLH1, and PMS2. They usually work in the form of the MLH1-PMS2 complex and MSH2-MSH6 complex. Previous studies have shown that the deletion rate of MMR protein is approximately 9.5%-34.3%, and the deletion rate of MLH1 is the most common[8,9]. In this study, the deletion rate of the MMR protein in elderly CRC patients was 9.79% (32/327), and the missing rate was slightly lower, which was consistent with previous studies on elderly patients with CRC. The missing rates of MSH2, MSH6, MLH1, and PMS2 were 1.83% (6/327), 3.06% (10/327), 7.65%

Table 2 Relationship between expression of mismatch repair protein and clinicopathological characteristics in elderly colorectal cancer patients

Clinical pathology data	*n*	MMR		χ^2	*P* value
		dMMR	pMMR		
Gender				0.005	0.945
Male	196	19	177		
Female	131	13	118		
Tumor size				3.907	0.048
≥ 5 cm	112	16	96		
< 5 cm	215	16	199		
Pathological type				0.456	0.796
Adenocarcinoma	281	27	254		
Mucinous adenocarcinoma	43	5	38		
Other	3	0	3		
Tumor morphology				1.768	0.622
Ulcer type	181	19	162		
Raised type	77	9	68		
Ulcer raised type	45	3	42		
Other	24	1	23		
Tumor site				32.368	0.000
Right colon	78	19	59		
Left colon	90	11	79		
Rectum	159	2	157		
Differentiation				8.181	0.085
High	4	1	3		
High-moderate	15	1	14		
Moderate	241	18	223		
Moderate-poor	59	10	49		
Poor	8	2	6		
Lymph node metastasis				2.003	0.157
Yes	130	9	121		
No	197	23	174		

MMR: Mismatch repair.

(25/327), and 7.65% (25/327). The missing rates of MLH1 and PMS2 were significantly higher than those of MSH2 and MSH6 ($P < 0.001$), which is consistent with previous research results.

Previous studies found that lack of the MMR protein is closely related to the clinicopathological characteristics of CRC[10-13], and some research results show that lack of the MMR protein is related to lymph node metastasis and differentiation[13].

In this study, there were no statistically significant differences between lack of the MMR protein and the patient's sex, pathological type, tumor morphology, degree of differentiation, and lymph node metastasis ($P > 0.05$), which may have been related to the patient's age and sample data volume. At the same time, there were significant differences in the tumor diameter and tumor location ($P = 0.048$/$P = 0.000$). Patients with tumor diameters ≥ 5 cm were more likely to have MMR protein loss or dMMR, and the right colon was more likely to develop dMMR than the left colon and rectum. These results are consistent with previous research results.

Table 3 Relationship between *KRAS, NRAS, BRAF, PIK3CA* gene expression and clinicopathological characteristics in elderly colorectal cancer patients

Clinical pathology data	*n*	*KRAS*		χ^2	*P*	*NRAS*		χ^2	*P*	*BRAF*		χ^2	*P*	*PIK3CA*		χ^2	*P* value
		Wild	Mutant			Wild	Mutant			Wild	Mutant			Wild	Mutant		
Gender																	
Male	196	108	88	0.001	0.98	192	4	0.046	0.829	189	7	0	1	191	5	0	1
Female	131	72	59			127	4			127	4			127	4		
Tumor size																	
≥ 5 cm	112	70	42	3.825	0.051	109	3	0	1	106	6	1.254	0.263	110	2	0.124	0.725
< 5 cm	215	110	105			210	5			210	5			218	7		
Pathological type																	
Adenocarcinoma	281	157	124	0.908	0.635	273	8	1.338	0.512	273	8	8.712	0.013	274	7	0.734	0.693
Mucinous adenocarcinoma	43	22	21			43	0			41	2			41	2		
Other	3	1	2			3	0			2	1			3	0		
Morphology																	
Ulcer	181	89	92	14.972	0.002	177	4	1.328	0.723	177	4	3.279	0.351	176	5	1.1	0.777
Raised	77	57	20			74	3			72	5			74	3		
Ulcer-raised	45	21	24			44	1			44	1			44	1		
Other	24	13	11			24	0			23	1			24	0		
Site																	
Right colon	78	38	40	3.184	0.203	77	1	0.784	0.676	71	7	11.004	0.004	75	3	5.652	0.059
Left colon	90	56	34			88	2			90	0			85	5		
Rectum	159	86	73			154	5			155	4			158	1		
Differentiation																	
High	4	2	2	1.791	0.774	4	0	5.926	0.205	3	1	18.408	0.001	4	0	4.738	0.315
High-moderate	15	8	7			15	0			15	0			15	0		
Moderate	241	134	107			237	4			234	7			236	5		
Moderate-poor	59	30	29			55	4			58	1			55	4		
Poor	8	6	2			8	0			6	2			8	0		

Lymph																
Yes	130	71	59	0.016	0.899	124	6	2.879	0.09	124	6	0.499	0.48	127	3	0.003	0.957
No	197	109	88			195	2			192	5			191	6		

Many previous studies have shown that *KRAS, NRAS, BRAF*, and *PIK3CA* gene mutations are closely related to the prognosis of CRC[6,7,14-16]. Of these mutations, the frequency of *KRAS* gene mutations is highest (approximately 30%-50%). When the *KRAS* gene is mutated, the RAS-RAF-MAPK signal transduction pathway can be activated, resulting in ineffective anti-EGFR inhibitor therapy[14]. In this study, the mutation frequency of *KRAS* was 44.95% (147/327), which was consistent with the results of previous studies, and we found that the mutation rate of *KRAS* (26.00%, 20/77) in late-stage CRC was significantly lower (P = 0.002). In previous studies, the mutation frequency of NRAS was below 5%[7,15], and the mutation rate of the *BRAF* gene was 2%-15%[17]. The *PIK3CA* gene belongs to the PI3K/AKT/mTOR signaling pathway and is also an EGFR signal. One of the pathways is related to cell proliferation. When *PIK3CA* is mutated, tumors are more aggressive and have a worse prognosis[6,18,19]. In this study, the mutation rates of the *NRAS, BRAF* and *PIK3CA* genes were 2.45% (8/327), 3.36% (11/327), and 2.75% (9/327), respectively, which are consistent with previous research results. In addition, we found that *NRAS* and *PIK3CA* were not related to the sex, tumor type, location, tumor size, morphology, and lymph node metastasis of CRC patients, while the *BRAF* gene is more common in the right colon, lymph node metastasis, mucinous carcinoma, and less differentiated tumors.

In this study, we analyzed the relationship between MMR protein deletion and the *KRAS, NRAS, BRAF*, and *PIK3CA* genes. We found that there was a significant correlation between deletion of the MMR protein in elderly CRC patients and *KRAS, BRAF*, and *PIK3CA* gene mutations and that there were no correlations between the MMR protein and *NRAS* mutations (P > 0.05). There was a significant negative correlation between MMR protein deletion and *KRAS* gene mutation. The mutation rate of the *KRAS* gene in dMMR was significantly lower than that in pMMR (P = 0.044). *KRAS* gene mutations can lead to ineffective anti-EGFR inhibitor therapy, which also confirms the results of previous studies on MMR and KRAS in CRC[20,21]. In addition, we also found that the mutation rate of the *BRAF* and *PIK3CA* genes was significantly higher than that of pMMR when dMMR occurred (P = 0.000/P = 0.003), which is consistent with the reports of Poulsen *et al*[22].

CONCLUSION

In summary, this study retrospectively analyzed several indicators of the MMR, *KRAS*, *NRAS, BRAF*, and *PIK3CA* genes in elderly CRC patients and found that they

Table 4 Relationship between mismatch repair protein and *KRAS, NRAS, BRAF, PIK3CA* genes in elderly colorectal cancer patients

Gene name	*n*	MMR		χ^2	*P* value
		dMMR	pMMR		
KRAS				4.06	0.044
Wild	180	23	157		
Mutant	147	9	138		
NRAS				0.116	0.733
Wild	319	32	287		
Mutant	8	0	8		
BRAF				12.489	0.000
Wild	316	27	289		
Mutant	11	5	6		
PIK3CA				8.879	0.003
Wild	318	28	290		
Mutant	9	4	5		

MMR: Mismatch repair.

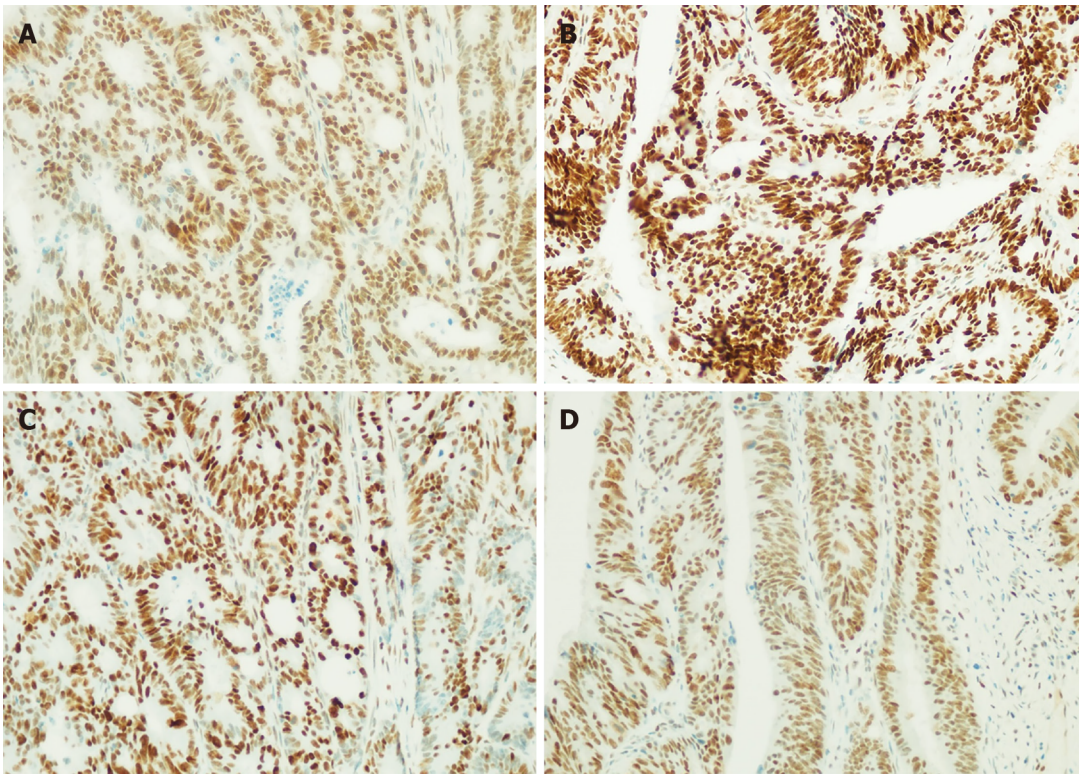

Figure 1 Immunohistochemical positive expression of four proteins in elderly colorectal cancer patients, MSH2, MSH6, MLH1, and PMS2, was observed under microscope. A: Immunohistochemical positive expression of MLH1; B: Immunohistochemical positive expression of MSH2; C: Immunohistochemical positive expression of MSH6; D: Immunohistochemical positive expression of PMS2. The brown-yellow particles were positive (10 × 20 light microscopy).

are related to multiple clinicopathological features, and there are also correlations between them. These findings provide additional support for the clinical diagnosis and treatment of CRC. The disadvantage of this study is that we do not have more data on the clinical treatment and prognosis of these patients and cannot explain the relationship between these indicators and the treatment or prognosis; these

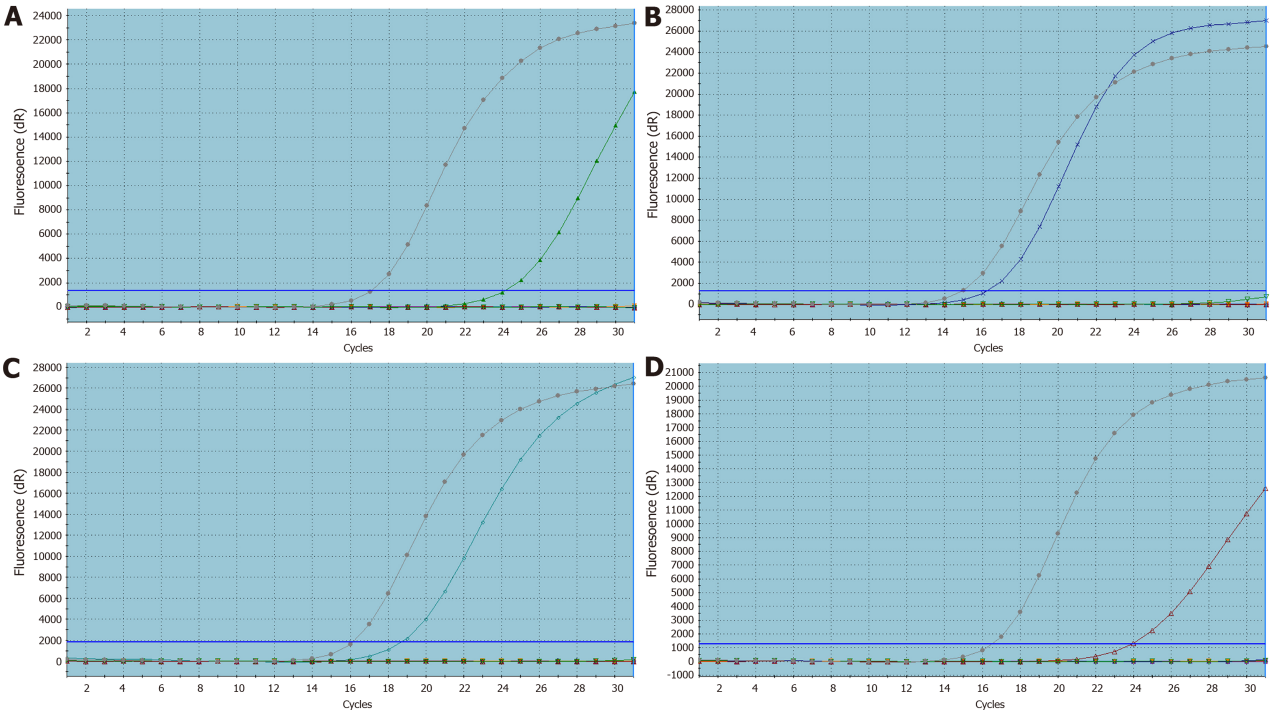

Figure 2 Polymerase chain reaction mutation curves of the four genes. A: Gene *KRAS-EXON-2-G13D* mutation; B: Gene *NRAS-EXON2-G12* mutation; C: Gene *PIK3CA-EXON20* mutation; D: Gene *BRAF-EXON15* mutation.

relationships need to be further investigated.

ARTICLE HIGHLIGHTS

Research background

Mismatch repair (MMR) protein deletion is one of the causes of colorectal cancer (CRC). The *RAS* (*KRAS/NRAS*), *BRAF*, and *PIK3CA* genes are important gene targets in CRC treatment and are closely related to the prognosis and survival of patients.

Research motivation

This study provides a further theoretical basis for clinicians in the diagnosis, treatment and prognosis of CRC.

Research objectives

This study aimed to explore the relationship between MMR, *RAS* (*KRAS/NRAS*), *BRAF*, *PIK3CA* and clinicopathological characteristics, and the relationship between MMR and the four target genes.

Research methods

The MMR protein was detected by immunohistochemistry, and real-time polymerase chain reaction was performed to detect *KRAS, NRAS, BRAF, PIK3CA* genes.

Research results

There were no significant differences between MMR protein deletion and sex, pathological type, tumor morphology, differentiation degree or lymph node metastasis, but there was a significant difference between MMR protein deletion and tumor diameter and tumor location. The *KRAS* gene mutation was closely related to tumor morphology, but not to other clinicopathological features, and there were no significant differences between *NRAS* gene mutation and clinicopathological features, MMR protein deletion and *NRAS* mutation.

Research conclusions

In elderly CRC patients, the deletion rate of MLH1 and PMS2 is more common; the

mutation rate of *KRAS* gene is higher than that of the *NRAS, BRAF* and *PIK3CA* genes.

Research perspectives

The relationship between these indicators and the treatment or prognosis requires further investigation.

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