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Original Article

Correlation of MLH1 and MSH2 levels with clinicopathologic characteristics in colorectal cancer

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Abstract: Objective: To determine the correlation of MLH1 and MSH2 expressions with clinicopathologic characteristics in colorectal cancer (CC). Methods: Clinical data, CC tissue, and paracancerous tissue from 88 patients treated in Baoji City People's Hospital from February 2015 to February 2017 were analyzed retrospectively. The relative expression levels of MLH1 and MSH2 in the tissues were measured with qRT-PCR, and the relationship of MLH1 and MSH2 with the pathological data of patients was analyzed. The value of MLH1 and MSH2 in the diagnosis of clinical stage, lymph node metastasis, and degree of differentiation in CC patients was analyzed by receiver operating curve (ROC). Cox regression analysis was applied to identify factors affecting prognosis. Results: The relative expression levels of MLH1 and MSH2 in CC tissue were lower than those in paracancerous tissue ($P < 0.001$). Tumor node metastasis stage (III + IV), poor differentiation, and lymph node metastasis were significantly increased in patients with low MLH1 and MSH2 expressions ($P < 0.05$). The levels of MLH1 and MSH2 in CC tissue of patients at stage I with moderately- or well-differentiated non-metastatic disease were higher than those in patients at stage II-IV with poor differentiation and lymph node metastasis, showing a good predictive ability. The 5-year survival rate of patients with low MLH1 and MSH2 expressions was lower as compared to its counterpart ($P < 0.01$). Conclusion: The low expressions of MSH2 and MLH1 in CC tissue have a correlation with pathological characteristics and survival, so they can be used as auxiliary references for the prognosis in CC patients.

Keywords: Colorectal cancer, MLH1, MSH2, clinicopathologic characteristics, survival

Introduction

Colorectal cancer (CC) is the most commonly known malignant tumor of the digestive tract [1]. According to the statistics from WHO in 2018 [2], there were more than 1.8 million CC cases and a total of 881,000 CC-related deaths, with an average of about 1 in every 10 cancer cases. Its incidence and mortality rank top three worldwide, making it a risk factor that seriously threatens people's physical and mental health. Therefore, in-depth research on CC is necessary [3, 4]. The early CC diagnosis rate is generally low in China, and most cases were diagnosed in the middle or late stages [5]. The postoperative recurrence and metastasis of CC are affected by factors including lymph node metastasis, tumor type, growth location and degree of invasion, which are also the key factors for prognosis [6].

In the *NCCN Guidelines for the Diagnosis and Treatment of CC (2017)*, PD-1 monoclonal antibody was recommended for the treatment of CC with mismatch repair defect phenotype or high microsatellite instability. It is believed that [7], according to tumor histopathologic examinations, CC patients with mismatch repair defect phenotype or high microsatellite instability phenotype in stage II have a better prognosis in clinical follow-up [8]. Therefore, the detection of microsatellite instability plays an essential role

Table 1. Primer sequences

Gene	Primer sequence	
	Upstream primers (5'-3')	Downstream primers (5'-3')
MLH1	AGTGGCTGGACAGAGAGACA	GATCAGGCAGGTTAGCAAGC
MSH2	GTCGGCTTCGTGCGCTTCTTT	TCTCTGGCCATCAACTGCGGA
β-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

in the diagnosis, postoperative treatment, prognosis and follow-up plans for CC patients [9].

Mismatch repair genes, which are specific molecular markers of CC, have also attracted wide attention [10]. Previous studies have found that germline mutations in mismatch repair genes lead to inactivation of mismatch repair genes, and then lead to incorrect DNA replication that cannot be repaired, resulting in genomic changes in normal cells and transformation into tumor cells [11, 12]. As the two most substantial genes in the mismatch repair system, mutS homolog 2 (MSH2) and mutL homolog 1 (MLH1) account for over 90% of mismatch repair gene deletions [13]. Research found that [14] MLH1 and MSH2 were expressed in CC, but their prognostic value remains to be clarified.

In this study, we aimed to analyze the relationship between MLH1 and MSH2 and the prognosis of CC patients, so as to provide a reference for evaluating clinical prognosis.

Methods and materials

Clinical information

This is a retrospective analysis of data from 88 patients with CC admitted to Baoji City People's Hospital from February 2015 to February 2017. The CC tissue was obtained from the patient during the surgery, and the normal tissue 5 cm next to the surgical site was taken as control. The tissues were transported in liquid nitrogen to the laboratory for detection. This study was approved by the medical ethics committee of Baoji City People's Hospital (2022-003).

Inclusion and exclusion criteria

Inclusion criteria: Patients' conditions were in line with the tumor node metastasis (TNM) staging criteria for CC according to the 8th edition of the American Association for Cancer Staging Manual [15]; patients with tumor lesions located in the colon which had been treated with radical surgery; patients who were diagnosed with colon cancer by pathological examination of the resected specimens; patients with complete clinical data and pathological data.

Exclusion criteria: Patients who did not receive preoperative neoadjuvant tumor therapy (such as radiotherapy and chemotherapy, immunotherapy, traditional Chinese medicine, etc.); patients with a history of other malignant tumors; patients with severe respiratory, liver, kidney or cardiovascular diseases; pregnant women.

Detection methods

Real-time quantitative PCR (RT-qPCR) was utilized to determine the levels of MLH1 and MSH2 in both CC tissue and adjacent tissue from patients. To be specific, postoperative CC tissue and adjacent tissue were collected, and the total RNA of the tissue was extracted by Trizol one-step kit (Invitrogen). Then, agarose gel electrophoresis was used to ensure the concentration, integrity and purity of total RNA. The template cDNA was obtained with RNA reverse transcription kit (Takara), and then diluted to 100 ng/μl. Then, RT-PCR kit (Takara) was used to quantify primers with MLH1, MSH2 and internal reference gene β-actin (see **Table 1**). Amplification was performed on an ABI 7500 PCR amplifier. The reaction conditions were as follows pre-denaturation at 95°C for 30 s, denaturation at 95°C for 5 s, annealing at 55°C for 25 s and extension at 72°C for 20 s, for 40 cycles, followed by extension at 72°C for 5 min. Data reading was automatically completed by real-time quantitative PCR, and the relative mRNA levels of MLH1, MSH2, and β-actin were calculated using the $2^{-\Delta\Delta Ct}$ method [16].

Observation indicators

Main outcome measures: The levels of MLH1 and MSH2 in CC tissue and paracancerous tissue were compared. The relationship of MLH1 and MSH2 with the pathological data of

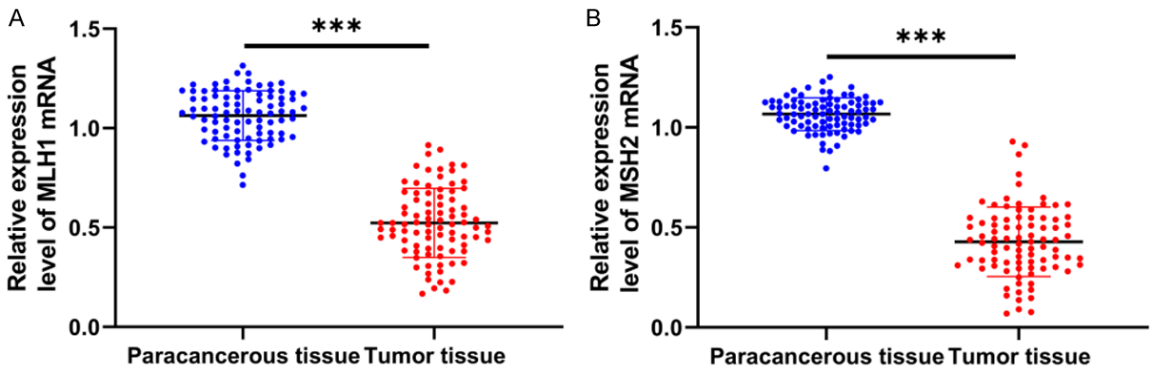

Figure 1. Expression levels of MLH1 and MSH2 in tumor tissues of colorectal cancer patients. A. qRT-PCR detection of relative expression levels of MLH1 in tumor tissue and adjacent tissue from colorectal cancer patients; B. qRT-PCR detection of relative expression levels of MSH2 in tumor tissue and adjacent tissue from colorectal cancer patients. Note: *** means $P < 0.001$.

patients was analyzed. The patient's electronic medical records, outpatient review records, and telephone follow-up data were collected to analyze patients' survival, which was analyzed based on the median value of MLH1 and MSH2 in each group.

Secondary outcome measures: Diagnostic value of MLH1 and MSH2 in TNM stages, differentiation, and lymph node metastasis were analyzed. Cox regression analysis was used to determine the prognostic factors affecting patients' 5-year survival.

Statistical analysis

SPSS 20.0 software was utilized for analyzing the collected data. The Shapiro-Wilk test was used to test for normality first, and measured data were denoted in the form of mean ± standard deviation (means ± SD). Kaplan-Meier survival curve was used to analyze the survival rate. Association between patient mortality and each latent variable was determined with univariate Cox regression analysis, and independent variables predicting patient mortality were identified with multivariate Cox regression analysis. Receiver operating characteristic (ROC) curve was drawn for the indicators with statistical significance in multivariate regression analysis, and the area under the ROC curve (AUC) was calculated. In addition, the value of MLH1 and MSH2 in diagnosing tumor stage, differentiation degree, and lymph node metastasis was calculated by ROC curve to evaluate the ability of the indicators to predict mortality. Significance was defined when $P < 0.05$.

Results

Low expressions of MLH1 and MSH2 in CC tissue

We detected the relative expression of MLH1 and MSH2 in CC tissue and paracancerous tissue of patients by qRT-PCR. It was revealed that the expressions of MLH1 and MSH2 were lower in tumor tissue than those in paracancerous tissue ($P < 0.001$, **Figure 1**).

Relationship of MLH1 and MSH2 to pathological data of CC patients

To better understand the value of MLH1 and MSH2 in CC, we assigned patients into low and high expression groups based on the median value of MLH1 and MSH2, and compared the relationship of the two indicators with patients' pathologic data. Our results showed that patients with low expressions of MLH1 and MSH2 held a markedly higher TNM stage (III + IV), poorer differentiation, and more lymph node metastasis ($P < 0.05$, **Tables 2**, **3**).

Expression and diagnostic ability of MLH1 and MSH2 for early-stage tumors

In the above results, MLH1 and MSH2 were found to be related to TNM staging. In attempts to further determine the value of the two in TNM staging, we analyzed the expressions in the CC tissue of patients with different stages.

Table 2. Relationship of MLH1 with pathological data of colorectal cancer patients

Factors	MLH1		X^2	P
	Low expression group (n=44)	High expression group (n=44)		
Sex			1.212	0.270
Male (n=55)	25	30		
Female (n=33)	19	14		
Age			0.409	0.522
> 60 years old (n=43)	20	23		
≤ 60 years old (n=45)	24	21		
Tumor diameter			0.733	0.392
> 5 cm (n=40)	22	18		
≤ 5 cm (n=48)	22	26		
Pathologic type			0.448	0.503
Ulcer type + infiltrating line (n=57)	27	30		
Massive type (n=31)	17	14		
Differentiation			6.175	0.013
Well differentiated + moderately differentiated (n=76)	34	42		
Poor differentiation (n=12)	10	2		
TNM staging			6.559	0.010
Stage I + II (n=42)	15	27		
Stage III + IV (n=46)	29	17		
Lymph node metastasis			6.600	0.010
Yes (n=40)	26	14		
No (n=48)	18	30		

Note: tumor node metastasis (TNM).

It was found that the levels of MLH1 and MSH2 in patients at stage I were higher than those in patients with stage II-IV ($P < 0.05$, **Figure 2A**, **2B**). Then through an ROC curve, we found that the AUCs to distinguish patients with stage I and stage II-IV were 0.916 and 0.894, respectively (**Figure 2C**; **Table 4**).

Expression and diagnostic ability of MLH1 and MSH2 for differentiation degrees

To further determine the value of MLH1 and MSH2 in identifying differentiation degree, we compared the expressions in poorly differentiated patients and moderately to well differentiated patients. It was revealed that the levels of the two in poorly differentiated patients were lower than those in moderately to well differentiated ones ($P < 0.05$, **Figure 3A**, **3B**). ROC curve showed that the AUCs to distinguish poorly differentiated and moderately to well differentiated patients were 0.772 and 0.739, respectively (**Figure 3C**; **Table 5**).

Expression and diagnostic ability of MLH1 and MSH2 for lymph node metastasis

In an attempt to further determine the value of MLH1 and MSH2 in lymph node metastasis, we compared their expressions between patients with and without metastasis. It was found that patients with lymph node metastasis had lower MLH1 and MSH2 than patients without metastasis ($P < 0.05$, **Figure 4A**, **4B**). The AUCs to distinguish patients with or without metastasis were both 0.634 (**Figure 4C**; **Table 6**).

Patients with low MLH1 and MSH2 levels had shorter 5-year survival

The 5-year survival rates of patients were compared between low-and high-expression groups according to the median values of MLH1 and MSH2. In our results, the low-expression group showed a lower 5-year survival rate than the high-expression group, suggesting that MLH1 and MSH2 expressions can be used as indica-

Table 3. Relationship of MSH2 to pathologic data of colorectal cancer patients

Factor	MSH2		X^2	P
	Low expression group (n=44)	High expression group (n=44)		
Sex			1.137	0.286
Male (n=55)	20	25		
Female (n=33)	24	19		
Age			3.684	0.054
> 60 years old (n=43)	17	26		
≤ 60 years old (n=45)	27	18		
Tumor diameter			2.933	0.086
> 5 cm (n=40)	24	16		
≤ 5 cm (n=48)	20	28		
Pathologic type			1.245	0.264
Ulcer type + infiltrating line (n=57)	31	26		
Massive type (n=31)	13	18		
Differentiation			6.175	0.013
Well differentiated + moderately differentiated (n=76)	34	42		
Poor differentiation (n=12)	10	2		
TNM staging			8.928	0.002
Stage I + II (n=42)	14	28		
Stage III + IV (n=46)	30	16		
Lymph node metastasis			4.583	0.032
Yes (n=40)	25	15		
No (n=48)	19	29		

Note: tumor node metastasis (TNM).

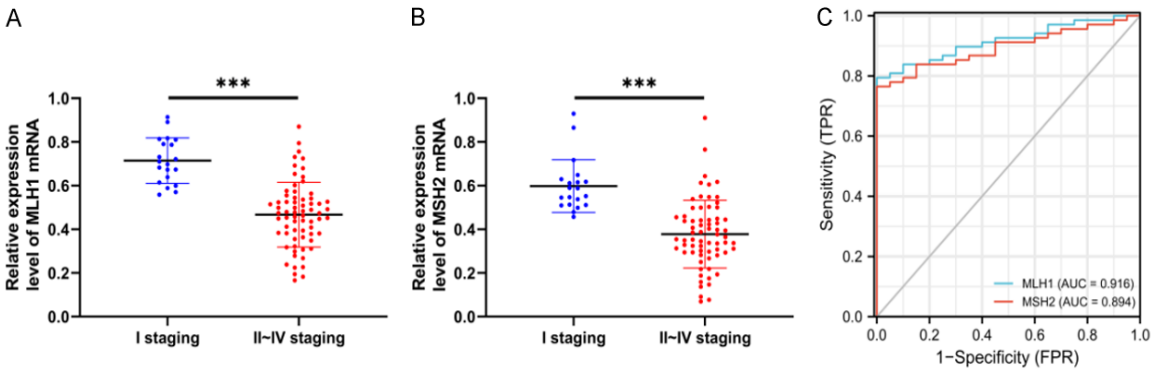

Figure 2. Expression and diagnostic value of MLH1 and MSH2 in patients with early tumor stage. A. MLH1 expression in patients at stage I and at stage II~IV; B. MSH2 expression in patients at stage I and at stage II~IV; C. ROC curve analysis of MLH1 and MSH2 in patients diagnosed with early-stage colorectal cancer. Note: *** means P < 0.001.

Table 4. ROC curve values of MLH1 and MSH2 in tumor staging

Diagnostic Gene	Area Under the Curve	Confidence Interval	Specificity	Sensitivity	Youden Index	Cut-off
MLH1	0.916	0.860-0.972	100.00%	79.40%	79.40%	0.557
MSH2	0.894	0.830-0.958	100.00%	76.50%	76.50%	0.456

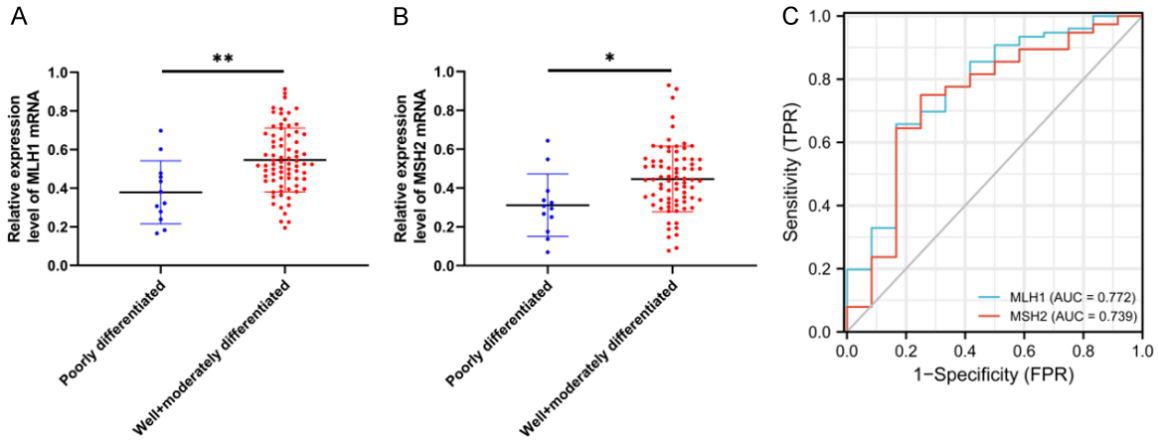

Figure 3. Expression and diagnostic value of MLH1 and MSH2 in various degrees of differentiation. A. MLH1 expression in patients with different differentiation degrees; B. MSH2 expression in patients with different differentiation degrees; C. ROC curve analysis of MLH1 and MSH2 in the diagnosis of differentiation degrees.

Table 5. ROC curve values of MLH1 and MSH2 in degrees of differentiation

Diagnostic Gene	Area Under the Curve	Confidence Interval	Specificity	Sensitivity	Youden Index	Cut-off
MLH1	0.772	0.615-0.929	83.00%	65.80%	49.10%	0.479
MSH2	0.739	0.566-0.912	75.00%	75.00%	50.00%	0.338

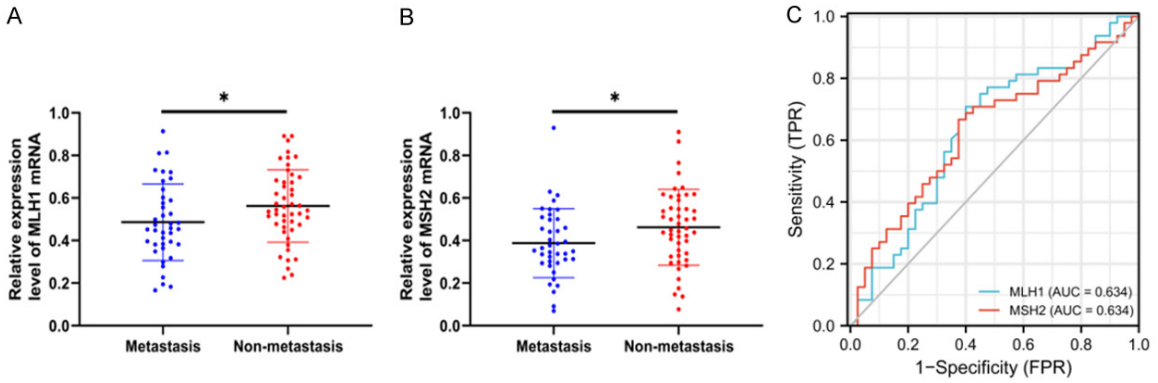

Figure 4. Expression and diagnostic value of MLH1 and MSH2 in lymph node metastasis. A. MLH1 expression in patients with lymph node metastasis; B. MSH2 expression in patients with lymph node metastasis; C. ROC curve analysis of MLH1 and MSH2 in the diagnosis of lymph node metastasis.

Table 6. ROC curve values of MLH1 and MSH2 in lymph node metastasis

Diagnostic Gene	Area Under the Curve	Confidence Interval	Specificity	Sensitivity	Youden Index	Cut-off
MLH1	0.634	0.515-0.754	60.00%	70.80%	30.80%	0.487
MSH2	0.634	0.517-0.751	62.50%	66.70%	29.20%	0.405

tors to predict patient survival ($P < 0.01$, **Figure 5**).

Cox regression analysis of prognostic factors affecting patients' 5-year survival

We analyzed the factors affecting patients' 5-year survival. Univariate Cox regression analysis found that differentiation degree, TNM stage, lymph node metastasis, MLH1, and MSH2 were factors affecting patients' prognosis ($P < 0.05$, **Table 7**). Further multivariate Cox regression analysis revealed that four factors mentioned above, except MLH1, were independent factors affecting the 5-year prognosis of patients ($P < 0.05$, **Table 7**).

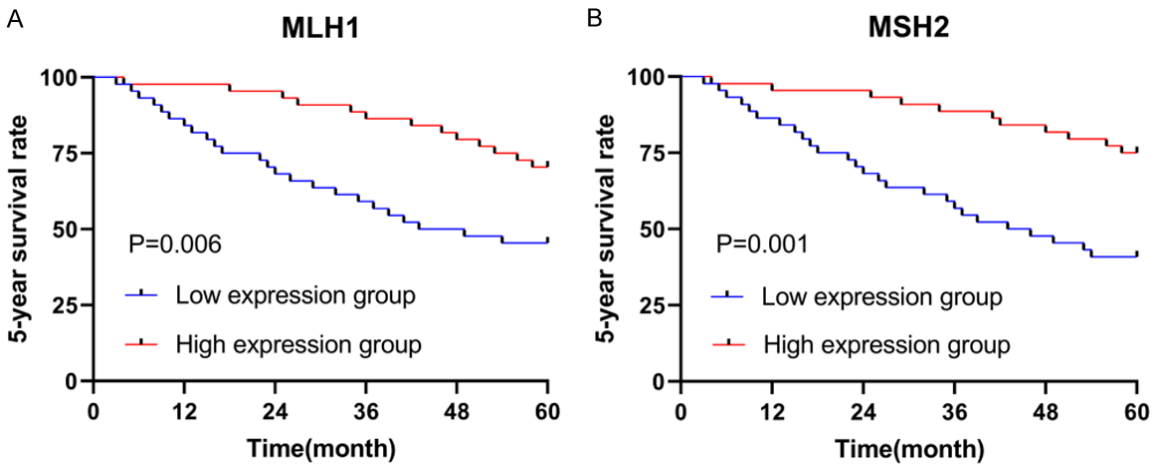

Figure 5. Association of MLH1 and MSH2 with 5-year survival in colorectal cancer patients. A. Comparison of 5-year survival rate of patients with high and low MLH1 expressions; B. Comparison of 5-year survival rate of patients with high and low MSH2 expressions.

Table 7. Cox regression analysis of factors affecting 5-year survival of patients

Factors	Univariate Cox			Multifactor Cox		
	HR Value	*P* Value	Confidence Interval	HR Value	*P* Value	Confidence Interval
Sex	0.630	0.161	0.330-1.203			
Age	0.742	0.369	0.387-1.423			
Tumor Diameter	0.784	0.468	0.407-1.512			
Pathological Type	1.322	0.438	0.653-2.676			
Differentiation	0.326	0.005	0.149-0.715	0.399	0.01	1.427-14.143
TNM staging	0.326	0.002	0.161-0.662	0.312	0.031	0.173-0.918
Lymph Node Metastasis	3.216	0.001	1.612-6.417	3.187	0.001	0.153-0.638
MLH1	2.510	0.008	1.275-4.939	0.397	0.121	0.124-1.275
MSH2	3.296	0.001	1.624-6.690	2.266	0.029	1.087-4.727

Note: tumor node metastasis (TNM).

Discussion

The occurrence and development of colorectal cancer (CC) is the result of the joint action of multiple genes and factors [17-19], and its pathogenesis is complicated. In addition to normal conditions related to recognized oncogenes and tumor suppressor genes, the body's cellular DNA is in a state of continuous synthesis, replication, and division. The mismatch repair system can repair damaged DNA in time to ensure the stability of genetic information [20]. When the mismatch repair system is defective, however, damaged DNA cannot be repaired in time as a result of weakened or absent proofreading repair function, resulting in the accumulation of DNA mismatches. This leads to the widespread distribution of short tandem repeats in the genome of the body and causes genetic instability, that is, microsatellite instability, ultimately leading to cell cancerization [21]. Researchers claimed [22] that microsatellite instability is a molecular phenotype of mismatch repair system defects, which indicates the efficacy of chemotherapeutic drugs and is of great significance in the treatment and prognosis of CC.

MLH1 can heterodimerize with the mismatch repair endonuclease PMS2 to form MutLα, which is involved in the repair system of DNA mismatch. Also, the protein encoded by MLH1 is part of DNA damage signaling. It can heterodimerize with DNA mismatch repair protein MLH3 to form MutLγ, and gets involved in meiosis [23, 24]. MSH2, homologous to the E. coli

MutS gene, is involved in DNA mismatch repair. Human MSH2 can form complexes with BLM-p53-RAD51 dealing with repair of DNA damage [25, 26], during which apoptosis was promoted by MSH2 by regulating ATR/Chk2/p53 signaling [27]. Previous studies have found that MSH2 missense mutations affected splicing, which may regulate cancer initiation and progression in a tissue-specific manner [28]. Another study revealed [29] a marked increase in the probability of poorly differentiated lymph node metastasis in patients with mismatch repair protein deletions. However, given that the results obtained by immunohistochemical methods are mostly negative or positive, quantitative analysis cannot be carried out at present. In this study, RT-qPCR was used to detect the CC tissue to quantify the expressions of MLH1 and MSH2, which were lower than in adjacent tissues. In the previous study by Ismael et al. [30], MLH1 and MSH2 in the lesions of CC patients were downregulated, which is consistent with our findings, suggesting that there is microsatellite instability in the genome of CC patients.

In the study of Wang et al. [31], MLH1/MSH2-positive tumors were significantly more frequent in the colon than in the rectum, and had the characteristics of less mucin production and poor differentiation. An earlier study by Lanza et al. [32] stated that MLH1 and MSH2 expressions could predict clinical outcomes of CC patients in stage II and III. In this study, in addition to the detection of MLH1 and MSH2 expressions in tumor tissue, we also analyzed the relationship of MLH1 and MSH2 with the pathological data of CC patients. We found that colon cancer patients with low expressions of MLH1 and MSH2 had significantly higher TNM stage, poorer differentiation, and more lymph node metastasis, which indicated that MLH1 and MSH2 were involved in the occurrence of CC. To further verify the value of MLH1 and MSH2 in CC patients, we analyzed the relationship of MLH1 and MSH2 with early CC, differentiation degree, and lymph node metastasis. It was found that the expressions of MLH1 and MSH2 were enhanced in CC patients at stage I, with poor differentiation and lymph node metastasis. This suggests that MLH1 and MSH2 have potential diagnostic value in the clinical staging, differentiation and lymph node metastasis in patients with CC. However, ROC analysis found that MLH1 and MSH2 only have good performance in the early diagnosis of CC, but not in differentiation degree and lymph node metastasis.

This study further analyzed the relationship of MLH1 and MSH2 with the 5-year survival of CC patients. As demonstrated in **Figure 5**, a reduction was observed in the 5-year survival rate in patients with low MLH1 and MSH2. By Cox regression analysis, however, only MSH2 was determined to be an independent prognostic factor for CC patients, MLH1 was not. Russo [33] reported that the 5-year survival rate of MLH1-positive and MSH2-positive patients was markedly lower, suggesting that MSH2 was an independent indicator for the prognosis of CC patients.

Nevertheless, the results of this study need to be optimized due to the following limitations. As a result of lacking of peripheral blood samples and normal controls, the diagnostic value of MLH1 and MSH2 in CC could not be analyzed. Second, real-time follow-up was not able to be conducted as in a prospective study, so that the data collected are quite limited. Finally, the mechanism of MLH1 and MSH2 in colon cancer still needs further research. We hope to carry out more experiments in follow-up studies to refine our conclusions.

To sum up, the low expression of MLH1 and MSH2 in CC tissue has a correlation with its pathological characteristics and survival, and can be used as an auxiliary reference for the prognosis of CC patients.

Disclosure of conflict of interest

None.

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