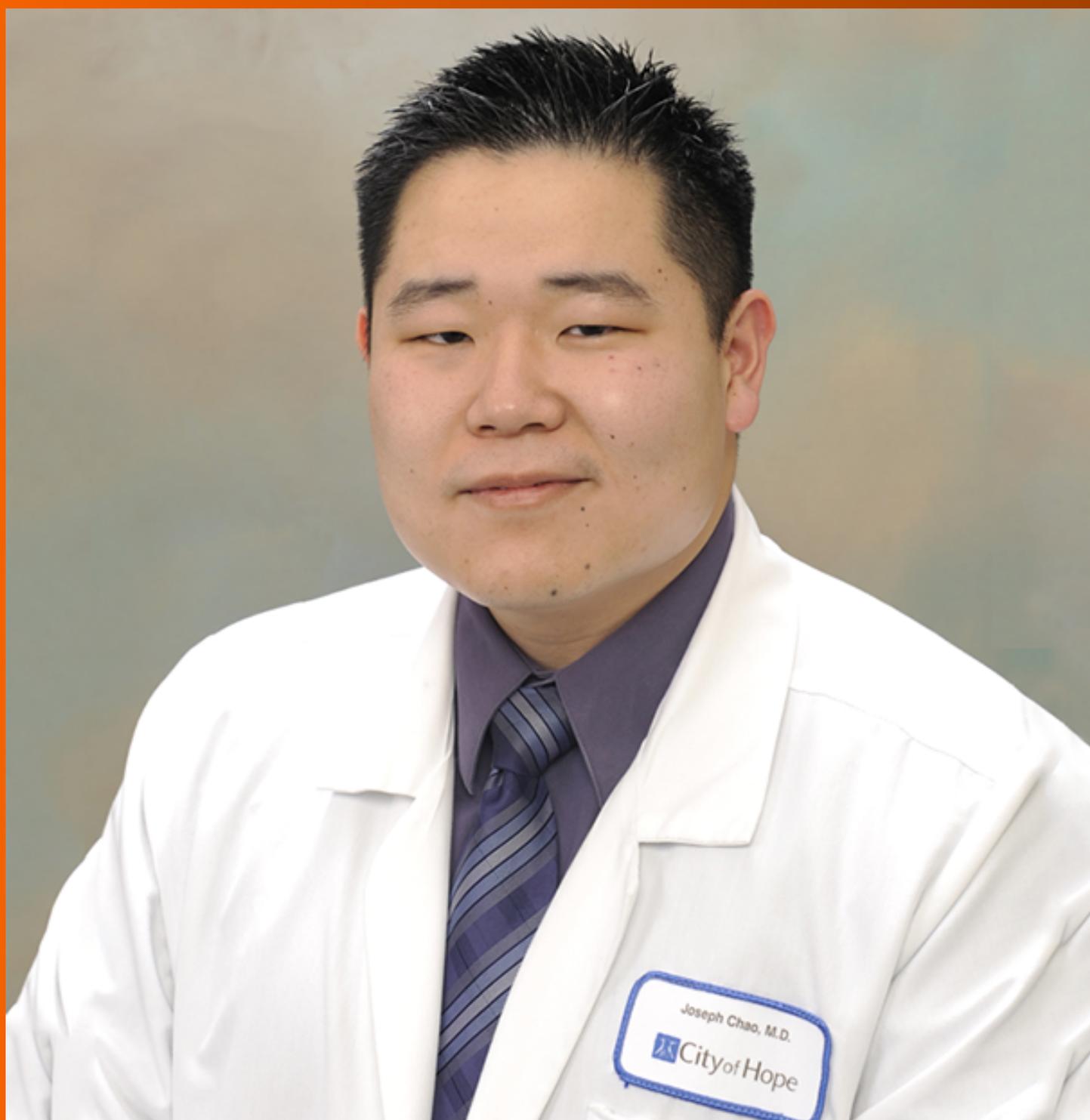


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*World J Gastrointest Oncol* 2019 November 15; 11(11): 933-1091



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**RESPONSIBLE EDITORS FOR THIS ISSUE**

Responsible Electronic Editor: *Lu-Lu Qi*  
 Proofing Production Department Director: *Yun-Xiaojuan Wu*

**NAME OF JOURNAL**

*World Journal of Gastrointestinal Oncology*

**ISSN**

ISSN 1948-5204 (online)

**LAUNCH DATE**

February 15, 2009

**FREQUENCY**

Monthly

**EDITORS-IN-CHIEF**

Monjur Ahmed, Rosa M Jimenez Rodriguez, Pashtoon Kasi

**EDITORIAL BOARD MEMBERS**

<https://www.wjgnet.com/1948-5204/editorialboard.htm>

**EDITORIAL OFFICE**

Jin-Lei Wang, Director

**PUBLICATION DATE**

November 15, 2019

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<https://www.wjgnet.com/bpg/gerinfo/242>

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**ONLINE SUBMISSION**

<https://www.f6publishing.com>

## Observational Study

Clinical significance of *MLH1/MSH2* for stage II/III sporadic colorectal cancer

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**Supported by** Medical Science and Technology Development Foundation, Nanjing Department of Health, No. YKK14140 (to Shui-Ming Wang) and No. ZKX15040 (to Bin Jiang); Project of Administration of Traditional Chinese Medicine of Jiangsu Province of China, No. LZ11101 (to Zhi-Ming Fang).

**Institutional review board**

**statement:** The study was reviewed and approved by the Institutional Review Board of Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine.

**Informed consent statement:** All participants provided informed consent prior to study enrollment.

**Conflict-of-interest statement:** All authors have no conflict of interest to disclose.

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**Abstract****BACKGROUND**

The development of colorectal cancer (CRC) is a complicated multistep process that involves an accumulation of mutations in tumor suppressor genes and oncogenes. In the process of DNA replication, base mismatch often occurs due to various factors leading to abnormal expression of mismatch repair genes (MMR), among which *MLH1* and *MSH2* are the most important. Recently, numerous studies indicated that *MLH1/MSH2* phenotype is associated with CRC. We wanted to elucidate the role of *MLH1/MSH2* in the prediction and prognosis of CRC through long-term clinical observation.

**AIM**

To evaluate the prognostic and predictive significance of *MLH1/MSH2* in patients with stage II-III CRC using immunohistochemical analysis and GeneScan.

**METHODS**

Specimens from 681 patients with CRC (395 stage II and 286 stage III, 387 males and 294 females) who underwent curative surgical resection from 2013 to 2016 were tested. Immunohistochemistry was used to analyze MMR status and the

**Data sharing statement:** No additional data are available.

**STROBE statement:** The authors have read the STROBE Statement checklist of items, and the manuscript was prepared and revised according to the STROBE Statement checklist of items.

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**Manuscript source:** Unsolicited manuscript

**Received:** April 29, 2019

**Peer-review started:** May 9, 2019

**First decision:** July 31, 2019

**Revised:** August 10, 2019

**Accepted:** September 10, 2019

**Article in press:** September 10, 2019

**Published online:** November 15, 2019

**P-Reviewer:** Seow-Choen F, Vynios D

**S-Editor:** Zhang L

**L-Editor:** Wang TQ

**E-Editor:** Wu YXJ



microsatellite status of 133 patients was determined by GeneScan analysis.

## RESULTS

Five hundred and fifty (80.76%) patients were *MLH1/MSH2* positive and 131 (19.24%) were negative by immunohistochemistry. *MLH1/MSH2*-positive tumors were significantly more frequent in the colon than in the rectum, and had poor differentiation and less mucin production ( $P < 0.05$ ). Patients of different groups did not differ in terms of age, gender, tumor size, tumor stage, lymphocytic infiltration, or circumscribed margin. *MLH1/MSH2*-negative patients had a more favorable OS than *MLH1/MSH2*-positive patients ( $P < 0.001$ ). Univariate and multivariate analyses demonstrated *MLH1/MSH2* expression as an independent prognostic and predictive factor for stage II/III CRC. *MLH1/MSH2* expression was a strong prognostic factor in all patients [ $P < 0.001$ , hazard ratio (HR) = 4.064, 95% CI: 2.241–7.369]. Adjuvant chemotherapy had a greater correlation with survival advantage in *MLH1/MSH2*-negative patients with stage III disease ( $P < 0.001$ , HR = 7.660, 95% CI: 2.974–15.883). However, patients with stage II disease or *MLH1/MSH2*-positive patients with stage III disease did not benefit from adjuvant chemotherapy. GeneScan analysis demonstrated that among 133 patients, 105 (78.95%) were microsatellite stable, and 28 (21.05%) had microsatellite instability (MSI), including 18 (13.53%) with high MSI and 10 (7.52%) with low MSI. This is consistent with the immunohistochemical results.

## CONCLUSION

*MLH1/MSH2* phenotype constitutes a pathologically and clinically distinct subtype of sporadic CRC. *MLH1/MSH2* is an independent prognostic and predictive factor for outcome of stage II-III CRC.

**Key words:** Colorectal cancer; Mismatch repair gene; *MLH1*; *MSH2*; Microsatellite instability

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**Core tip:** Mutation or methylation of mismatch repair gene leads to microsatellite instability (MSI), which is one of the most important mechanisms for the development of colorectal cancer (CRC). The purpose of this study was to collect data on *MLH1/MSH2* phenotype and MSI status in stage II-III CRC patients and to assess their predictive and prognostic value. This is the first large study in China to evaluate the role of *MLH1/MSH2* in CRC and its relationship with adjuvant chemotherapy.

**Citation:** Wang SM, Jiang B, Deng Y, Huang SL, Fang MZ, Wang Y. Clinical significance of *MLH1/MSH2* for stage II/III sporadic colorectal cancer. *World J Gastrointest Oncol* 2019; 11(11): 1065-1080

**URL:** <https://www.wjgnet.com/1948-5204/full/v11/i11/1065.htm>

**DOI:** <https://dx.doi.org/10.4251/wjgo.v11.i11.1065>

## INTRODUCTION

Colorectal cancer (CRC) is one of the most common malignancies of the digestive tract. In 2017, there were nearly 135430 newly diagnosed CRC cases with 50260 associated deaths in the United States<sup>[1]</sup>. CRC is mainly associated with at least three distinct genetic pathways: Microsatellite instability (MSI), chromosomal instability (CIN), and CpG Island methylator phenotype (CIMP)<sup>[2]</sup>. Most hereditary nonpolyposis CRC and 15%<sup>[3]</sup> of sporadic CRC are characterized by MSI<sup>[4,5]</sup>. In contrast, 85% of CRC develop from the CIN pathway and are characterized by aneuploidy, allelic losses, amplifications, and translocations<sup>[6]</sup>. Meanwhile, many sporadic MSI CRC are also CIMP positive. These three pathways are not mutually exclusive, and most tumors are characterized by multiple pathways. The mismatch repair (*MMR*) gene<sup>[7]</sup> is a housekeeping gene that is highly conserved. *MMR* maintains correct DNA replication and high fidelity by repairing DNA base mismatches, which allows for genomic stability and reduces spontaneous mutations<sup>[8]</sup>. MSI is characterized by the deletion of DNA methylation or *MMR* caused by genetic

mutation, which leads to widespread alterations in the length of short repeated sequences<sup>[9,10]</sup>. In China, the incidence of CRC has increased significantly in the last 10 years. While the mortality rate of male patients has increased annually, the mortality of female patients has tended to be relatively stable. CRC is the fifth leading cause of morbidity in men and fourth in women. The number of new cases of CRC in 2015 in China was 376300, including 215700 men and 160600 women. There were 191000 deaths due to CRC, including 111100 men and 80000 women<sup>[11]</sup>.

Most cases of MSI appear to result from MMR deficiency. At least six of the genes involved in MMR have now been identified, including MutL homolog 1 (*MLH1*), MutS homolog 2 (*MSH2*), MutS homolog 3 (*MSH3*), postmeiotic segregation increased 1 (*PMS1*), postmeiotic segregation increased 2 (*PMS2*), and MutS homolog 6 (*MSH6*)<sup>[12]</sup>. *MLH1* is located on chromosome 3p21–23 and is connected with the creation of *MLH3*, *PMS2*, and *PMS1*. *MSH2* is located on chromosome 2p21 and has been shown to form *MSH3* and *MSH6*<sup>[13]</sup>. Mutations in either *MLH1* or *MSH2* account for the majority of known germline mutations in CRC, and >90% of MMR deficiencies are deletions of *MLH1* or *MSH2* that rarely appear in other genes, which are the major causes leading to the mutated phenotype<sup>[14,15]</sup>. Mutations either in *MLH1* or *MSH2* or both of them are considered as *MLH1/MSH2* negative, and no mutations in either of them are considered as *MLH1/MSH2* positive. *MLH1* recruits and allocates other proteins to the mismatch repair system and *MSH2* can recognize any errors in DNA replication and replace the incorrect sequence using the parental strand sequence as the correct one<sup>[16]</sup>.

The purpose of the current study was to evaluate the prognostic significance of *MLH1/MSH2* status determined by immunohistochemical analysis in a large cohort of patients with stage II–III CRC. In particular, we sought to detect the relationship between *MLH1/MSH2* and overall survival (OS).

## MATERIALS AND METHODS

### Patients

Initially, we enrolled 836 consecutive patients who underwent curative-intent surgical resection between January 2013 and December 2016 at the Nanjing Hospital of Chinese Medicine Affiliated to Nanjing University of Chinese Medicine (Nanjing, China). One hundred and fifty-five patients were excluded due to loss to follow-up or because their specimens were not available for immunohistochemical analysis. There were at least 681 patients who had all data available at the time of follow-up (387 males and 294 females with a median age of 63 years; range 22–87 years). Diagnosis of CRC was confirmed according to World Health Organization (WHO) criteria and the TNM stage classification<sup>[17]</sup>. Clinical data were obtained from hospital medical records and included details pertaining to patient gender and age; tumor differentiation, location, and size; and mucin, surgical margin, TNM stage, lymph node (LN) metastasis, and histopathological grade. Follow-up of all cases started from the postoperative period to December 2018. Of the 681 patients with CRC, 300 underwent surgery only and 381 surgery plus adjuvant chemotherapy. One hundred and twenty-two (30.9%) of the 395 patients with stage II CRC and 259 (90.6%) of 286 with stage III CRC received postoperative adjuvant chemotherapy. Patients with stage II CRC were subdivided into high- and low-risk categories according to poor prognostic features. The National Comprehensive Cancer Network (NCCN) guidelines<sup>[18]</sup> consider the following high-risk factors for recurrence: Poorly differentiated histology [exclusive of those cancers that have MSI (MSI-H)] or undifferentiated tumors; pathological T4 (pT4) disease; perineural invasion; bowel obstruction; indeterminate or positive margins or localized perforating tumors; and inadequate LN sampling (<12 LNs).

### Treatment

Combining the NCCN guidelines with patients' personal wishes, for stage II CRC, all patients with no high-risk factors, 90% (36/40) of those with high-risk factors combined with *MLH1/MSH2* negativity, and 6.56% (12/183) of those with high-risk factors combined with *MLH1/MSH2* positivity received only regular follow-up. The remaining stage II patients received the CAPEOX regimen (oxaliplatin 130 mg/m<sup>2</sup> for 1 d, intravenous drip 2 h; capecitabine 1 g/m<sup>2</sup>/d for 14 d, oral administration, every 21 d for 6 cycles). For stage IIIA<sup>[19]</sup> (T1–2N1M0 or T1N2M0) CRC, 69.91% (79/113) of patients received the CAPEOX regimen, and for stage IIIB–IIIC (T3–4N1M0 or T1–4N2M0), 80.92% (140/173) of patients with high-risk factors for recurrence received the FOLFOX6 regimen (oxaliplatin 85 mg/m<sup>2</sup> for 1 d, intravenous drip 2 h; calcium folinate 400 mg/m<sup>2</sup> for 1 d, intravenous drip; 5-fluorouracil (5-FU) 400 mg/m<sup>2</sup> for 1 d, intravenous injection; 5-FU 1.2 g/m<sup>2</sup> for 46 h, intravenous drip, every 14 d for 12

cycles), and the remaining ones received only regular follow-up. No patients received radiotherapy, neoadjuvant chemotherapy, or immunotherapy before surgery. Written informed consent was obtained from each patient. Ethical approval to perform this research was issued by the Human Research Ethics Committee of the Nanjing Hospital of Chinese Medicine affiliated to Nanjing University of Chinese Medicine.

### **Histopathological and immunohistochemical analyses**

According to histopathological and immunohistochemical detection, tumor types were classified into adenocarcinoma with or without mucin production. Three hundred and ninety-five patients had stage II CRC and 286 had stage III on the basis of the WHO criteria.

The tumor tissue specimens were immersed in 4% paraformaldehyde for 4 h, and transferred to 70% ethanol. Individual lobes of tumor tissue biopsy material were placed in processing cassettes, dehydrated through a graded series of ethanol, and embedded in paraffin wax blocks. Before immunostaining, 5- $\mu$ m-thick colorectal tissue sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, washed in phosphate-buffered saline, and stained with hematoxylin and eosin. After staining, sections were dehydrated through increasing concentrations of ethanol and xylene. Staining was carried out using the BenchMark XT system (Roche, Shanghai, China).

The specimens were observed under a light microscope, with 10 fields with no fewer than 100 cells per field observed by a double-blind method. At least 5% of the tumor cells were stained positive, otherwise they were considered negative. The final result was reviewed by more than two senior pathologists. Complete loss of *MLH1* or *MSH2* expression was classified as *MLH1* or *MSH2* negative, which formed the *MLH1/MSH2*-negative group. Normal expression of *MLH1* and *MSH2* was classified as *MLH1* positive or *MSH2* positive, which formed the *MLH1/MSH2*-positive group<sup>[20]</sup>.

### **Microsatellite analysis**

One hundred and thirty-three fresh CRC tissues and matched tumor-adjacent normal tissues were collected, frozen in liquid nitrogen, and stored at -80 °C. DNA was extracted by a standard phenol-chloroform procedure. Before DNA extraction, frozen sections were cut from each tumor sample, which were stained with hematoxylin and eosin to verify by microscopic examination the presence of adequate neoplastic material (60%-70% of tumor cells). The primers, location, and sequence of MSI are listed in [Table 1](#).

In all 133 cases, MSI was evaluated at five microsatellite loci (*BAT26*, *BAT25*, *D2S123*, *D5S346*, and *D17S250*) using fluorescence-based polymerase chain reaction (PCR). Analysis of the PCR products was performed with an automated laser-activated fluorescent DNA sequencer using the Applied Biosystems 3130XL and analyzed with GenScan 3.1 software. MSI-H was defined as  $\geq 2$  mutation sites; low MSI (MSI-L) was defined as only one mutation site; and microsatellite stability (MSS) was defined as no mutations.

### **Statistical analysis**

The relationship between *MLH1/MSH2* expression and clinicopathological factors was analyzed by the  $\chi^2$  test. Clinical factors that were analyzed included age, gender, tumor stage, differentiation, lymphocytic infiltration, tumor size, mucin, and tumor margin. Survival was estimated by the Kaplan-Meier method. Univariate and multivariate analyses were carried out using Cox's proportional hazards regression models.  $P < 0.05$  was defined as significant. The statistical analyses were performed using SPSS version 20.0.

## **RESULTS**

### ***MLH1/MSH2* expression and clinicopathological features**

Among the 681 patients, 131 (19.24%) were *MLH1/MSH2*-negative and 550 (80.76%) were *MLH1/MSH2*-positive. The relationship between clinicopathological characteristics and *MLH1/MSH2* expression is shown in [Table 2](#) and [Figure 1](#). *MLH1/MSH2*-negative CRC occurred more frequently in the right than in the left colon (27.88% vs 17.86%,  $P = 0.029$ ). It also occurred more frequently in the colon than in the rectum (22.82% vs 15.80%,  $P = 0.025$ ) and in poorly differentiated than well-moderately differentiated CRC (33.33% vs 18.31%,  $P = 0.017$ ). In addition, *MLH1/MSH2*-negative CRC was characterized by LN metastasis and mucinous tumor. *MLH1/MSH2*-negative tumors were more likely to contain mucin ( $P = 0.024$ ). *MLH1/MSH2* expression was not associated with age, gender, tumor stage, tumor size, lymphocytic infiltration, or circumscribed margin ( $P > 0.05$ ).

**Table 1** Name of primers, location, and the sequence of microsatellite instability

SNP	Location	Primer sequence
BAT26	2p16	p1 TGACTACTTTTGACTTCAGCC p2 AACCATTC AACATTTTAAACCC
D2S123	2p21-2p16	p1 AAACAGGATGCCTGCCTTTA p2 GGACTTTCCACCTATGGGAC
D5S346	5q21-5q22	p1 ACTCACTCTAGTGATAAATCGGG p2 CAGATAAGACAGTATTACTAGTT
BAT25	4q12	p1 TCGCCTCCAAGAATGTAAGT p2 TCTGCATTTTAACTATGGCTC
D17S250	17q11.2-17q12	p1 GGAAGAATCAAATAGACAAT p2 GCTGGCCATATATATTTAAACCC

SNP: Single nucleotide polymorphism.

### Survival analysis

With a median follow-up period of 56 mo (range 8.0–72.0 mo), 36 of 395 (9.11%) patients with stage II CRC died and 42 (10.63%) had recurrence or metastasis during the study. For stage III CRC, 90 of 286 (31.47%) patients died and 107 (37.41%) had recurrence or metastasis. Causes of death included cancer recurrence ( $n = 46$ ), metastasis to other organs ( $n = 51$ ), pulmonary infection ( $n = 15$ ), heart disease ( $n = 11$ ), second primary cancer ( $n = 17$ ), multiple organ failure ( $n = 7$ ), and unspecified reasons ( $n = 8$ ). Some patients had more than one cause of death.

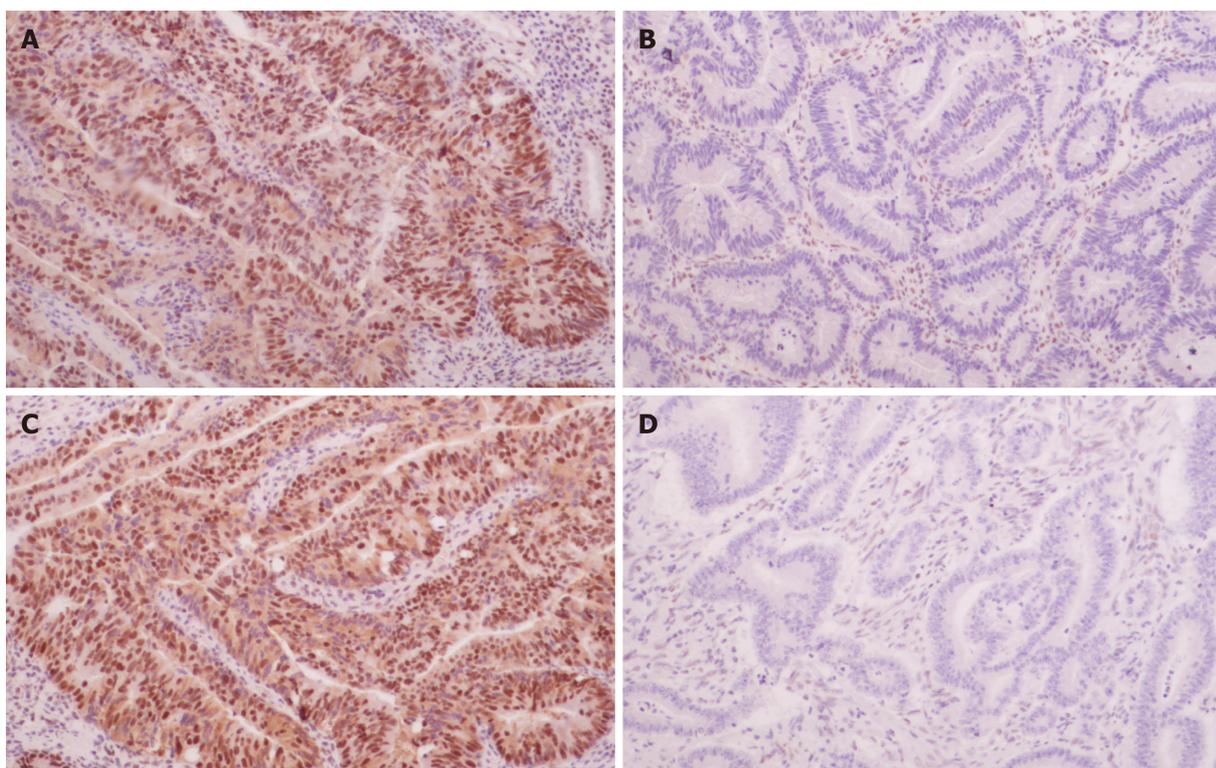
Patients with *MLH1/MSH2*-negative stage II or III CRC showed a favorable trend for OS ( $68.62 \pm 0.83$  vs  $62.11 \pm 1.07$  mo,  $P < 0.001$ ). Stratified analyses showed that patients with *MLH1/MSH2*-negative stage II CRC had longer OS than those with *MLH1/MSH2*-positive CRC ( $70.67 \pm 0.65$  vs  $66.02 \pm 1.01$  mo,  $P = 0.011$ ). Patients with *MLH1/MSH2*-negative stage III CRC also had a longer OS than those with *MLH1/MSH2*-positive ( $66.05 \pm 1.62$  vs  $63.40 \pm 1.15$  mo,  $P = 0.023$ ). In patients who received adjuvant chemotherapy, those with *MLH1/MSH2*-negative CRC had an OS of  $64.02 \pm 1.61$  mo compared with  $62.11 \pm 1.07$  mo in those with *MLH1/MSH2*-positive CRC ( $P = 0.015$ ). The 5-year survival rate for patients with *MLH1/MSH2*-negative CRC was 86.9%, compared with 59.1% for patients with *MLH1/MSH2*-positive CRC. The data for OS are listed in [Figure 2](#).

### Univariate and multivariate analyses

In univariate analysis, patients with *MLH1/MSH2*-positive CRC had a significantly worse OS than those with *MLH1/MSH2*-negative CRC [ $P < 0.001$ , hazard ratio (HR) = 3.799, 95%CI: 2.205–6.546]. Several other factors were also associated with disease survival, such as age, tumor differentiation, tumor stage, lymphocytic infiltration, mucin, and circumscribed margin ( $P < 0.05$ ).

In multivariate survival analysis incorporating status of *MLH1/MSH2*, gender, age, tumor location, tumor differentiation, tumor stage, tumor size, lymphocytic infiltration, mucin, and tumor margin, the status of *MLH1/MSH2* was an independent prognostic factor for OS ( $P < 0.001$ , HR = 4.064, 95%CI: 1.241–7.369). Besides that, age, tumor location, tumor stage, lymphocytic infiltration, mucin, and circumscribed margin were also independent prognostic factors for OS ( $P < 0.05$ ). In the subgroup analysis of stage II CRC, patients with *MLH1/MSH2*-negative tumor demonstrated a better OS than those with *MLH1/MSH2*-positive tumor (multivariate  $P < 0.011$ , HR = 5.583, 95%CI: 1.478–21.092). Patients with stage III disease had similar results but *MLH1/MSH2* status was less significant than in patients with stage II disease (multivariate  $P = 0.023$ , HR 2.289, 95%CI: 1.270–4.125) ([Table 3](#)).

We observed no significant benefit from adjuvant chemotherapy for patients with *MLH1/MSH2*-negative (multivariate  $P = 0.147$ , HR = 1.563, 95%CI: 0.481–4.441) or *MLH1/MSH2*-positive (multivariate  $P = 0.070$ , HR = 1.267, 95%CI: 0.212–5.052) stage II CRC. However, a better survival was observed for patients with *MLH1/MSH2*-negative stage III CRC who received adjuvant chemotherapy (multivariate  $P < 0.001$ , HR = 7.660, 95%CI: 2.974–15.883). But a nonsignificant trend for survival benefit from adjuvant chemotherapy was observed in patients with *MLH1/MSH2*-positive stage III disease (multivariate  $P = 0.052$ , HR = 2.817, 95%CI 0.223–6.671) ([Table 4](#)). All findings are consistent for the OS end point.



**Figure 1** Expression of *MLH1* and *MSH2* in colorectal cancer tissues detected by immunohistochemistry (DAB staining, ×100). A: *MLH1* positivity; B: *MLH1* negativity; C: *MSH2* positivity; D: *MSH2* negativity.

### MSI

Microsatellite analysis was performed in 133 CRC patients (71 stage II and 62 stage III) using GenScan, and 105 had MSS (78.95%) and 28 (21.05%) had MSI, including 18 (13.53%) cases of MSI-H and 10 (7.52%) cases of MSI-L. All patients were detected by immunohistochemical analysis, which confirmed that patients with MSI-H carcinomas included 17 (94.4%) who were *MLH1/MSH2*-negative and one (5.6%) who was *MLH1/MSH2*-positive ( $P < 0.001$ ). Patients with MSS and MSI-L who had *MLH1* or *MSH2* positivity were classified as *MLH1/MSH2*-positive. According to our data analysis, MSI-H was more frequent in patients aged  $> 50$  years ( $P = 0.048$ ), in the right colon ( $P < 0.001$ ), in tumors with poor differentiation ( $P = 0.028$ ), and in tumors with mucin ( $P = 0.037$ ). The clinicopathological features of MSI are consistent with previous immunohistochemical results of *MLH1/MSH2* expression. The analysis of MSI is shown in Figure 3.

## DISCUSSION

Defective DNA MMR is most often associated with loss of *MLH1* and *MSH2* gene functions and results in MSI mutation. *MLH1* and *MSH2* promoter hypermethylation is an important DNA MMR pathway in sporadic proximal CRC<sup>[21]</sup>. Abnormal methylation, causing alteration of *MLH1/MSH2*, can form transcriptional target genes for silencing<sup>[22]</sup>. Several studies<sup>[23-25]</sup> have revealed that *MLH1* and *MSH2* play a critical role, and mutations in either gene result in complete loss of function, with tumor formation preferentially in the proximal colon, and this hypermethylation is significantly more common in sporadic than in hereditary MSI-positive tumors. Thibodeau *et al*<sup>[26,27]</sup> reported that in most colorectal carcinomas with MSI-H phenotype, 91% of cases are confirmed as *MLH1*-negative. In another study, Herman *et al*<sup>[28]</sup> reported that hypermethylation of *MLH1* in sporadic CRC with MSI-H was as high as 84%. By contrast, Vasen *et al*<sup>[29]</sup> demonstrated that *MSH2* mutation is associated with a higher risk of developing cancer than *MLH1* mutation. *MSH2* generally forms a connection with *MSH6* or *MSH3*, so it can control most of the hypermethylation that occurs with different bound proteins. And *MLH1* forms with *PMS2*, and goes through one pathway to combine with other proteins<sup>[30,31]</sup>. Gene mutation is the key reason for the decrease of *MLH1* and *MSH2* expression and both of them are the most dominant parts of the MMR system, so detection for those two

**Table 2 Relationship between clinicopathological features and *MutL homolog1/MutS homolog2* expression in 681 patients, n (%)**

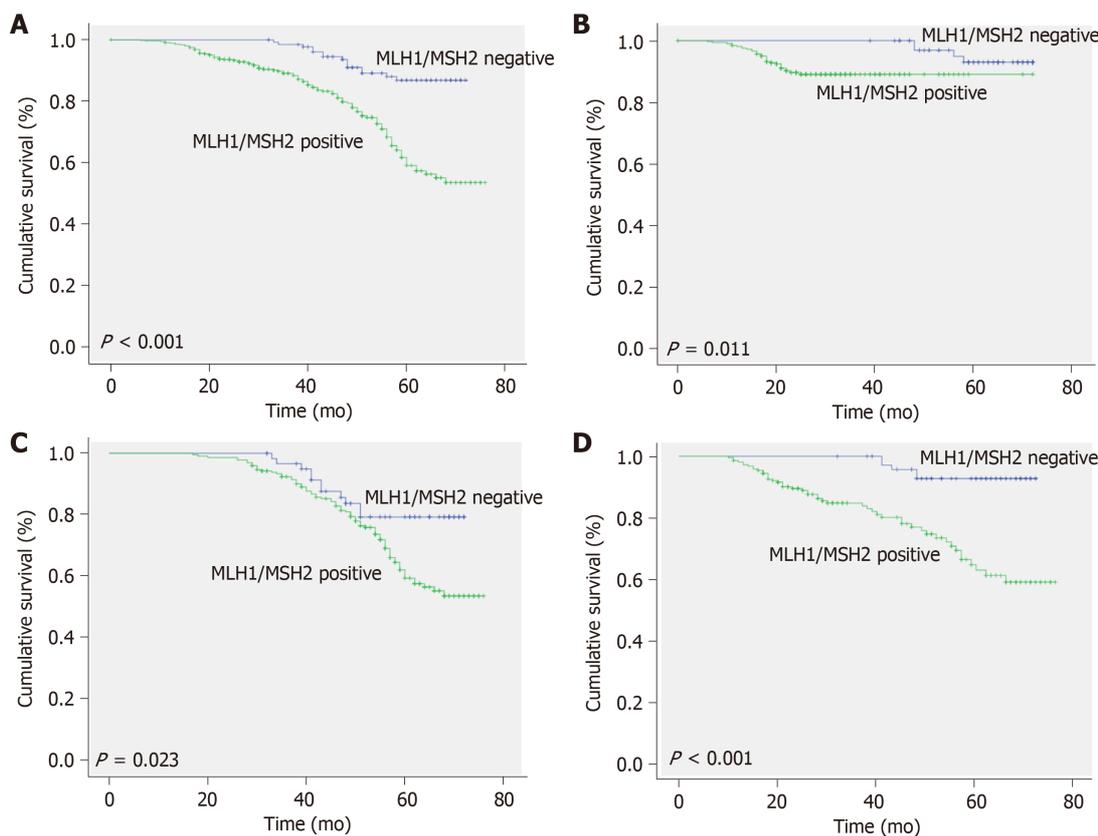
Observation	Positive <sup>a</sup>	Negative <sup>b</sup>	Total	$\chi^2$	P value
Gender					
Male	310 (80.10)	77 (19.90)	387	0.252	0.625
Female	240 (81.63)	54 (18.37)	294		
Age (yr)					
≤50	63 (88.73)	8 (11.27)	71	3.240	0.072
>50	487 (79.84)	123 (20.16)	610		
Location in the colon					
Left	138 (82.14)	30 (17.86)	168	4.746	0.029
Right	119 (72.12)	46 (27.88)	165		
Location of tumor Colon	257 (77.18)	76 (22.82)	333	5.395	0.025
Rectum	293 (84.20)	55 (15.80)	348		
Grade of differentiation					
Poor	28 (66.67)	14 (33.33)	42	5.725	0.017
Well-moderate	522 (81.69)	117 (18.31)	639		
Tumor stage (TNM)					
II	324 (82.03)	71 (17.97)	395	0.964	0.327
III	226 (79.02)	60 (20.98)	286		
Tumor size (cm)					
≤4	210 (78.95)	56 (21.05)	266	0.927	0.370
>4	340 (81.93)	75 (18.07)	415		
Lymphocytic infiltration					
None or little	336 (82.76)	70 (17.24)	406	1.195	0.332
Marked or moderate	214 (75.76)	61 (27.24)	275		
Mucin					
Positive	108 (73.97)	38 (26.03)	146	5.517	0.024
Negative	442 (82.62)	93 (17.38)	535		
Circumscribed margin					
Negative	482 (79.80)	122 (20.20)	604	3.184	0.090
Positive	68 (88.31)	9 (11.69)	77		

<sup>a</sup>MLH1/MSH2 positive;<sup>b</sup>MLH1/MSH2 negative. MLH1: MutL homolog 1; MSH2: MutS homolog 2.

genes are important for discovering the pathogenesis of sporadic CRC.

In this study, we used immunohistochemistry to detect *MLH1* and *MSH2* expression in all postoperative patients because it is more accurate, rapid, and cost-effective for assessment of MMR status than other methods<sup>[32,33]</sup>. *MLH1/MSH2* status can verify MMR expression. In contrast, using GenScan to analyze MSI status can more accurately and directly demonstrate the difference between normal and abnormal loci, although the test is expensive and not easy to analyze in each postoperative patient<sup>[34]</sup>. So, immunohistochemical staining for MMR is now performed as part of routine processing in the department of pathology in almost all hospitals after surgery<sup>[35-37]</sup>. In our study, immunohistochemical analysis for MMR found that *MLH1/MSH2* negativity was more frequent in the right colon, in tumors with poor differentiation, and in tumors with mucin production. With regard to MSI detection, MSI-H occurred mostly in patients aged > 50 years, in the right colon, in tumors with poor differentiation, and in tumors with mucin production. We confirmed that these two clinical assays have more consistency and accuracy than other detections.

The result of our study clearly showed that *MLH1/MSH2*-negative tumors were mostly in the right colon with poor differentiation and contained mucin. This conclusion is consistent with most of the published research. Benatti *et al*<sup>[38]</sup> reported that MSI-H occurred in 256 (20.3%) of 1263 patients, more frequently in tumors which were in the less advanced stage, right sided, poorly differentiated with mucinous phenotype, and had infiltrative growth than MSS<sup>[39]</sup>. Numerous studies have a similar conclusion that most MSI-H tumors are mucinous adenocarcinoma, located in the



**Figure 2** Kaplan-Meier survival curves for patients with colorectal cancer, stratified according to expression of *MLH1/MSH2*. A: Overall survival of total colorectal cancer (CRC) patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative subgroups; B: Overall survival of stage II CRC patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative subgroups; C: Overall survival of stage III CRC patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative subgroups; D: Overall survival of all adjuvant chemotherapy patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative subgroups.

right colon, and poorly differentiated ( $P < 0.05$ )<sup>[40,41]</sup>. Though in the early studies, CRC was divided into colonic and rectal by anatomical site, they had some differences in specific treatments even if they have been treated as the same disease. And in recent years, with the deepening of understanding of this disease and the increase of evidence-based medical proof, CRC in different parts was considered to have distinct clinical pathological expression and prognosis. So the location of tumor is instructive for prognosis and treatment<sup>[42,43]</sup>. More and more studies now tend to divide CRC into right site and left site according to the colonic splenic flexure<sup>[44]</sup>. It is based on the right site originating from the midgut of embryo, while the left site originates from the hindgut of embryo. Anatomically, the right site was supplied by the superior mesenteric artery, and the left site was supplied by the inferior mesenteric artery<sup>[45,46]</sup>. Therefore, the right-sided tumor has poorer differentiation, worse pathological stage, and earlier metastasis than the left-sided tumor<sup>[47]</sup>. Since the rectal blood is supplied from the internal iliac artery, and rectal cancer is different from the colonic cancer in clinical treatment, in our study we divided CRC into the right colon, left colon, and rectum parts. As found in other studies<sup>[48,49]</sup>, the frequency of mismatch repair deficiency (dMMR) in right-sided tumors with poor differentiation was significantly higher than that in tumors of the left colon and rectum in our study, indicating that MSI is mainly involved in the development of right colon cancer. Thus, the occurrence of CRC in different parts is not the same at the genetic level.

In terms of prognostic value of MMR phenotype in CRC, abundant studies<sup>[50-52]</sup> have acquired positive results. Gryfe *et al*<sup>[53]</sup> analyzed 607 patients with CRC and divided them into TNM stages I-IV. All patients with MSI-H had a survival advantage compared with MSS patients ( $P < 0.001$ , HR 0.42, 95% CI 0.27-0.67). The incidences of distant metastasis ( $P = 0.02$ , HR 0.49) and regional LN metastasis ( $P < 0.001$ , HR 0.33) in MSI-H patients were lower than those in patients who had MSS tumors. Roth *et al*<sup>[54]</sup> investigated the effect of MMR at different stages. In 1404 patients with stage II or III CRC, the prognostic advantage conferred by MSI was more evident in stage II than in stage III ( $P = 0.04$ ). In a large meta-analysis<sup>[55]</sup> of 12782 patients, there was a clear correlation between MSI-H tumors and improved OS. The data demonstrated that the OS of patients with MSI-H was significantly better than that of MSI-L and MSS

**Table 3** Prognostic factors for survival in univariate and multivariate analyses

Observation	Univariate			Multivariate		
	P	HR	95%CI	P	HR	95%CI
<b>Model 1<sup>a</sup></b>						
Gender						
Male vs Female	0.932	0.984	0.684-1.417	0.991	0.998	0.685-1.454
Age/yr						
≤50 vs >50	< 0.001	0.288	0.189-0.438	0.025	0.508	0.213-0.995
Location in the colon						
Left vs Right	0.730	1.086	0.678-1.740	0.440	1.211	0.745-1.967
Location of tumor						
Rectum vs Colon	0.232	1.311	0.840-2.047	0.017	1.795	1.111-2.902
Differentiation						
Poor vs Well-moderate	0.002	0.426	0.472-0.734	0.923	0.972	0.542-1.741
Tumor stage						
II vs III	0.034	0.651	0.437-0.968	0.041	0.601	0.321-0.932
Tumor size						
<4 vs ≥4 cm	0.646	0.921	0.647-1.311	0.421	0.861	0.598-1.240
Lymphocytic infiltration						
Positive vs Negative	< 0.001	2.282	1.092-5.756	0.022	3.665	1.207-7.128
Mucin						
Positive vs Negative	0.001	2.361	1.647-3.383	< 0.001	2.512	1.714-4.682
Circumscribed margin						
Positive vs Negative	< 0.001	3.908	2.654-5.755	0.011	2.474	1.433-4.270
<i>MLH1/MSH2</i>						
Positive vs Negative	< 0.001	3.799	2.205-6.546	< 0.001	4.064	2.241-7.369
<b>Model 2<sup>b</sup></b>						
Gender						
Male vs Female	0.761	0.896	0.441-1.821	0.207	0.622	0.298-1.300
Age/yr						
≤50 vs >50	< 0.001	0.150	0.075-0.302	< 0.001	0.131	0.063-0.271
<i>MLH1/MSH2</i>						
Positive vs Negative	0.014	4.833	1.382-16.899	0.011	5.583	1.478-21.092
Therapeutic regimen						
Operation vs Operation + Chemotherapy	0.176	0.821	0.520-1.233	0.063	0.901	0.899-2.312
<b>Model 3<sup>c</sup></b>						
Gender						
Male vs Female	0.964	0.990	0.647-1.517	0.748	0.932	0.607-1.432
Age/yr						
≤50 vs >50	0.002	0.424	0.247-0.728	0.004	0.446	0.258-0.769
<i>MLH1/MSH2</i>						
Positive vs Negative	0.041	1.625	1.042-2.803	0.023	2.289	1.270-4.125
Therapeutic regimen						
Operation vs Operation + Chemotherapy	0.028	2.891	1.209-6.372	< 0.001	4.002	1.929-9.425

<sup>a</sup>All 681 patients;<sup>b</sup>395 Patients with stage II CRC;<sup>c</sup>286 Patients with stage III CRC. HR: hazard ratio; CI: confidence interval; CRC: Colorectal cancer; MLH1: MutL homolog 1; MSH2: MutS homolog 2.

patients ( $P < 0.001$ ), with an overall odds ratio of 0.6 (95%CI 0.53–0.69). In addition, disease-free survival (DFS) was also significantly different ( $P < 0.001$ ). In our study, patients with *MLH1/MSH2*-negative stage II-III CRC had a better clinical outcome than those with *MLH1/MSH2*-positive CRC. Moreover, in multivariate analysis, the survival advantage for *MLH1/MSH2*-negative patients was independent from several other clinical and pathological parameters. These conclusions are consistent with

**Table 4 Predictive factors for survival in univariate and multivariate analyses**

Observation	Univariate			Multivariate		
	P	HR	95%CI	P	HR	95%CI
Model 1 <sup>a</sup>						
<i>MLH1/MSH2</i> negative						
Operation <i>vs</i> Operation + Chemotherapy	0.098	1.021	0.342-2.741	0.147	1.563	0.481-4.441
<i>MLH1/MSH2</i> positive						
Operation <i>vs</i> Operation + Chemotherapy	0.081	1.899	0.127-4.114	0.070	1.267	0.212-5.052
Model 2 <sup>b</sup>						
<i>MLH1/MSH2</i> negative						
Operation <i>vs</i> Operation + Chemotherapy	0.001	4.393	2.068-12.316	< 0.001	7.660	2.974-15.883
<i>MLH1/MSH2</i> positive						
Operation <i>vs</i> Operation + Chemotherapy	0.063	2.015	0.648-5.997	0.052	2.817	0.223-6.671

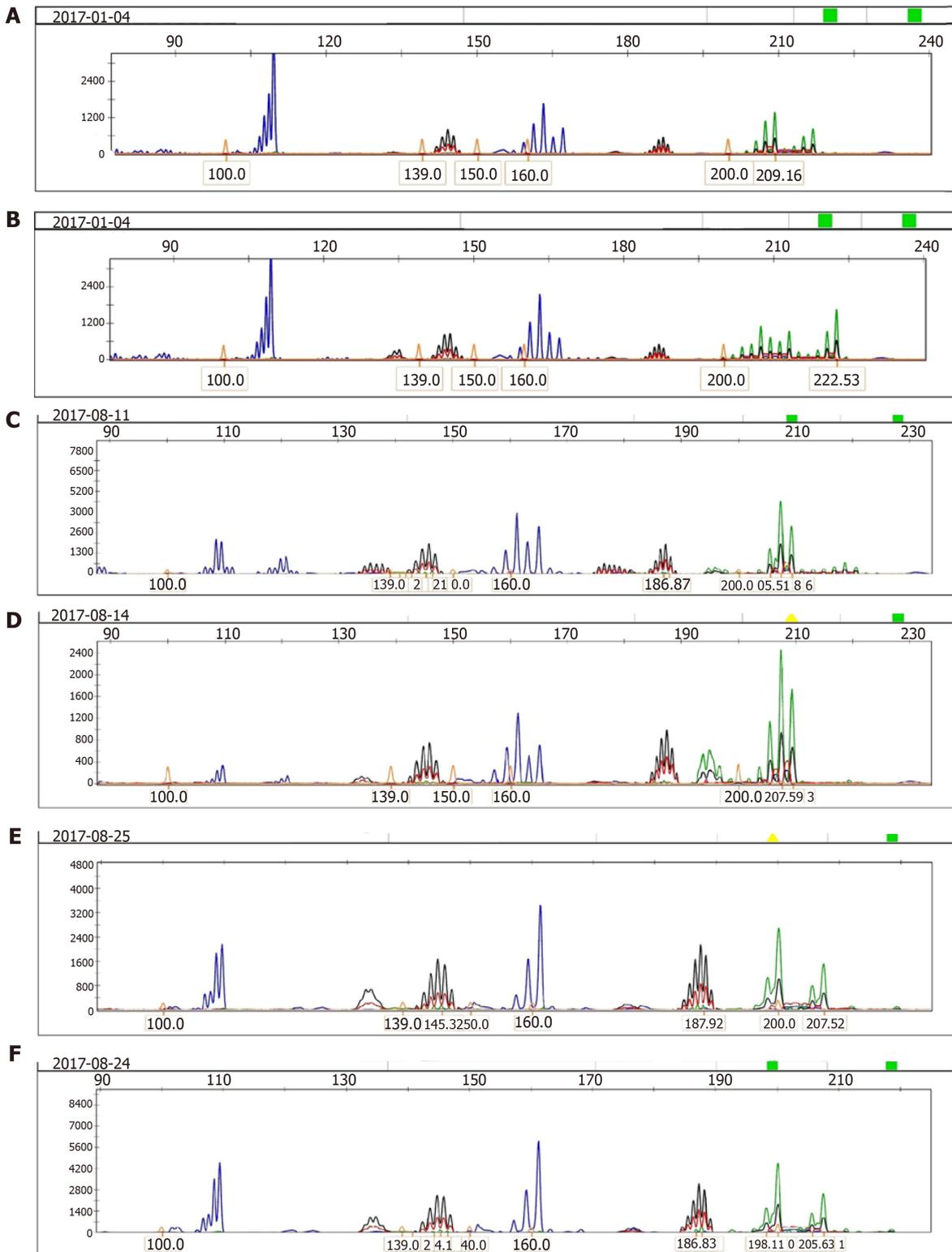
<sup>a</sup>395 Patients with stage II CRC;

<sup>b</sup>286 Patients with stage III CRC. HR: hazard ratio; CI: confidence interval; MLH1: MutL homolog 1; MSH2: MutS homolog 2.

almost all related studies because of the difference in histology, anatomy, and accompanying degree of differentiation, histopathology in different locations of the intestine.

Adjuvant chemotherapy is considered as the gold standard for treatment of patients with stage III CRC. However, there was controversy in a previous study as to whether patients with stage II CRC should take adjuvant chemotherapy after surgery. The 2013 NCCN guidelines<sup>[48]</sup> suggested to test for MMR proteins for all patients < 50 years of age or with stage II disease. Stage II MSI-H patients may have a good prognosis and do not benefit from 5-FU adjuvant therapy<sup>[56]</sup>. Our study followed this guideline and respected patients' wishes to formulate their treatment schedule. We found that stage II-III CRC had different results on adjuvant chemotherapy. In stage II CRC, OS was not strongly associated with adjuvant chemotherapy either in *MLH1/MSH2*-negative (multivariate  $P = 0.147$ , HR = 1.563, 95%CI: 0.481-4.441) or *MLH1/MSH2*-positive patients (multivariate  $P = 0.070$ , HR = 1.267, 95%CI: 0.212-5.052). However, in stage III CRC, we found that OS of the *MLH1/MSH2*-negative patients (multivariate  $P < 0.001$ , HR = 7.660, 95%CI: 2.974-15.883) was more strongly associated with adjuvant chemotherapy than that of *MLH1/MSH2*-positive patients (multivariate  $P = 0.052$ , HR = 2.817, 95%CI: 0.223-6.671). This is similar to the study by Elsaleh *et al*<sup>[57]</sup> which revealed that patients with stage III colon cancer with MSI had improved survival when treated with 5-FU-based chemotherapy (fluorouracil and levamisole) compared with no chemotherapy (HR = 0.07, 95%CI: 0.01-0.53 *vs* HR = 1.06, 95%CI: 0.65-1.72). In the study of Sinicrope *et al*<sup>[58]</sup> (NCCTG N0147) of 2720 stage III colon cancer patients for 5-year disease-free survival, it was found that MMR proficient (pMMR) patients had statistically shorter survival time than dMMR patients ( $P < 0.0001$ ). But in patients with stage III CRC, the predictive function of MMR for adjuvant chemotherapy remains controversial because there are some studies supporting the opposite conclusion. Ribic *et al*<sup>[59]</sup> showed no benefit from adjuvant chemotherapy in stage II-III patients with MSI. Sargent *et al*<sup>[60]</sup> enrolled 457 stage II-III CRC patients divided into 5-FU-based therapy ( $n = 229$ ) and postsurgical treatment groups ( $n = 228$ ). Patients with MSI who received 5-FU had no improvement in DFS ( $P = 0.85$ , HR 1.10; 95%CI 0.42-2.91) compared with the postsurgical treatment group. Jover *et al*<sup>[61]</sup> confirmed that patients with dMMR colon cancer do not benefit from adjuvant 5-FU/leucovorin. Many studies that analyzed the relationship between MMR and prognosis enrolled patients with all TNM stages of disease rather than stage II- III patients. Some studies included fewer patients with MSI-H, which made it difficult or inaccurate to evaluate 5-FU-based chemotherapy regimens. Those are the main reasons leading to the inconsistency of the final results. We encouraged to make risk-stratification for patients in evaluating the effect of adjuvant chemotherapy for patients with dMMR colon cancer.

Tumor immunotherapy has greatly advanced in recent years, especially, PD-1/PD-L1 blocking therapy has shown encouraging effects and become a major pillar of immunotherapy. Patients with metastatic colorectal cancer also gain new hopes from immunotherapy, particularly in dMMR patients for whom immune checkpoint inhibitor antibody can achieve a 40% objective response rate (ORR) and up to 78%



**Figure 3** Analysis of microsatellite instability in colorectal cancer tissues and corresponding normal mucosa using the five markers of the international workshop of Bethesda and fluorescence-based multiplex polymerase chain reaction. Fragment pattern of a high-frequency microsatellite instability tumor showing instability at all five loci examined is shown. A: Low microsatellite instability (MSI-L) of tumor tissue; B: MSI-L of corresponding normal tissue; C: High microsatellite instability (MSI-H) of tumor tissue; D: MSI-H of corresponding normal tissue; E: Microsatellite stability (MSS) of tumor tissue; F: MSS of corresponding normal tissue.

clinical benefit rate<sup>[62]</sup>. The clinical trial KEYNOTE-028<sup>[63]</sup> enrolled advanced colorectal adenocarcinoma patients who failed standard therapy and had PD-L1 expression in  $\geq 1\%$  of cells in tumor nests. The primary endpoints were ORR, safety, and tolerability. Patients received pembrolizumab 10 mg/kg every 2 wk and lasted more than 2 years or until confirmed unacceptable toxicity or progression. The results showed that in

the 156 advanced CRC patients, 23 were PD-L1 positive, 1 gained complete remission (CR), and 1 experienced a partial response (ORR, 4%; 95% CI: 0–22%) who was confirmed as MSI-H. This trial revealed that PD-L1 expression cannot screen out the dominant population of anti-PD-1 immunotherapy. Based on the KEYNOTE-028 and several other clinical trial results, Le *et al*<sup>[64]</sup> designed a single-arm, phase II clinical study (NCT01876511) which aimed to explore the predictive value of MMR status in the treatment of PD-1. The investigator divided 41 patients with progressive metastatic carcinoma with or without dMMR into three groups, namely, 11 with dMMR CRC, 21 with pMMR CRC, and 9 with dMMR non-CRC (4 ampullary /cholangiocarcinoma, 2 endometrial cancer, 2 small intestine cancer, and 1 gastric cancer) and every patient was administered pembrolizumab 10 mg per kilogram of body weight every 14 d. The primary endpoints were ORR at 20 wk and progression-free survival (PFS). The results showed that the ORR for the three groups were 40%, 0, and 71%, respectively, and the PFS rates were 78%, 11%, and 67%, respectively, at 20 wk. Interestingly, the investigators used whole-exome sequencing to check somatic mutations and found that the mutation rate was higher in dMMR than in pMMR ( $P = 0.007$ ). Moreover, the study demonstrated that high somatic mutation loads were associated with prolonged PMS ( $P = 0.02$ ) and dMMR patients received clinical benefit of immune checkpoint blockade with pembrolizumab.

The data of these innovative single-arm clinical studies<sup>[65-67]</sup> led to accelerated approval of the United States FDA for pembrolizumab in patients with dMMR/MSI-H solid tumors (including CRC) who failed previous treatment in May 2017<sup>[68]</sup>. Subsequently, pembrolizumab and nivolumab were recommended for second-line or later treatment of dMMR/MSI-H CRC in the 2017 NCCN guidelines<sup>[69]</sup>, but were approved in refractory or metastatic CRC, so there is just little evidence or trial using the new immunotherapeutic drugs, alone or in combination with chemotherapy, in patients with stage III CRC. Although checkpoint inhibitors (CPIs) have achieved remarkable efficacy in CRC, they still face the dilemma of limited effective drugs and limited access to dMMR patients. The proportion of dMMR with advanced CRC was less than 5%, so how to make the majority of pMMR patients benefit from immunotherapy in the future is an important problem to be solved. We may need to face the following questions: (A) How to screen out patients who may be effective in immunotherapy in a large pMMR population and then expand the indications for CPIs? (B) How to more optimize the treatment strategy to overcome the primary resistance of pMMR population to immunotherapy and improve the response to immunotherapy? (C) How to control the immune-related events more effectively? (D) Is there any other new targeted immune checkpoints?

In conclusion, this study demonstrated the function of *MLH1/MSH2* expression in sporadic CRC, including its effect on prognostic and predictive factors, but we have few details about the correlation between MMR and tumorigenesis, loss of heterozygosity, and immunotherapy<sup>[70,71]</sup>. Further studies should clarify the cause and mechanisms of hypermethylation in *MLH1/MSH2* and antineoplastic immunity. Data from the current study may be helpful to understand the roles of *MLH1/MSH2* in the development and progression of CRC. They also suggest a new therapeutic strategy by regulating MMR expression to slow down the malignant progression of CRC and to improve the prognosis of CRC patients.

## ARTICLE HIGHLIGHTS

### Research background

Colorectal cancer (CRC) can arise through three distinct mutational pathways: Microsatellite instability, chromosomal instability, and CpG island methylator phenotype. We tested the hypothesis that CRC arising from the microsatellite-instability pathway through *MLH1/MSH2*-negative expression can lead a more favorable overall survival (OS) than *MLH1/MSH2*-positive patients. We also made an in-depth observation of the correlation between adjuvant chemotherapy and *MLH1/MSH2* expression in different stages of CRC.

### Research motivation

A larger sample size with a longer follow-up period was included to assess the effect of *MLH1/MSH2* status on the prediction and prognosis of stage II-III CRC and its association with adjuvant chemotherapy. It is important for clinical doctors to choose optimal treatment regimen, especially adjuvant chemotherapy, for patients.

### Research objectives

To evaluate the predictive and prognostic effects of *MLH1/MSH2* status in stage II-III CRC patients and its significance in guiding adjuvant chemotherapy.

### Research methods

We analyzed 681 postoperative patients with CRC with a median follow-up period of 56 mo (range, 8.0–72.0 mo) between January 2013 and December 2016. The main outcome data included MLH1/MSH2-positive rate, MLH1/MSH2-negative rate, and long-term follow-up outcomes.

### Research results

The outcomes showed that 550 patients were MLH1/MSH2-positive and 131 were MLH1/MSH2-negative. MLH1/MSH2-positive tumors were significantly more frequent in the colon than in the rectum, and with poor differentiation and less mucin production ( $P < 0.05$ ). Patients did not differ in terms of age, gender, tumor size, tumor stage, lymphocytic infiltration, or circumscribed margin. MLH1/MSH2-negative patients had a more favorable OS than MLH1/MSH2-positive patients ( $P < 0.001$ ). In both stages II and III, MLH1/MSH2 expression was a strong prognostic factor in all patients [ $P < 0.001$ , hazard ratio (HR) = 4.064, 95% confidence interval (CI): 2.241–7.369]. Adjuvant chemotherapy had a greater correlation with survival advantage in MLH1/MSH2-negative patients with stage III disease ( $P < 0.001$ , HR = 7.660, 95% CI: 2.974–15.883). Patients with stage II disease or MLH1/MSH2-positive stage III patients did not benefit from adjuvant chemotherapy.

### Research conclusions

MLH1/MSH2 phenotype constitutes a pathologically and clinically distinct subtype of sporadic CRC. MLH1/MSH2 is an independent prognostic and predictive factor for outcome of stage II-III CRC.

### Research perspectives

Our study demonstrated mismatch repair (MMR) is an important prognostic and predictive biomarker for stage II-III CRC, but we did not enroll the patients who used any PD-1/PD-L1 blocking therapy and had no data for survival improvement with different MMR statuses. In addition to detecting MMR status and tumor mutational burden, are there any indicators that are more sensitive to immunotherapy? The currently found immunologic drugs are only effective for high microsatellite instability (MSI-H)/dMMR population, but are ineffective for most patients with microsatellite stability (MSS). Would any new effective immune drugs be found for MSS patients? Is it possible to subdivide MSI-H (through the number of mutation sites) for enriching the dominant population in future? These issues will be the focal points and difficulty in our later research.

## ACKNOWLEDGEMENTS

We thank Ya-Nan Shi, Xiao Chen, Xin-Yang Wu, Jing-Yu Shen, and Zong-Lan Zhang for data collection and Youping Deng's team for excellent collaboration. We also thank Chris K Deng for his extensively revising the article.

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