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***Observational Study***

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

Wang SM *et al*. Relationship between MLH1/MSH2 and CRC

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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 gene (MMR) that *MLH1* and *MSH2* are most important in it. Recently abundant of studies indicate *MLH1/MSH2* phenotype is associated with CRC. We want 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 CRCwere tested (395 stage II and 286 stage III, 387 males and 294 females)which underwent curative surgical resection from 2013 to 2016 enrolled to this study. Immunohistochemistry was used toanalyze MMR status and confirmed the microsatellite status of 133 patients by GeneScan.

***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 rectum, and with poor differentiation and mucin (all *P* < 0.05). Patients did not differ in terms of age, gender, tumor size, tumor stage, lymphocytic infiltration and circumscribed margin. Patients with *MLH1/MSH2*-negative had more favorable OS than *MLH1/MSH2*-positive patients (*P* < 0.001). Either in stage II or III, univariate and multivariate analysis demonstrated *MLH1/MSH2* expression as an independent prognostic and predictive factor for 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 of stage III (*P* < 0.001, HR 7.660, 95%CI: 2.974–15.883). However, patients with stage II or *MLH1/MSH2*-positive of stage III did not benefit from adjuvant chemotherapy. GeneScan analysis demonstrated that among 133 patients, 105(78.95%) were microsatellite stable, 28(21.05%) were microsatellite instability (MSI) including 18(13.53%) high MSI and 10(7.52%) low MSI. This was consistent with the immunohistochemical results.

**Conclusion**

*MLH1/MSH2* phenotype constitutes a pathologically and clinically distinct subtype of sporadic CRC. *MLH1/MSH2*is independently prognostic and predictive of 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 of colorectal cancer (CRC). The purpose of this study was to collect data of MLH1/MSH2 and MSI status in stage II-III CRC and to assess the predictive and prognostic value of this analysis. This is the first large study in China to evaluate the role of MLH1/MSH2 in CRC and its relationship with adjuvant chemotherapy.

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**INTRODUCTION**

Colorectal cancer (CRC) is one of the most common malignancies of the digestive tract. In 2017, the incidence of CRC in the United States was nearly 135430 newly diagnosed cases with 50260 associated deaths[1]. CRC is mainly associated with at least three distinct genetic pathways: microsatellite instability (MSI), chromosomal instability (CIN) and CpG Island methylator phenotype (CIMP)[2]. Most hereditary nonpolyposis CRC and 15%[3] of sporadic CRC are characterized by MSI[4,5].In contrast, 85%of CRC develops from the CIN pathway and is characterized by aneuploidy, allelic losses, amplifications, and translocations[6]. Meanwhile, many sporadic MSI colorectal cancers are also CIMP positive ones. These three pathways are not mutually exclusive, and most tumors are characterized by multiple pathways. Mismatch repair (*MMR*)[7] is a housekeeping gene and has highly conserved sequences. *MMR* maintains correct DNA replication and high fidelity by repairing DNA base mismatching that allows for genomic stability and reduces spontaneous mutations[8]. 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[9,10]. In China, the incidence of CRC has increased significantly in the last 10 years. While the mortality rate of male patients has increased annually, mortality of female patients has tended to be relatively stable. CRC is fifth leading cause of morbidity in men and fourth in women. The number of new cases of CRC in 2015 of China was 376300, including 215700 men and 160600 women. There were 191000 deaths due to CRC, including 111100 men and 80000 women[11].

Most cases of MSI appear to result from *MMR* deficiency. At least six of the genes in *MMR* have now been identified including *MutL homolog 1* (*MLH1*), *MutS homolog 2* (*MSH2*), *MutS homolog 3* (*MSH3*), *Postmeiotic segregation increased 1 (PMS1)*, *PMS2* *Postmeiotic segregation increased 2* (*PMS2)*, and *MutS homolog 6* (*MSH6)*[12]. *MLH1* is located on chromosome 3p21–23 and is connected with the creation o*f MLH3, PMS2 and PMS1. MSH2* is located on chromosome 2p21 and has been shown to form *MSH3* and *MSH6*[13]. 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[14,15]. Mutations either on *MLH1* or *MSH2* or both of them are considered as *MLH1*/*MSH2* negative, and both are not mutated 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[16].

The purpose of the current study was to evaluate the prognostic significance of *MLH1/MSH2* status determined by immunohistochemical analyses 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 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[17]. 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 through to December 2018. Of the 681 patients with CRC, 300 underwent surgery only and 381 surgery plus adjuvant chemotherapy. One hundred and twenty-two of the 395 (30.9%) patients with stage II CRC and 259 of 286 (90.6%) 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. National Comprehensive Cancer Network (NCCN) guidelines[18] consider the following high-risk factors for recurrence: poorly differentiated histology [exclusive of those cancers that are high MSI (MSI-H)] or undifferentiated tumors; pathological T4 (pT4) disease; perineural invasion; bowel obstruction; close indeterminate, positive margins or localized perforating tumors; and inadequate LN sampling (< 12 LNs).

***Treatment***

Combined with NCCN guideline and 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 CAPEOX regimen (oxaliplatin 130 mg/m2 for 1 d, intravenous drip 2 h; capecitabine 1 g/m2/d for 14 d, oral administration, every 21 d for 6 cycles). For stage IIIA[19] (T1-2N1M0 or T1N2M0) CRC, 69.91%(79/113) of patients received 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 FOLFOX6 regimen (Oxaliplatin 85 mg/m2 for 1 d, intravenous drip 2 h: calcium folinate 400 mg/m2 for 1 d, intravenous drip; 5-fluorouracil (5-FU) 400 mg/m2 for 1 d, intravenous injection; 5-FU 1.2 g/m2 for 46 h, intravenous drip, every 14 d for 12 cycles), and 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 approved by the Human Research Ethics Committee of the Nanjing Hospital of Chinese Medicine affiliated to Nanjing University of Chinese Medicine.

***Histopathological and immunohistochemical analysis***

According to histopathological and immunohistochemical detection, tumor types were classified into adenocarcinoma with or without mucinous. Three hundred and ninety-five patients had stage II CRC and 286 had stage III on the basis of 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-μm-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; 10 fields with no fewer than 100 cells per field by 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* negative 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[20].

***Microsatellite analysis***

One hundred and thirty-three samples of 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% tumor cells). The primers, location, and sequence of MSI are noted in Table 1.

In all 133 cases, MSI was evaluated at five microsatellite loci (*BAT26*, *BAT25*, *D2S123*, *D5S346* and *D17S250*) using a 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 by GenScan 3.1 software. MSI-H was defined as ≥ 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 clinicopathologicalfactors was analyzed by *χ*2 test. Clinical factors that were analyzed included: age, gender, tumor stage**,** differentiation, lymphocytic infiltration, tumor size, mucin, and tumor margin. Survivalwas estimated by Kaplan–Meier method.Univariate and multivariate analysis were carried out usingCox’s proportional hazards regression models. *P* < 0.05was defined as significant. The statistical analysis wasperformed 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 poor differentiated than well–moderate 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 and circumscribed margin (all *P* > 0.05).

***Survival analysis***

With a median follow-up time 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. In stage III CRC, 90 of 286 (31.47%) patients died and 107 (37.41%) had recurrence or metastases. 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 survival trend for OS (68.62 ± 0.83 *vs* 62.11 ± 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 ± 0.65 *vs* 66.02 ± 1.01 mo, *P* = 0.011). Patients with *MLH1/MSH2*-negative stage III CRC also had longer OS than those with *MLH1/MSH2*-positive (66.05 ± 1.62 *vs* 63.40 ± 1.15 mo, *P* = 0.023). In patients who received adjuvant chemotherapy, those with *MLH1/MSH2*-negative CRC had OS of 64.02 ± 1.61 mo compared with 62.11 ± 1.07 mo in those with *MLH1/MSH2*-positive CRC (*P* = 0.015). In conclusion, 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 was listed in Figure 2.

***Univariate and multivariate analysis***

In univariate analysis, patients with *MLH1/MSH2*-positive CRC had 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 (all *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 (all *P* < 0.05). In the subgroup analysis of stage II CRC, patients with *MLH1/MSH2*-negative tumor demonstrated better OS than those with *MLH1/MSH2*-positive (multivariate *P* < 0.011, HR 5.583, 95%CI: 1.478–21.092). Patients with stage III had similar results but *MLH1/MSH2* status was less significant than in patients with stage II (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) in stage II CRC. However, a better survival for patients with *MLH1/MSH2*-negative was observed in stage III 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 stage III with *MLH1/MSH2*-positive(multivariate *P* = 0.052, HR 2.817, 95%CI 0.223-6.671) (Table 4). All findings were consistent for the OS end point.

***MSI***

Microsatellite analysis was performed in 133 CRC patients (71 stage II and 62 stage III) tested by GenScan, and 105 were MSS (78.95%), 28 (21.05%) MSI, including 18 (13.53%) MSI-H and 10 (7.52%) MSI-L. All patients were detected by immunohistochemical analysis that 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 were 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 CRC with proximal tumor[21]. Abnormal methylation, causing alteration of *MLH1/MSH2*,can form transcriptional target genes for silencing[22]. Several studies[23-25] 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*[26,27] reported that in most MSI-H phenotype colorectal carcinomas, 91% of cases are confirmed as *MLH1* negative. In another study, Herman *et al*[28] reported that hypermethylation of *MLH1* in sporadic CRC with MSI-H was as high as 84%. By contrast, Vasen *et al*[29] demonstrated that *MSH2* mutation is associated with a higher risk of developing cancer than *MLH1* mutation is *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 protein[30,31]. Gene mutation is the key reason for the decrease of *MLH1* and *MSH2* expression and both of them are the most dominant parts of MMR system, so detection for those two 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[32,33]. *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[34]. So, immunohistochemical staining for MMR is now performed as part of routine processing in the department of pathology in almost all hospitalsafter surgery[35-37]. 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. With MSI detection, MSI-H occurred mostly in patients aged > 50 years, in the right colon, and in tumors with poor differentiation and with mucin. We confirmed that these two clinical assays have more consistency and accuracy than other detections.

The result of our study clearly showed *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*[38] reported that MSI-H occurred in 256 (20.3%) of 1263 patients that more frequently in the less advanced stage, right sided, poorly differentiated with mucinous phenotype, and infiltrative growth than MSS[39]. Numerous studies have confirmed similarly conclusion that most MSI-H tumors are mucinous adenocarcinoma, located in the right colon, and poorly differentiated (*P* < 0.05)[40,41]. Though in the early researches, CRC was divided into colon and rectal by anatomical site, but they had some differences in specific treatments even if they have been treated as same disease. And in recent years, with the deepening of cognition in disease and the increase of evidence-based medical proof, colorectal cancer was considered in different parts has distinct clinical pathological expression and prognosis. So the location of tumor is instructive for prognosis and treatment[42,43]. More and more studies now tend to divide the colorectal cancer into right site and left site according to the colonic Splenic flexure[44]. It is based on the right site originating from the midgut of embryo, while the left site originating 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[45,46]. Therefore, the right colon has more poor differentiation, worse pathological stage and earlier metastasis than left colon[47]. Since the rectal blood supplied from the internal iliac artery, and rectal cancer is different from the colon in clinical treatment, so in our study we divided the colorectal into right colon, left colon and rectum parts. As found in other studies[48,49], the frequency of dMMR in right colon with poor differentiation was significantly higher than in left colon and rectum in our study, indicating that MSI was mainly involved in the development of right colon cancer. 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[50-52] have acquired positive results. Gryfe *et al*[53] 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 incidence 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 in patients who had MSS tumors. Roth *et al*[54] investigated the effect of MMR at different stages. In 1404 patients with stage II or III CRC, prognostic advantage conferred by MSI was more evident in stage II than III (*P* = 0.04). In a large meta-analysis[55] of 12 782 patients, there was a clear correlation between MSI-H tumors and improved OS. The data demonstrated that OS of patients with MSI-H was significantly higher than in MSI-L and MSS patients (*P* < 0.001). The overall odds ratio was 0.6 (95%CI 0.53–0.69) and disease-free survival (DFS) was also significantly different (*P* < 0.001). In our study, patients with *MLH1/MSH2*-negative had a better clinical outcome than *MLH1/MSH2*-positive in stage II-III. Moreover, in multivariate analysis, the survival advantage for patients with *MLH1/MSH2*-negative was independent from several other clinical and pathological parameters. These conclusions were consistent with almost all related researches because of the difference in histology, anatomy, and accompanying degree of differentiation, histopathology in different location of intestine.

Adjuvant chemotherapy is considered as a 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[18] 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[56]. Our study followed this guideline and respected patients’ wishes to formulate their treatment schedule. We found stage II and stage III CRC have different results in adjuvant chemotherapy. In stage II, 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 (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 (multivariate *P* < 0.001, HR 7.660, 95%CI 2.974–15.883) was more strongly associated with adjuvant chemotherapy than *MLH1/MSH2*-positive (multivariate *P* = 0.052, HR 2.817, 95%CI 0.223–6.671). This was similar to the study by Elsaleh *et al*[57] that 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 Sinicrope *et al*[58] observation (NCCTG N0147) of 2720 stage III Colon cancer patients for 5-year disease-free survival. It showed 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 cause there were some studies support the opposite conclusion. Ribic *et al*[59]showed no benefit from adjuvant chemotherapy with stage II and III MSI patients. Sargent *et al*[60] enrolled 457 stage II and III CRC patients grouped into 5-FU-based therapy (*n* = 229) and postsurgical treatment (*n* = 228). Patients who received 5-FU with MSI had no improvement in DFS (*P* = 0.85, HR 1.10; 95%CI 0.42-2.91) compared with postsurgical treatment group. Jover *et al*[61] confirmed that patients with dMMR colon cancer do not benefit from adjuvant 5-FU/leucovorin. Many studies have analyzed the relationship between MMR and prognosis just enrolled whole TNM stage I-IV rather than stageⅡ- III. 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 adjuvant chemotherapy effect of MMR.

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 that immune checkpoint inhibitor antibody can achieve 40% Objective Response Rate (ORR) and up to 78% clinical benefit rate[62]. The clinical trial KEYNOTE-028[63] enrolled advanced adenocarcinoma of CRC who failed of standard therapy and PD-L1 expression in ≥ 1% of cells in tumor nests. The primary endpoint was ORR, safety and tolerability. Patients received Pembrolizumab 10mg/kg every 2 wk and lasted more than 2 years or until confirmed unacceptable toxicity or progression. The result showed in the 156 advanced CRC, 23 were PD-L1 positive ones one gained complete remission (CR), but 1 experienced a partial response (ORR, 4%; 95%CI, 0–22%) who was confirmed as MSI-H. This trial revealed PD-L1 expression can’t screen out the dominant population of anti-PD-1 immunotherapy. Based on the KEYNOTE-028 and several other clinical trial results, Le *et al*[64] designed a single-arm, phase II clinical study (NCT01876511) which aims 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 3 groups, that was 11 dMMR CRC, 21 pMMR CRC and 9 dMMR non-CRC (4 ampullary/cholangiocarcinoma, 2 endometrial cancer, 2 small intestine cancer, 1 gastric cancer) and every patient administered Pembrolizumab 10 mg per kilogram of body weight every 14 d. The primary endpoint was ORR at 20 wk and the progression-free survival (PFS). The results showed that ORR for the three groups were 40%, 0 and 71% respectively and the PFS were 78%, 11%, and 67% respectively in the 20 wk. Interestingly, investigator used Whole-exome sequencing to check somatic mutations and found it is higher in dMMR than in pMMR (*P* = 0.007). Moreover, the study demonstrated high somatic mutation loads were associated with prolonged progression-free survival (*P* = 0.02) and dMMR patients received clinical benefit of immune checkpoint blockade with pembrolizumab.

These innovative single-arm clinical studies data[65-67] led to United States FDA granted accelerated approval for pembrolizumab in previous treatment failure of dMMR/MSI-H solid tumors including CRC in May 2017[68]. Subsequently, pembrolizumab or nivolumab were recommended for second-line or later treatment of dMMR/MSI-H CRC in 2017 NCCN Guidelines[69],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 patients with 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 of immunotherapy? (C) How to control the immune-related events more effectively? (D) Is there any other new targeted immune checkpoint existence?

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[70,71]. 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 colorectal cancers arising from the microsatellite-instability pathway through *MLH1/MSH2*-negative expression which can lead more favorable overall survival (OS) than *MLH1/MSH2*-positive. And 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 were 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 which treatment regimen and especially whether to choose adjuvant chemotherapy for patients.

***Research objectives***

Evaluate the predictive and prognostic effect of *MLH1/MSH2* status with stage II-III CRC patients and its significance of guiding adjuvant chemotherapy.

***Research methods***

We analyzed 681 postoperative patients of CRC with a follow-up at median time 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 in each stage of CRC and long-term follow-up outcomes.

***Research results***

The outcomes showed that 550 patients were *MLH1/MSH2*-positive ones and 131 were *MLH1/MSH2*-negative. *MLH1/MSH2*-positive tumors were significantly more frequent in the colon than rectum, and with poor differentiation and mucin (all *P* < 0.05). Patients did not differ in terms of age, gender, tumor size, tumor stage, lymphocytic infiltration and circumscribed margin. Patients with *MLH1/MSH2*-negative had more favorable OS than *MLH1/MSH2*-positive patients (*P* < 0.001). Either in stage II or 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 of stage III (*P* < 0.001, HR 7.660, 95%CI 2.974–15.883). Patients with stage II or *MLH1/MSH2*-positive of stage III did not benefit from adjuvant chemotherapy.

***Research conclusions***

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

***Research perspectives***

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

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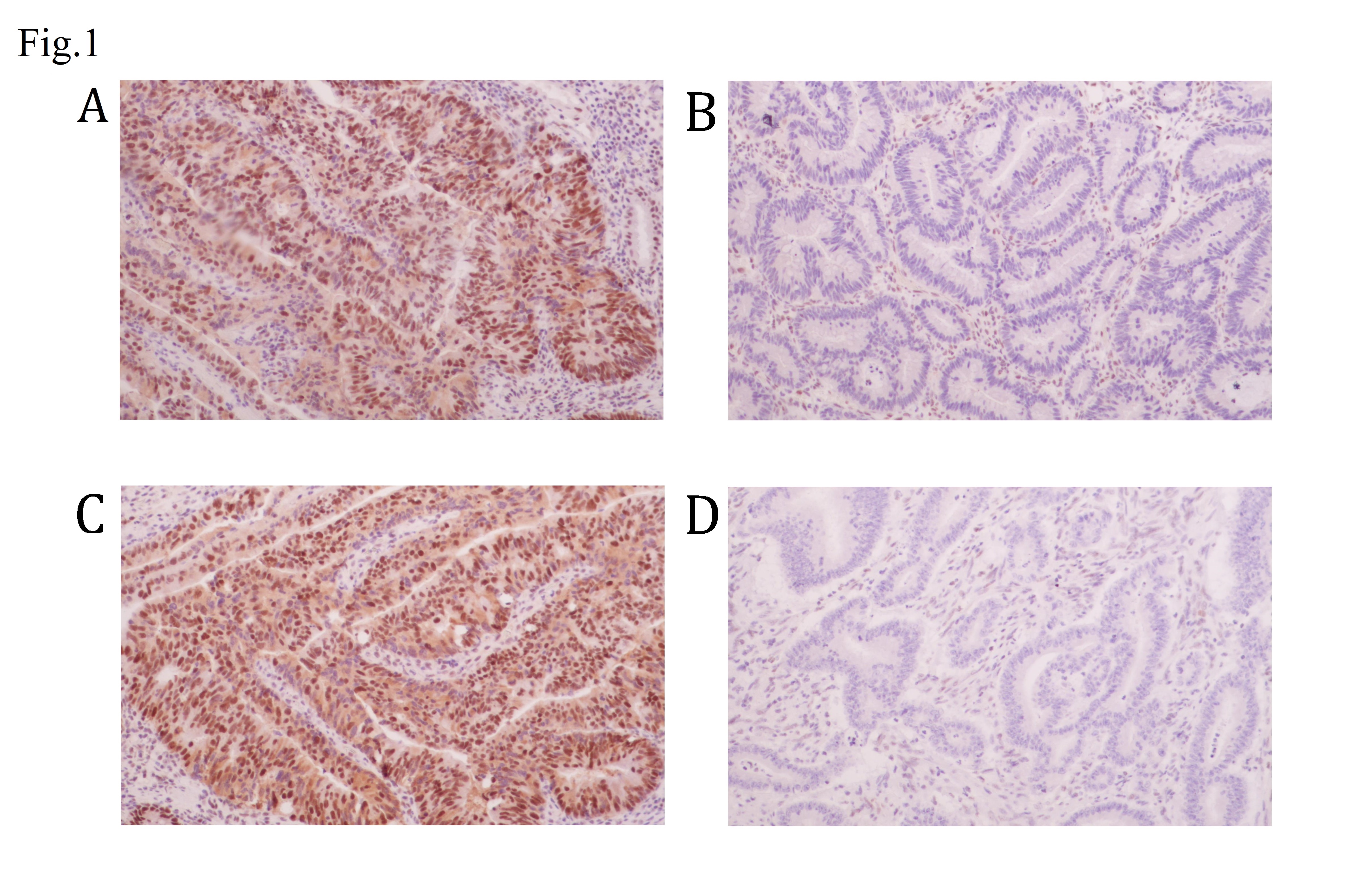
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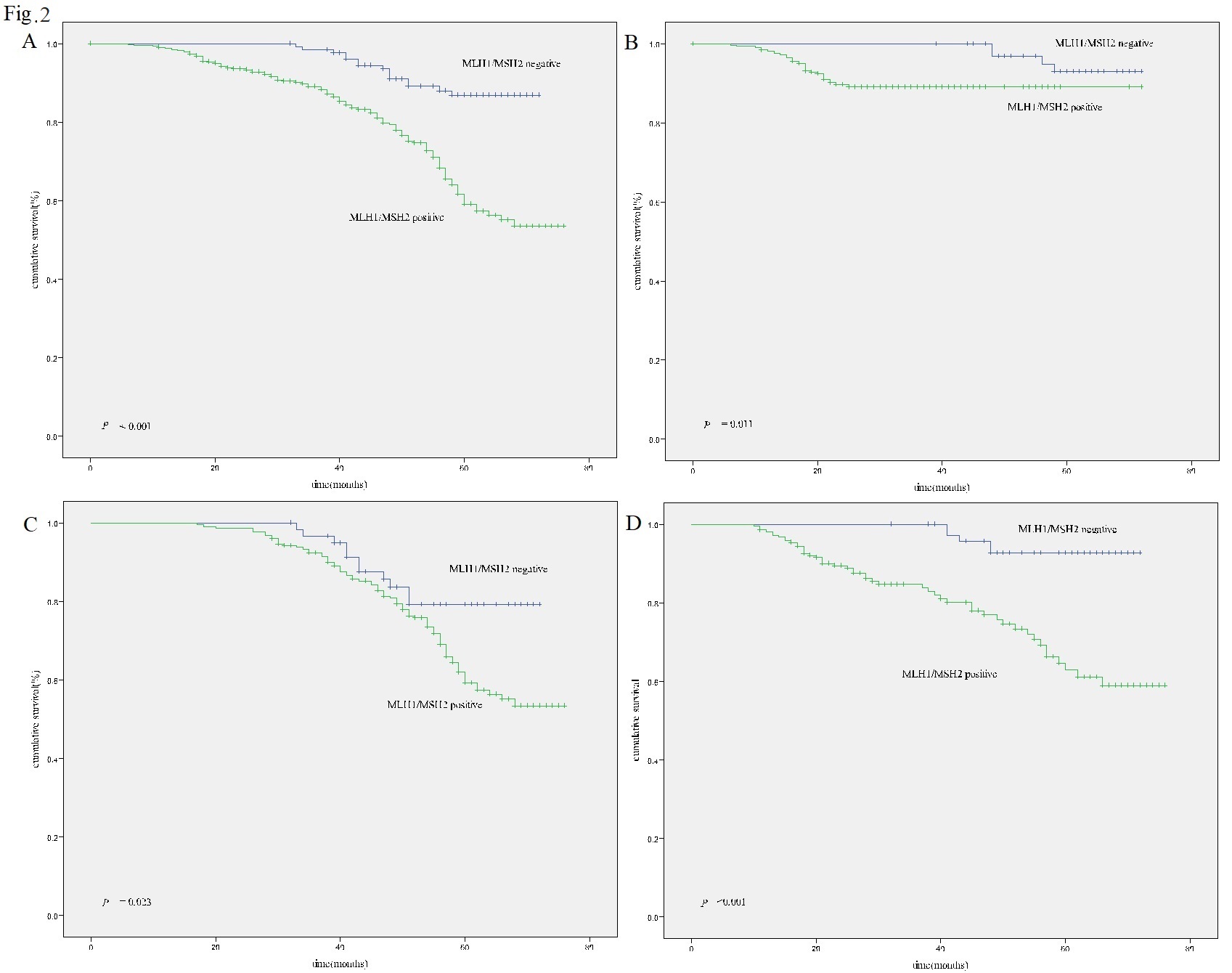
Grade C (Good): C

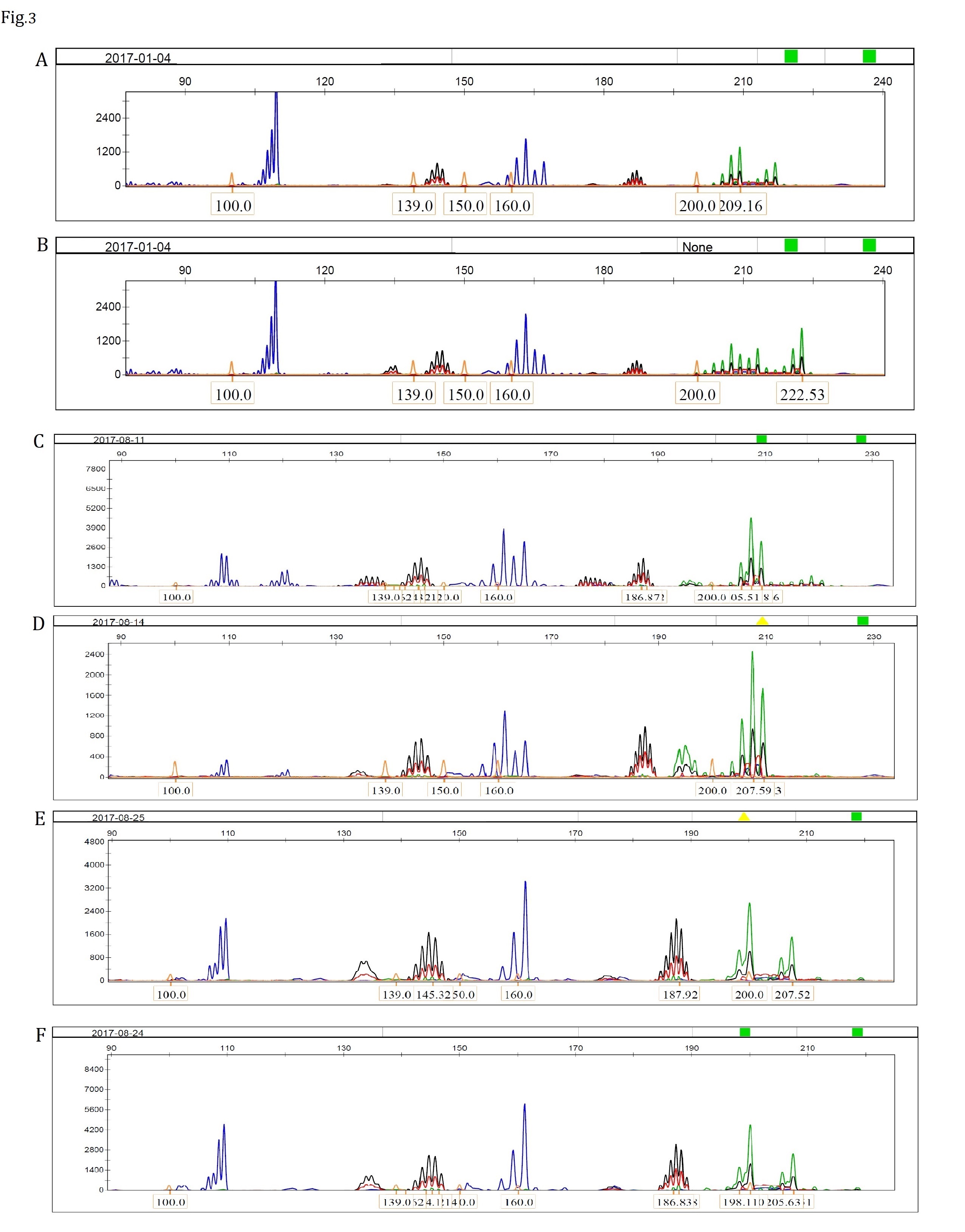
Grade D (Fair): D

Grade E (Poor): 0

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

** 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; B: Overall survival of stage II CRC patients classified as *MLH1/MSH*2-positive and *MLH1/MSH2*-negative; C: Overall survival of stage III CRC patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative; D: Overall survival of all adjuvant chemotherapy patients classified as *MLH1/MSH2*-positive and *MLH1/MSH2*-negative.

 **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. 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: tumor tissue microsatellite stability (MSS) of tumor tissue; F: MSS of corresponding normal tissue.

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

|  |  |  |
| --- | --- | --- |
| **SNP** | **Location** | **Primer sequence** |
| *BAT26* | 2p16 | p1 TGACTACTTTTGACTTCAGCC |
|  |  | p2 AACCATTCAACATTTTTAACCC |
| *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 GCTGGCCATATATATATTTAAACC |

SNP: Single nucleotide polymophisms.

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

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Observation** | **Positivea** | **Negativeb** | **Total** | ***χ*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 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 |

a*MLH1/MSH2* positive.b*MLH1/MSH2* negative. MLH1: MutL homolog 1; MSH2: MutS homolog 2.

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

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Observation** | **Univariate** | | | **Multivariate** | | |
| ***P*** | **HR** | **95%CI** | ***P*** | **HR** | **95%CI** |
| Model 1a | | | | | | |
| 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 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 |
| *MLH1/ MSH2* | | | | | | |
| Positive *vs* Negative | < 0.001 | 3.799 | 2.205-6.546 | < 0.001 | 4.064 | 2.241-7.369 |
| Model 2b | | | | | | |
| 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 |
| *MLH1/ MSH2* | | | | | | |
| 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 |
| Model 3c | | | | | | |
| 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 |
| *MLH1/ MSH2* | | | | | | |
| 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 |

aAll 681 patients. b395 Patients with stage II CRC. c286 Patients with stage III CRC. HR: hazard ratio; CI: confidence interval; CRC: Colorectal cancer; MLH1: MutL homolog 1; MSH2: MutS homolog 2.

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

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

a395 Patients with stage II CRC. b286 Patients with stage III CRC. HR: hazard ratio; CI: confidence interval; MLH1: MutL homolog 1; MSH2: MutS homolog 2.