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ORIGINAL PAPER

Prognostic significance of *hMLH1/hMSH2* gene mutations and *hMLH1* promoter methylation in sporadic colorectal cancer

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Abstract No study in China has focused on the relationships between germline and somatic *hMLH1/hMSH2* gene mutations, *hMLH1* promoter methylation, and the prognosis of colorectal cancer (CRC), especially in sporadic CRC. Therefore, we carried out this study with 433 primary sporadic CRC patients to investigate the associations between germline and somatic *hMLH1/hMSH2* gene mutations, *hMLH1* promoter methylation, and the overall survival (OS) of CRC; to evaluate the effect of interaction between gene mutation and methylation on the risk of CRC prognosis. As a result, the 3-, 5-, and 7-year survival of the sporadic CRC patients was 67, 57, and 50.0 %, respectively. There were no significant associations observed between germline and somatic *hMLH1/hMSH2* gene mutations after adjusted (HR = 1.37, 95 % CI 0.70–2.67, $p = 0.35$; HR = 1.31, 95 % CI 0.69–2.47, $p = 0.42$, respectively). When the analyses were stratified based on tumor stage, tumor location, and chemotherapy, no significant survival advantage of *hMLH1/hMSH2* gene mutation was illustrated. In addition, no significant association between germline and somatic *hMLH1* promoter methylation and OS of CRC was observed (HR = 1.46, 95 % CI 0.57–3.74, $p = 0.43$; HR = 0.70, 95 % CI 0.32–1.53, $p = 0.37$, respectively). In conclusion, the research did not find the significant association between germline and somatic *hMLH1/hMSH2* gene mutations, *hMLH1* promoter methylation, and sporadic CRC prognosis.

Keywords *hMLH1/hMSH2* · Mutation · Methylation · Colorectal cancer prognosis

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Introduction

Colorectal cancer (CRC) is one of the most common malignancies, representing the third most common cancer in men and the second in women worldwide [1]. Accounting for 8.5 % (6,94,000) of the total cancer deaths, CRC is the fourth leading cause of cancer death in 2012. [1]. Although the relative 5-year survival of CRC increased in Europe during 1995–2007 [2], it was only about 30–65 % worldwide [3]. Anatomic and pathological stages are still the most accurate predictors of CRC prognosis until now. Therefore, novel molecular prognostic markers for colorectal cancer are needed for the accurate prediction of prognosis.

One of the genetic pathways in the development of CRC is the failure of the DNA mismatch repair (MMR) system [4], which contributes to maintain the genomic stability by recognizing and removing insertion/deletion mutations that occur during DNA replication [5]. The two main mismatch repair genes are *hMLH1* [6] and *hMSH2* [7]. Germline mutations of them have been identified as the main cause of Lynch syndrome (LS) CRC (traditional HNPCC: meet the Amsterdam I or II criteria, Japanese criteria, or revised

Bethesda guidelines). LS CRC patients who carried with germline mutations of *hMLH1/hMSH2* gene were reported to have a better prognosis than non-carriers [8] and sporadic CRC patients (there is no obvious CRC family history) [9]. However, few studies have focused on the relationship between germline and somatic mutations of *hMLH1/hMSH2* gene and prognosis of sporadic CRC. Only one published paper [10] has not observed statistically significant different survival rates between carriers (74 %) and non-carriers (63 %) of *hMLH1/hMSH2/hMSH6* gene mutations in 870 consecutively ascertained sporadic CRC patients under the age of 55 in Scotland.

hMLH1 promoter methylation-induced transcriptional silencing of *hMLH1* gene has been proposed as an important mechanism in the development of sporadic CRC [11]. It also has been reported having potential application for treatment response prediction (5-FU-based chemotherapy) [12]. The predominant cause of sporadic CRC has been reported to be microsatellite instability (MSI) [13], which has been hypothesized to be the most promising molecular marker for CRC prognosis [14]. In sporadic CRC, MSI is mainly caused by methylation-induced silencing of the *hMLH1* gene [15]. Therefore, we hypothesized that *hMLH1* promoter methylation may be associated with the prognosis of sporadic CRC.

Since evidence suggests that rare mutations of severe effect are responsible for a substantial portion of complex human cancer [16]. Therefore, we conducted the study to investigate the associations between germline and somatic *hMLH1/hMSH2* gene mutations, *hMLH1* promoter methylation, their interactions, and the prognosis of CRC.

Methods

Study participants

After obtaining informed written consent from study subjects and approval from Ethics Committee of Harbin Medical University, we identified CRC patients who underwent surgery at the Cancer Hospital of Harbin Medical University and they were diagnosed with pathology. Patients with no family history of CRC were categorized as sporadic CRC. Totally, 433 primary sporadic CRC patients were recruited. A total of 418 blood and 329 tumor tissue DNA were collected for molecular genetic analysis.

We followed patients until Mar 2012 or death. After surgery, the clinical data of patients were collected based on the medical records, which included age at diagnosis, pathological diagnosis, and the level of serum carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) before surgery. During follow-up, chemotherapy and radiotherapy protocols were obtained. Meanwhile, we obtained information about disease progression, recurrence, and the date and cause of death (if deceased).

Screening for germline and somatic mutations of *hMLH1* and *hMSH2* genes

PCR-SSCP: sequencing analysis

The primers for 20 pairs of all the 19 exons in the *hMLH1* gene and 17 pairs of all the 16 exons in the *hMSH2* gene, including exon–intron boundaries, were synthesized for genomic PCR, SSCP, and sequencing [17].

Tumor MSI analysis

MSI status was determined using PCR-SSCP for the Bethesda markers: three dinucleotide (D5S346, D2S123, and D17S250) and two mononucleotide (BAT-25, BAT-26) markers. Three levels of MSI were identified as follows: high-level MSI (MSI-H), generally defined as MSI in two or more than two of the standard markers; low-level MSI (MSI-L), one of the standard markers exhibited MSI; and microsatellite stable (MSS) in the absence of microsatellite alterations [18].

Methylation-sensitive high-resolution melting (MS-HRM)

Genomic DNA was sodium bisulfite modified by the EZ Methylation Gold Kit (Zymo Research, Orange, CA). High-resolution melting (HRM) was used to assess the methylation status of the "A" region of *hMLH1* promoter [19]. Human methylated and unmethylated DNA set from Zymo Research was used as 100 % methylated and 0 % methylated controls, and the methylation percentage of 0, 1, 5, 25, 50, and 100 % were used as the standard curve. For germline and somatic *hMLH1* methylation, 5 % of methylation was used as cutoff value.

Statistical analysis

Calculating from the first diagnosis of colorectal cancer to the death from any cause or Mar 2012, OS was defined as the primary end point in the study. The survival curves were estimated using Kaplan–Meier product-limit method. Cumulative survival probability was calculated at the third, fifth, and seventh year, respectively. Proportional hazards regression models were fitted with computing hazard ratios (HR) and the corresponding 95 % confidence intervals (95 % CI). All statistical tests were 2 sided; p values < 0.05 were considered statistically significant. For multiple tests, α level of 0.05 was adjusted to α' divided by the number of multiple tests. All statistical analysis was conducted with SAS 9.1 (SAS Institute, Cary, NC, USA).

Results

Characteristics of CRC patients

The mean age of the 433 sporadic CRC patients was 58.61 years (range 24–82 years). Among the 433 CRC patients, 254 (58.66 %) were males and 179 (41.34 %) were females. Of the 432 patients with available information of TNM stage (UICC), 234 (54.17 %) were in early stage (stages I and II) and 198 (45.83 %) were in advanced stage (stages III and IV). Thirty-seven percent (159/432) tumors located at colon cancer; 63 % (271/432) tumors located at rectal cancer.

A total of 188 (43.42 %) CRC patients received 5-FU-based chemotherapy after surgery. A total of 19 (4.39 %) CRC patients received radiotherapy after surgery; 17 of the 19 patients received both chemotherapy and radiotherapy.

The median follow-up time was 52 months (range 1–87 months). During follow-up, 164 (37.88 %) CRC patients died and 29 (6.70 %) CRC patients were lost to follow-up.

Mutation of *hMLH1* and *hMSH2* genes and *hMLH1* promoter methylation

Germline mutations of *hMLH1/hMSH2* genes were identified in 12.44 % (52/418) CRC patients; somatic mutation frequencies of *hMLH1/hMSH2* genes were 14.59 % (48/329). Synonymous mutations were excluded from the mutation calculation, whereas a polymorphic mutation in exon 7 of *hMSH2* gene (4.07 %, 17/418) remained in the analysis.

Somatic mutation frequencies of *hMLH1/hMSH2* genes were 23.44 % (15/64) in proximal colon cancer, 18.03 % (11/61) in distal colon cancer, and 10.40 % (21/202) in rectal cancer ($p = 0.02$). There was no significant difference between germline mutation frequency of *hMLH1/hMSH2* genes and tumor location (proximal colon cancer, distal colon cancer, and rectal cancer) ($p = 0.07$). Both the germline and somatic mutation frequencies in MSI CRC were significantly higher than in MSS CRC (26.92 vs. 10.85 % for germline mutation; 35.19 vs. 10.57 % for somatic mutation) ($p = 0.0005$). Germline and somatic mutation frequencies of *hMLH1/hMSH2* genes were not significantly different in other clinicopathological characteristics (age, gender, BMI, tumor stage, tumor differentiation, histotypes, and pathological types) of CRC (Table 1). The details of the *hMLH1/hMSH2* gene mutations detected in the study have been described before [17].

The prevalence of germline and somatic *hMLH1* promoter methylation was 8.29 % (30/362) and 14.62 % (44/301), respectively.

Somatic *hMLH1* promoter methylation frequency was 26.22 % (16/61) in proximal colon cancer, 10.91 % (6/55) in distal colon cancer, and 11.96 % (22/184) in rectal cancer ($p = 0.02$). Somatic *hMLH1* promoter methylation frequency in female and male CRC was 20.00 % (25/125) and 10.80 % (19/176) ($p = 0.03$), respectively. Somatic *hMLH1* promoter methylation frequency was 11.38 % (28/246) in MSS CRC and 30.61 % (15/49) in MSI CRC ($p = 0.00$). Neither germline nor somatic *hMLH1* promoter methylation was significantly different in other clinicopathological characteristics (age, gender, BMI, tumor stage, tumor differentiation, histotypes, and pathological types) of CRC (Table 1).

Survival analysis

Overall survival analysis on clinical and pathological status

The 3-, 5-, and 7-year survival of the 433 CRC patients was 67, 57, and 50.0 %, respectively; the mean survival time was 62.56 months. In multivariate Cox regression analysis, tumor stage and CA19-9 level before surgery were significantly associated with the prognosis of colorectal cancer (data not shown).

Overall survival analysis on hMLH1/hMSH2 gene mutations, MSI, and hMLH1 promoter methylation

Neither germline nor somatic *hMLH1/hMSH2* gene mutation was associated with overall survival of CRC in the multivariate Cox regression analysis after adjusting by age, gender, tumor location, tumor differentiation, tumor stage, and CA19-9 level before surgery ($HR_{adj} = 1.37$, 95 % CI 0.70–2.67, $p = 0.35$; $HR_{adj} = 1.31$, 95 % CI 0.69–2.47, $p = 0.42$, respectively). For germline and somatic *hMLH1* promoter methylation, no significant association was observed between germline and somatic *hMLH1* promoter methylation and overall survival of CRC (Table 2).

When analyses stratified by tumor stage, there was no significant association between germline mutation of *hMLH1/hMSH2* gene and overall survival of stage I + II CRC ($HR_{adj} = 2.32$, 95 % CI 0.97–5.59, $p = 0.06$) (Fig. 1). No significant association was observed between germline mutation of *hMLH1/hMSH2* gene and overall survival of stage III + IV CRC ($HR_{adj} = 0.68$, 95 % CI 0.33–1.40, $p = 0.29$). For the somatic mutation of *hMLH1/hMSH2* gene, non-significant associations were reported (Table 3).

When analyses by tumor location, MSI status, and chemotherapy after surgery, no significant association was observed between the germline and somatic *hMLH1/*

Table 1 The relationships between mutations of *hMLH1/hMSH2* genes, *hMLH1* promoter methylation, MSI, *BRAF*, *K-ras,* and other clinical characteristics

	Germline mutations of *hMLH1/hMSH2* genes			Somatic mutations of *hMLH1/hMSH2* genes			Germline *hMLH1* methylation			Somatic *hMLH1* methylation		
	Wild type	Variant	*p* value	Wild type	Variant	*p* value	No	Yes	*p* value	No	Yes	*p* value
MSI												
MSS	230	28		237	28		210	21		218	28	
MSI	38	14	0.00	35	19	0.0005	44	4	0.87	34	15	0.0005
Gender												
Male	213	35		161	32		193	20		157	19	
Female	153	17	0.21	120	16	0.22	139	10	0.36	100	25	0.03
Age(year) at CRC diagnosis												
≤50	79	12		59	13		79	4		56	9	
>50	287	40	0.81	222	35	0.35	253	26	0.19	201	35	0.84
BMI												
<18.5	86	14		69	13		89	4		70	8	
18–23.9	161	15		122	19		137	11		105	23	
≥24	39	5	0.36	30	3	0.63	32	6	0.08	23	6	0.25
Location												
Proximal colon cancer	64	14		49	15		65	6		45	16	
Distal colon cancer	58	12		50	11		52	6		49	6	
Rectal cancer	241	26	0.07	181	21	0.02	214	17	0.75	162	22	0.02
Tumor stage												
I + II	201	26		156	24		173	19		140	25	
III + IV	164	26	0.49	124	24	0.46	158	11	0.25	116	19	0.79
Histotypes												
Adenocarcinoma	275	42		214	37		253	20		197	32	
Mucinous adenocarcinoma	16	1		12	2		15	2		12	2	
Others	12	2	0.67	6	2	0.72	10	2	0.42	7	1	0.99
Pathological types												
Protrude type	233	29		181	29		201	20		160	30	
Ulceration type	26	4		17	3		24	2		12	6	
Ulceration + infiltrating type	76	14		60	12		80	5		63	6	
Infiltrating type	12	1	0.67	7	1	0.94	8	1	0.82	6	1	0.07
Differentiated degree												
Poor	65	6		45	7		60	4		39	12	
Moderate	280	42		223	37		252	23		205	31	
Well	3	1	0.42	3	0	0.77	3	0	0.75	2	0	0.14

hMSH2 gene mutation, *hMLH1* promoter methylation, and the prognosis of CRC (data not shown).

The study has not found significant interaction between *hMLH1/hMSH2* gene mutation and *hMLH1* promoter methylation on the prognosis of CRC (data not shown).

Discussion

The aim of our study was to examine the prognostic significance of germline and somatic mutations of *hMLH1/hMSH2* genes and *hMLH1* promoter methylation in 433 sporadic CRC patients.

Germline mutations of *hMLH1/hMSH2* genes were reported to be associated with about one-third of LS CRC and 3–5 % of sporadic CRC patients [20]. Moreover, carriers of germline mutations in *hMLH1/hMSH2* reached up to 70–80 % probabilities of developing colorectal cancer [21, 22]. Whether germline and somatic *hMLH1/hMSH2* gene mutation carriers are associated with the prognosis of CRC patients, especially sporadic CRC patients, remain unclear. In our cohort, neither germline nor somatic mutation of

Table 2 The relationships between *hMLH1/hMSH2* mutation, *hMLH1* promoter methylation, and the overall survival of CRC patients

	No. of cases	7-year survival (%)	Survival time (mean ± SD, month)	Crude HR (95 % CI)	Adjusted HR (95 % CI)[a]	*p*
hMLH1 or *hMSH2* germline mutation						
Wild type	366	52.00	62.98 ± 1.67	1.00	1.00	
Mutant	52	42.00	60.59 ± 4.40	1.13 (0.73–1.77)	1.37 (0.70–2.67)	0.35
hMLH1 or *hMSH2* somatic mutation						
Wild type	281	52.00	64.88 ± 1.83	1.00	1.00	
Mutant	48	47.00	64.23 ± 4.50	1.02 (0.62–1.68)	1.31 (0.69–2.47)	0.42
Germline methylation of *hMLH1* promoter						
No	332	48.00	61.89 ± 1.77	1.00	1.00	
Yes	30	56.00	63.67 ± 5.32	0.95 (0.53–1.72)	1.46 (0.57–3.74)	0.43
Somatic methylation of *hMLH1* promoter						
No	257	49.00	64.33 ± 1.95	1.00	1.00	
Yes	44	66.00	67.19 ± 4.48	0.80 (0.46–1.41)	0.70 (0.32–1.53)	0.37

[a] Adjusted for age, gender, tumor location, tumor differentiation, tumor stage, and CA19-9 level before surgery

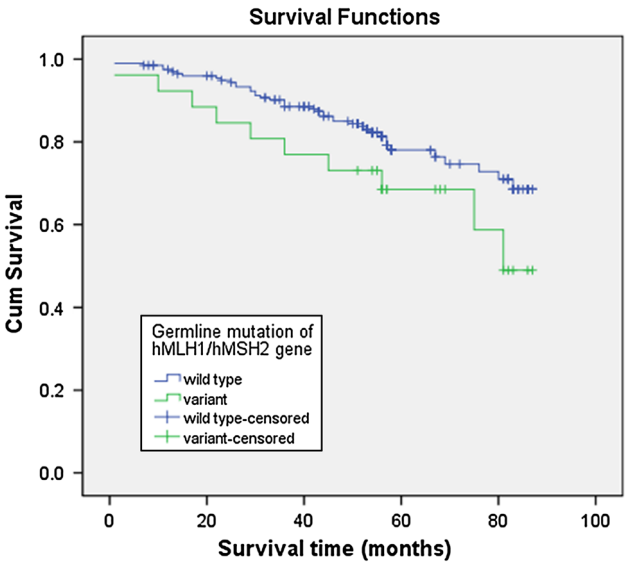

Fig. 1 The Kaplan–Meier survival curves for the stages I + II CRC overall survival according to the germline mutation of *hMLH1/hMSH2* genes

hMLH1/hMSH2 genes were associated with the prognosis of CRC, which was in concordance with the non-significant result by Barnetson et al. [10] in 870 consecutively ascertained sporadic CRC patients under 55 years old in Scotland. LS CRC patients who carried with MMR gene mutations were reported to have a better prognosis than non-carriers [8], the different survival advantage of mutations of *hMLH1/hMSH2* genes in LS and sporadic CRC patients may be due to the different cause of MSI in LS and sporadic CRC: in sporadic CRC, MSI was mainly caused by *hMLH1* promoter methylation [23, 24]; whereas, in LS CRC, MSI was mainly caused by MMR inactivation because of germline mutation [25]. In addition, according to the report in the InSiGHT database and the function prediction by any two of the PolyPhen/SIFT/MMP-MMR software, most of the mutations detected in our cohort were non-pathological or uncertain, and only two mutations of *hMLH1* gene (c.1845_1847 del GAA and c.1742 CCG > CTG) in two patients were pathological [17]. This may also explain the non-significant survival advantage of *hMLH1/hMSH2* gene mutation carriers.

In our study, germline mutation of *hMLH1/hMSH2* genes marginally increased the hazard risk by 2.32-folds in stages I + II CRC patients ($p = 0.06$). In stages III + IV CRC patients, neither germline nor somatic mutation of *hMLH1/hMSH2* gene had significant effect on the prognosis of CRC. The association between germline mutation of *hMLH1/hMSH2* gene and CRC survival may be affected by tumor stage. However, the underlying mechanisms need to be investigated.

Until now, six published studies have concerned the association between *hMLH1* promoter methylation in tumor DNA and CRC prognosis [26–28] or CpG island methylator phenotype (CIMP) and CRC prognosis [29–31], which provided the information on the association between *hMLH1* promoter methylation and CRC prognosis. Four of the six studies suggested that there was no significant association between *hMLH1* promoter methylation in tumor tissue and CRC prognosis (one paper provided information without sample size in sporadic MSS CRC patients [29], one paper concerned 199 sporadic CRC patients with MSI/*BRAF* alteration [26], one focused on 188 advanced CRC patients treated with 5-FU-based chemotherapy [30], and the other paper related 35 CRC patients treated with 5-FU-based chemotherapy [27]). Nevertheless, significant association between *hMLH1* promoter methylation in tumor tissue and CRC prognosis was reported in two studies with 72 sporadic CRC patients [28] and 130 sporadic CRC patients [31], respectively. In addition, the study by Ogino et al. [32] also reported a significant association in 30 metastatic MSS CRC patients treated with combination of

Table 3 The relationships between *hMLH1/hMSH2* mutation, *hMLH1* promoter methylation, and the overall survival of CRC patients in different tumor stages

	Stages I + II						Stages III + IV					
	No.	7-year survival (%)	Survival time (mean ± SD, month)	Crude HR (95 % CI)	Adjusted HR (95 % CI)[a]	*p*	No.	7-year survival (%)	Survival time (mean ± SD, month)	Crude HR (95 % CI)	Adjusted HR (95 % CI)	*p*
hMLH1 or *hMSH2* germline mutation												
Wild type	201	68.00	74.60 ± 1.73	1.00	1.00		164	33.00	49.04 ± 2.64	1.00	1.00	
Mutant	26	43.00	66.59 ± 5.64	1.75 (0.88–3.50)	2.32 (0.97–5.59)	0.06	26	38.00	52.69 ± 6.28	0.80 (0.45–1.42)	0.68 (0.33–1.40)	0.29
hMLH1 or *hMSH2* somatic mutation												
Wild type	156	67.00	74.84 ± 1.94	1.00	1.00		124	34.00	52.62 ± 2.98	1.00	1.00	
Mutant	24	42.00	67.09 ± 5.93	1.74 (0.83–3.64)	2.04 (0.70–5.99)	0.19	24	53.00	59.59 ± 6.48	0.63 (0.32–1.28)	0.74(0.32–1.68)	0.47
Germline methylation of *hMLH1* promoter												
No	173	62.00	73.76 ± 1.92	1.00	1.00		158	33.00	49.12 ± 2.69	1.00	1.00	
Yes	19	64.00	69.24 ± 6.23	1.34 (0.57–3.16)	1.81 (0.51–6.41)	0.36	11	44.00	53.46 ± 8.48	0.88 (0.39–2.02)	0.51 (0.14–1.86)	0.31
Somatic methylation of *hMLH1* promoter												
No	140	58.00	72.94 ± 2.17	1.00	1.00		116	38.00	54.32 ± 3.17	1.00	1.00	
Yes	25	90.00	82.64 ± 2.93	0.25 (0.06–1.06)	0.41 (0.09–1.82)	0.24	19	35.00	46.90 ± 7.38	1.37 (0.73–2.55)	0.76 (0.27–2.18)	0.61

[a] Adjusted for age, gender, tumor location, tumor differentiation, and CA19-9 level before surgery

5-Fu and other chemotherapy (with only one methylated tumors). Since the inconsistent study subjects, no sufficient data to pool the HR, and 95 % CI, we could not evaluate the association between *hMLH1* promoter methylation and CRC prognosis upon meta-analysis. No study has investigated the association between *hMLH1* promoter methylation in blood DNA and CRC prognosis. Therefore, we evaluated the association between *hMLH1* promoter methylation in blood sample and CRC prognosis. However, no significant association was observed.

Although *hMLH1* promoter methylation-induced transcriptional silencing of *hMLH1* gene has been proposed as an important mechanism in the development of colorectal cancer [11], its prognostic significance needs to be further studied and confirmed. *hMLH1* promoter methylation has been reported having resistance to 5-FU-based chemotherapy [12]. We conducted subgroup analysis based on chemotherapy; however, no significant survival advantage of *hMLH1* methylation was observed in patients treated with chemotherapy. The small sample size may explain the non-significant result. Furthermore, we determined methylation by the percentage of *hMLH1* promoter methylation. However, it is unclear to what extent promoter methylation of *hMLH1* might affect *hMLH1* expression and function. Further studies are needed to confirm which percentage of *hMLH1* promoter methylation was pathogenic methylation.

Tumor MSI has been hypothesized to be the most promising molecular marker for CRC prognosis; the two published meta-analyses confirmed the significant association between MSI and favorable prognosis [14, 33]. MSI was reported to be mainly (about 67 %) caused by *hMLH1* promoter methylation in sporadic CRC [13]. Whereas, only 30.6 % (15/49) MSI patients presented with somatic *hMLH1* promoter methylation and 8.3 % (4/44) MSI patients presented with germline *hMLH1* promoter methylation in our study. Neither MSI patients with germline *hMLH1* promoter methylation nor MSI patients with somatic *hMLH1* promoter methylation had significant survival advantage compared with MSS patients without *hMLH1* promoter methylation (HR = 0.95, 95 % CI 0.13–6.93, $p = 0.96$; HR = 0.18, 95 % CI 0.03–1.32, $p = 0.09$, respectively). Small sample size of MSI and germline *hMLH1* promoter methylation carriers ($n = 4$) and MSI and somatic *hMLH1* promoter methylation carriers ($n = 15$) limited the statistical power. Retrospective analysis was another limitation of the research, and larger number of sporadic CRC patients may needed to confirm the relationship between MSI, *hMLH1* promoter methylation, and CRC prognosis.

Our study could provide references in searching molecular markers to predict the prognosis of colorectal cancer. However, limitations such as different time to initiation of adjuvant chemotherapy and no unified period of chemotherapy should be considered in deriving conclusion.

In conclusion, no significant association was observed between *hMLH1/hMSH2* gene mutations, *hMLH1* promoter methylation, and CRC prognosis.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical standard All the subjects included in the study have been approved by the Ethics Committee of Harbin Medical University.

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