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ARTICLE

Comparisons of screening strategies for identifying Lynch syndrome among patients with MLH1-deficient colorectal cancer

Binyi Xiao[1] · Jun Luo[2] · E. Xie[3] · Lingheng Kong[1] · Jinghua Tang[1] · Dingxin Liu[1] · Linlin Mao[4] · Qiaoqi Sui[1] · Weirong Li[1] · Zhigang Hong[1] · Zhizhong Pan[1] · Wu Jiang[1] · Pei-Rong Ding[1]

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Abstract

BRAF and *MLH1* promoter methylation testings have been proven effective prescreens for Lynch Syndrome. We aimed to compare different screening strategies for Lynch Syndrome in patients with MLH1(−) CRC. Patients with MLH1(−) CRC who had been tested for *BRAF* mutation and germline variants of DNA mismatch repair genes were included. We compared the sensitivities and specificities for identifying Lynch Syndrome and the cost-effectiveness of four screening approaches that used the following tests as prescreens: *BRAF* testing, *MLH1* methylation testing, *MLH1* methylation & *BRAF* testing, and *MLH1* methylation testing & Revised Bethesda Criteria. Of 109 patients included, 23 (21.1%) were Lynch Syndrome patients. *BRAF* mutation and *MLH1* methylation occurred in 6 (5.5%) and 40 (36.7%) patients, respectively. The sensitivity for identifying Lynch syndrome of *BRAF* testing was 100%, but the specificity was only 7%. *MLH1* methylation testing had a lower sensitivity than *BRAF* testing (97.5% vs 100%), but had a markedly higher specificity (45.3% vs 7%). The combination of the two testings had a slightly higher specificity than *MLH1* methylation testing alone (47.7% vs 45.3%). The *MLH1* methylation testing approach had a 10% lower cost of identifying MLH1(−) Lynch syndrome carriers per case than universal genetic testing, but it missed 4.5% of patients. *BRAF* and *MLH1* promoter methylation testings as prescreens for Lynch syndrome are less effective in Chinese patients with MLH1(−) CRC than in their Western counterparts. Universal genetic testing could be considered an up-front option for this population.

Introduction

Lynch Syndrome is the most common hereditary colorectal cancer (CRC) syndrome. Although it accounts for only 2%–3% of all CRCs, more than 50% of its carriers develop CRC or other tumors during their lifetimes [1]. Screening for Lynch Syndrome among CRC patients is important for early diagnosis and disease management. Indeed, a universal screening approach has been implemented for newly diagnosed CRC patients, which involves the analysis of miscrosatellite instability (MSI) or of the expression of DNA mismatch repair (MMR) proteins (MLH1, MSH2, MSH6, and PMS2) by immunohistochemistry [2]. About 15% of CRCs show high microsatellite instability (MSI-H) or do not express one or more of the four MMR proteins, leading to a status called MMR deficiency (dMMR).

These authors contributed equally: Binyi Xiao, Jun Luo and E. Xie

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✉ Zhizhong Pan
panzhzh@sysucc.org.cn

✉ Wu Jiang
jiangwu@sysucc.org.cn

✉ Pei-Rong Ding
dingpr@sysucc.org.cn

1 Sun Yat-sen University Cancer Center; State Key Laboratory of Oncology in South China; Collaborative Innovation Center of Cancer Medicine, Guangzhou 510060, China

2 Institute of Cancer and Basic Medicine (ICBM), Chinese Academy of Sciences; Cancer Hospital of the University of Chinese Academy of Sciences; Zhejiang Cancer Hospital, Zhejiang, China

3 Department of General Surgery, the Affiliated Shantou Hospital of Sun Yat-sen University, Guangdong 515000, China

4 Guangzhou Kingmed Diagnostics Group Co., Ltd. Clinical Genome Center, KingMed Center for Clinical Laboratory Co., Ltd., Guangzhou, China

Of the four MMR deficiencies, MLH1 deficiency represents a special subtype. It is the most common type of dMMR, accounting for more than 70% of the total [3, 4]. In addition to germline variants in MMR genes, MLH1 deficiency can also result from double somatic mutations in *MLH1* and, more often, from the methylation of *MLH1* promoter [5, 6]. *MLH1* methylation is responsible for most of MLH1 deficiency observed in sporadic CRC cases; it can be used as a marker to exclude this population from unnecessary genetic testing. In addition, *MLH1* promoter methylation is strongly associated with *BRAF* mutation, which makes *BRAF* testing an alternative tool for screening out Lynch Syndrome [7–11].

However, the value of these tests can be affected by the genetic disparity of CRCs between populations. For example, *BRAF* V600E mutation occurs in 15%-20% of Western CRC patients, but in less than 5% of Chinese CRC patients [12–14]. This low frequency is likely to reduce the value of *BRAF* testing for Chinese CRC patients, but there is no study evaluating these screening methods or comparing them with universal genetic testing among this population. Our study aims to compare different screening strategies for Lynch Syndrome in Chinese patients with MLH1(−) CRC.

Methods

Data collection

We reviewed a consecutive series of 3330 patients who were diagnosed as CRC in Sun Yat-sen University Cancer Center between November 1, 2011 and December 31, 2015. Immunotaining for MLH1, MSH2, MSH6, and PMS2 was performed for 3250 patients, 330 of whom were MMR deficient. Of the 330 patients, 170 were MLH1-deficient. Of them, 136 had undergone genetic testing with a panel that included *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM* in our previous study [15]. 109/136 patients had viable tumor tissue, and were included in the study.

Clinical data were collected from hospital records, including sex, age, personal history of cancer, family history of cancer, and pathology of tumors. This retrospective study was approved by the institutional review board of Sun Yat-sen University Cancer Center, and written informed consent was obtained from all patients.

Testing protocols

BRAF V600E testing

Somatic testing for *BRAF* mutation had been carried out for 165/170 patients with MLH1 deficiency by using formalin-fixed paraffin-embedded (FFPE) tissue blocks, according to the screening algorithm in our previous study [15]. All patients included in this study ($n = 109$) had undergone the test. Briefly, the *BRAF* mutation within exon 15 was tested using fluorescent real-time polymerase chain reaction (PCR). Genomic DNA was amplified in a 24-μl PCR reaction with 7500 real-time fluorescence quantitative PCR system (Applied Biosystems, Foster City, CA). Mutations were confirmed with independent duplicate analyses.

MLH1 promoter methylation testing

MLH1 promoter methylation testing was carried out by pyrosequencing for all 109 patients. Tumor DNA was extracted by QIAamp DNA FFPE Tissue Kit (QIAGEN, US), and then converted into bisulfite using EpiTect Bisulfite Kits (QIAGEN, US). After tumor DNA was purified, *MLH1* promoter methylation testing was carried out with validated PCR primers specific to methylated sequences: MLH1-F: GTATTTTTGTTTTTATTGGTTGGATA;MLH1-R: CCAA TCAAATTTCTCAACTCTATA. Fifty-cycle PCR was performed as follows: denaturation 95 °C, 15 s; annealing 54 °C, 20 s; extension 72 °C, 30 s. After DNA amplification, the DNA sequence was analyzed by PyroMark Q96 ID. The sequencing section contains 5 CpG: AGAGCGGAC AGCGATCTCTAACGCGCAA-GCGCA.

Genetic testing of MMR genes

Genetic testing for hereditary colorectal syndromes had been undertaken for 109 patients, as previously described [15]. In brief, DNAs extracted from peripheral blood samples were used to construct libraries, which were then sequenced by a multigene panel covering *MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*. Lynch Syndrome was diagnosed when pathogenic or likely pathogenic variants in one of the five genes were identified.

Screening approaches and cost-effectiveness analyses

As shown in Fig. 1, our study used four approaches to screen for Lynch Syndrome. They were as follows:

Approach 1: *BRAF* mutation-germline testing. *BRAF* testing was used as a primary screening tool. Patients with *BRAF* mutation tumors were considered sporadic and were excluded, while patients without *BRAF* mutation proceeded with genetic testing for MMR genes.

Approach 2: *MLH1* methylation-germline testing. Patients were screened for *MLH1* promoter methylation before germline sequencing. Patients with methylated tumors were considered sporadic and excluded, while those without methylation were tested for germline variants.

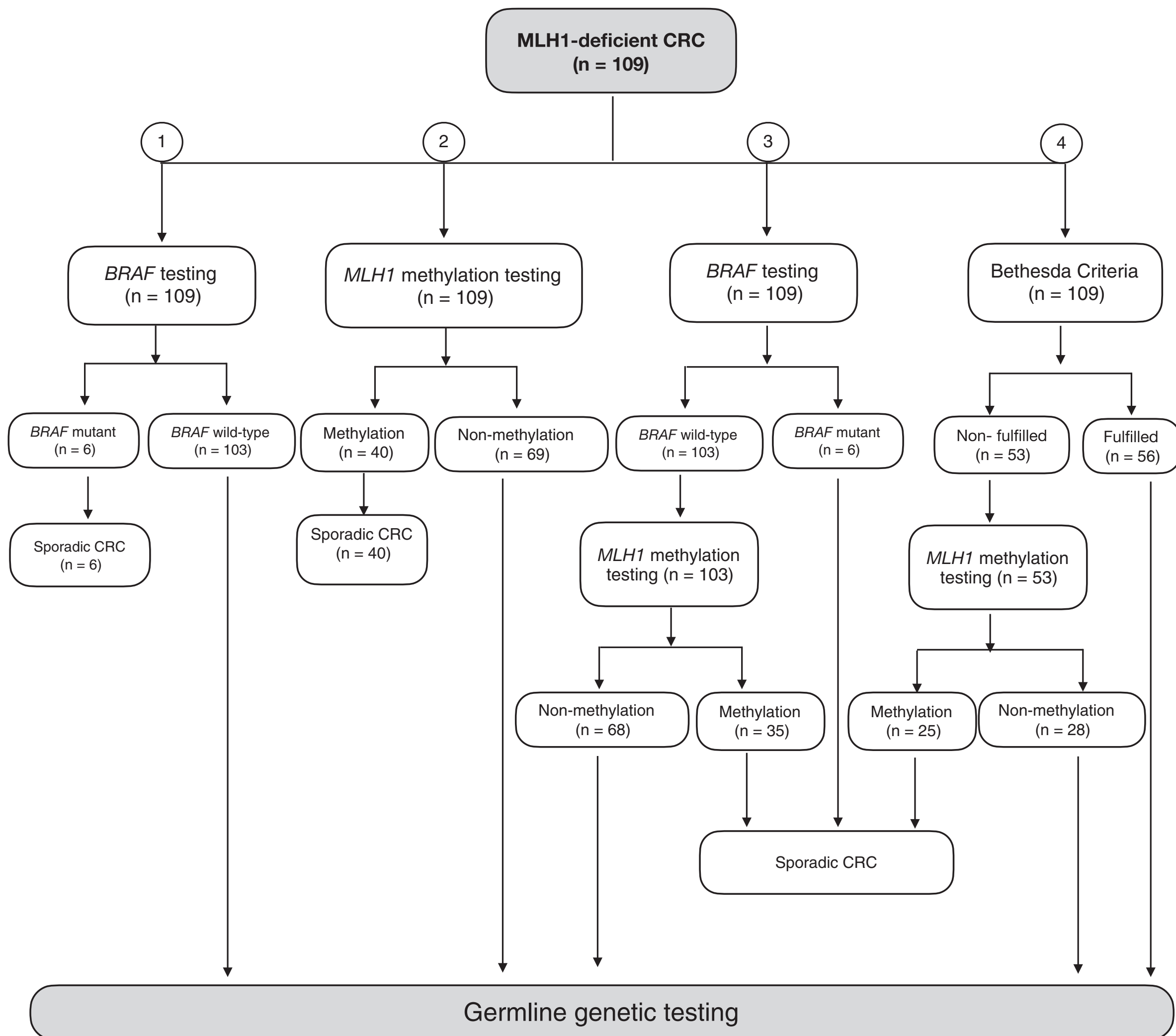

Fig. 1 Four screening approaches for identifying Lynch syndrome among patients with MLH1-deficient CRC.

Approach 3: *BRAF* mutation & *MLH1* methylation-germline testing. Patients were pre-screened for *BRAF* mutation, and those without mutation were tested for *MLH1* methylation. Germline sequencing was reserved for patients negative for both tests.

Approach 4: Bethesda Criteria & *MLH1* methylation-germline testing. Patients were prescreened with the Revised Bethesda Criteria. Patients who did not met the criteria underwent *MLH1* methylation testing. Patients who met the criteria and patients without *MLH1* methylation were recommended for genetic testing.

We then performed cost-effectiveness analyses for the four approaches and compared their costs of identifying MLH1(−) Lynch syndrome patients per case. The prices of *BRAF* testing and *MLH1* methylation testing were 472 RMB and 708 RMB per case, respectively, and the price of genetic testing with a 14-gene panel was 3220 RMB.

Statistics

Associations between categorical variables were assessed by chi-square test, Fisher's exact test, or Wilcoxon's rank-sum test, as appropriate. We compared the sensitivities and specificities for identifying Lynch Syndrome, positive predictive values (PPVs), and negative predictive values (NPVs) of the four screening approaches. All P values were two-sided, and $P < 0.05$ was considered statistically significant. All of the analyses were performed in R (version 3.5.1).

Results

Baseline information

Table 1 shows the baseline information. Of the 109 MLH1-deficient patients, 49 (45.0%) were female; the median age

Table 1 Baseline characteristics.

Factors		*n* (109)	percentage
Sex			
	Female	49	45.0
	Male	60	55.0
Locations			
	Ascending colon	53	48.6
	Transverse colon	15	13.8
	Descending colon	9	8.3
	Sigmoid colon	14	12.8
	Rectum	13	11.9
	Multiple	5	4.6
IHC staining patterns			
	MLH1-, PMS2-	94	86.2
	MLH1-, MSH2-, PMS2-	2	1.8
	MLH1-, MSH2-	1	0.9
	MLH1-, MSH6-, PMS2-	4	3.7
	MLH1-	8	7.3
Mucinous			
	Yes	42	38.5
	No	67	61.5
MLH1 methylation			
	Methylated	40	36.7
	Nonmethylated	69	63.3
BRAF mutation			
	Mutant-type	6	5.5
	Wild-type	103	94.5
Pathogenicity[a]			
	Pathogenic	11	22.9
	Likely Pathogenic	12	25.0
	VUS	25	52.1
Revised Bethesda Criteria			
	Fulfilled	56	51.4
	Nonfulfilled	53	48.6
Lynch Syndrome			
	Lynch	23	21.1
	Sporadic	86	78.9

IHC Immunohistochemistry, *VUS* variation of unknown significance, *FDR* first-degree relatives.

[a]61 patients did not harbor any genetic mutation in detected genes.

at diagnosis was 54.0 years. *BRAF* mutation and *MLH1* methylation were observed in 6 (5.5%) and 40 (36.7%) patients, respectively. Germline variants in MMR-related genes were found in 49 (45.0%) patients; among those, 11 were pathogenic, 12 were likely pathogenic, and 26 were variants of unknown significance (VUS). All pathogenic and likely pathogenic variants occurred in *MLH1* (see Supplementary Table 1). In total, 23 (21.1%) patients were diagnosed with Lynch Syndrome, and the other 86 (78.9%) with sporadic CRC.

Table 2 Comparisons of clinicopathological features between lynch and sporadic CRC.

Factors		Lynch ($n = 23$)	Sporadic ($n = 86$)	*P* value
Age	Mean	46.8	55.8	0.001
Sex				0.755
	Female	11 (47.8)	38 (44.2)	
	Male	12 (52.2)	48 (55.8)	
Sideness				0.993
	Left	8 (34.8)	30 (34.9)	
	Right	15 (65.2)	56 (65.1)	
Mucinous				0.019
	Yes	4 (17.4)	38 (44.2)	
	No	19 (82.6)	48 (55.8)	
BRAF mutation				0.193
	Mutant-type	0 (0.0)	6 (7.0)	
	Wild-type	23 (100.0)	80 (93.0)	
MLH1 methylation				<0.001
	Methylated	1 (4.3)	39 (45.3)	
	Nonmethylated	22 (95.7)	47 (54.7)	
Revised Bethesda Criteria				
	Met	20 (87.0)	36 (41.9)	<0.001
	Unmet	3 (13.0)	50 (58.1)	
History of polyps				0.484
	Yes	9 (39.1)	27 (31.4)	
	No	14 (60.9)	59 (68.6)	
Cancer history of FDR				<0.001
	Yes	15 (65.2)	20 (23.3)	
	No	8 (34.8)	66 (76.7)	

FDR first-degree relatives.

Comparison of characteristics between Lynch and sporadic CRCs

Table 2 shows the comparisons between Lynch and sporadic CRC. Compared with sporadic CRC, Lynch Syndrome related CRC was associated with a younger age (46.8 vs 55.8 years), fewer mucinous carcinomas (17.4% vs 44.2%), and higher prevalence of cancer history among first-degree relatives (65.2% vs 23.3%). *BRAF* mutation was observed only in sporadic CRC. Most of *MLH1* methylation occurred in sporadic CRC, with one exception (39 vs 1, $P < 0.001$). This patient was a 47-year-old male, with personal and family histories of CRC, fulfilling revised Bethesda Criteria; he harbored pathogenic variant in MLH1 (Exon 8, c.677G>A). There were no differences in terms of sex, sideness of primary tumor, and history of polyps.

Table 3 Sensitivities and specificities of different strategies for Lynch Syndrome.

Methods	Lynch	Sporadic	Sensitivities % (95% Confidence Interval)	Specificities % (95% Confidence Interval)	PPVs % (95% Confidence Interval)	NPVs % (95% Confidence Interval)
BRAF wt	23	80	100% (82.2–100%)	7.0% (2.9–15.1%)	22.3% (15.0–31.8%)	100% (51.7–100%)
BRAF mt	0	6				
MLH1 methylation (−)	22	47	95.7% (76.0–99.8%)	45.3% (34.7–56.4%)	31.9% (21.5–44.3%)	97.5% (85.3–99.9%)
MLH1 methylation (+)	1	39				
BRAF wt + *MLH1* methylation (−)	22	46	95.7% (76.0–99.8%)	47.7% (36.9–58.7%)	32.8% (22.1–45.5%)	97.6% (85.9–99.9%)
BRAF mt or/and *MLH1* methylation (+)	1	40				
RBC (−) + *MLH1* methylation (−)	23	59	100% (82.2–100%)	29.1% (20.0–40.0%)	27.4% (18.5–38.4%)	100% (83.4–100%)
RBC (+) or *MLH1* methylation (+)	0	27				

wt wild-type, *mt* mutant-type, *PPVs* positive predictive values, *NPVs* negative predictive values, *RBC* the Revised Bethesda Criteria.

Table 4 Costs of different screening approaches (RMB).

	BRAF testing	*MLH1* methylation testing	*BRAF* & *MLH1* methylation testing	Universal genetic testing
Cases	109	109	109	109
Sensitivity	100%	95.7%	95.7%	–
Costs per case	472	708	1180	3220[a]
Cases excluded from genetic testing	6 (5.5%)	40 (36.7%)	41 (37.6%)	–
Costs of excluding sporadic CRC from genetic testing	51448	77172	124372	–
Costs of exclusion per case	8575	1929	3033	–
Costs of identifying all MLH1(−) Lynch syndrome	383108	299352	343332	–
Cases of MLH1(−) identified	23 (100%)	22 (95.7%)	22 (95.7%)	23 (100%)
Omission of Lynch syndrome	0	1 (4.3%)	1 (4.3%)	0
Costs of identifying MLH1(−) Lynch syndrome per case	16657	13607	15606	15260

[a]The costs of a 14-gene panel (*MLH1*, *MLH3*, *MSH2*, *MSH6*, *PMS1*, *PMS2*, *APC*, *AXIN2*, *STK11*, *EPCAM*, *PTEN*, *SMAD4*, *MUTYH*, *BMPR1A*).

Performance of different screening approaches

BRAF mutation-genetic testing

BRAF mutation was identified in six sporadic CRC patients. The sensitivity for identifying Lynch Syndrome of this approach was 100%, but the specificity was only 7%. The positive predictive value (PPV) and negative predictive value (NPV) of this test were 22.3% and 100%, respectively (Table 3).

MLH1 methylation-genetic testing

MLH1 promoter methylation was identified in 40 (36.7%) patients, with 39 (97.5%) being sporadic. The sensitivity and specificity for identifying Lynch Syndrome of this approach were 95.7% and 45.3%, respectively (Table 3). This approach missed 4.3% of Lynch Syndrome carriers. The PPV of this test was 31.9%, slightly higher than that of the *BRAF* testing approach. *MLH1* methylation and germline variants together account for 56.9% of MLH1 deficiency, while the other 43.1% remained unexplained.

Combination of *MLH1* promoter methylation testing with *BRAF* testing and with Bethesda criteria

Five (4.6%) patients with *MLH1* methylation were *BRAF* mutant-type; 68 (62.4%) patients without *MLH1* methylation were *BRAF* wild-type. The concordance rate of the two tests was 67.0%. When *BRAF* and *MLH1* methylation tests were combined, the addition of *BRAF* testing did not

improved the sensitivity for identifying Lynch Syndrome of *MLH1* methylation testing, with 4.3% of Lynch Syndrome carriers remaining missed. When the Revised Bethesda Criteria and *MLH1* methylation testing were combined, the sensitivity increased to 100%, with no Lynch Syndrome patient being missed, but the PPV declined from 31.9% to 27.4%, suggesting that an additional 4.5% patients would be referred for unnecessary genetic testing.

Cost-effectiveness analyses

Cost-effectiveness analyses are showed in Table 4. Among the four pre-screening approaches, *MLH1* methylation testing had the lowest costs of identifying MLH1(−) Lynch syndrome per case. The costs of this approach were lower than those of universal genetic testing (13,607 RMB vs 15,260 RMB), but its omission rate was higher (4.3% vs 0).

Discussion

Our study found that *BRAF* mutation was rare among MLH1 deficient CRCs in the Chinese population. Germline variants in MMR genes and *MLH1* promoter methylation together accounted for only 56.9% of MLH1 deficiency. *BRAF* testing and *MLH1* promoter methylation testing as prescreens to exclude Lynch Syndrome were less effective in Chinese CRC patients than in Western CRC patients.

MLH1 methylation has been proposed as a screening marker for excluding patients from germline testing, because it occurs mostly in non-Lynch MLH1(−) CRCs. Tomer et al. found that *MLH1* methylation existed in 85.5% of MLH1(−) CRCs, and its detection helped to reduce the rate of referral to genetic testing from 31% to 13.5% [16]. In our study, however, *MLH1* methylation was identified in less than 40% of the MLH1(−) CRCs, leading to a significant reduction in its utility as an exclusion criterion for Lynch syndrome.

MLH1 methylation can coexist with Lynch Syndrome. A previous study found that *MLH1* methylation occurred in 15% of Lynch Syndrome carriers, who would be missed when using *MLH1* methylation as an exclusion criterion [17]. In our study, 39/40 (97.5%) patients with *MLH1* methylation were sporadic; only one (2.5%) patient with Lynch syndrome was missed. It appears that Chinese Lynch Syndrome carriers are less likely to harbor *MLH1* methylation compared with their Western counterparts, which explains the high sensitivity for identifying Lynch syndrome of this approach in our study.

A simpler screening method is to detect *BRAF* mutation. *BRAF* mutation is highly concordant with *MLH1* methylation, and can also be used as an exclusion criterion. Bessa et al. found that *BRAF* testing could improve the cost-effectiveness of genetic testing for Lynch Syndrome [18]. Moreover, in a Spanish study, *BRAF* testing was more sensitive than *MLH1* methylation testing for ruling out Lynch Syndrome [17]. However, in some studies, *BRAF* testing was less effective than *MLH1* methylation testing, identifying only 20–40% of sporadic CRCs [18, 19]. In Chinese CRCs, this number may further shrink because *BRAF* mutation occurs in less than 5% of Chinese CRCs [13, 20]. In our study, *BRAF* mutation was observed in 5.5% of the patients and in 15% (6/40) of the *MLH1*-methylated cases; therefore *BRAF* testing spared only a small number of patients from unnecessary genetic testing. The results are consistent with a previous Chinese study [21], in which *BRAF* mutation was found in only 15% of Chinese patients with MLH1-/PMS2- CRC, significantly lower than that in their Western counterparts (63%) [22]. Therefore, *BRAF* mutation testing had little to offer as a prescreen to rule out sporadic cases in Chinese patients with MLH1(−) CRC.

A previous study found that compared with *MLH1* methylation testing alone, the combination of *BRAF* testing and *MLH1* methylation testing could slightly reduce the rate of referral to genetic counseling [16]. In our study, although the addition of *BRAF* testing slightly increased the specificity of *MLH1* methylation testing (from 45.3% to 47.7%), it failed to improve the sensitivity, with one Lynch Syndrome carrier remaining missed. Therefore, the performance of this combination is similar to that of *MLH1* methylation testing alone.

To improve the performance of *MLH1* methylation testing, we introduced the Revised Bethesda Criteria as a first-step screening to select eligible patients. As expected, the criteria helped to include the Lynch Syndrome case missed by *MLH1* methylation testing, increasing its sensitivity to 100%. However, the criteria also included a considerable number of low-risk cases for genetic testing, leading to a drop in PPV from 31.9% to 27.4%. Although this combination increased the sensitivity for identifying Lynch Syndrome, it also increased the costs.

Genetic testing is the gold standard for diagnosing Lynch Syndrome, but it is used mainly as a diagnostic tool rather than a screening one because of its high costs. However, as the technology matures and the price goes down, it can also be used in screening programs. In 2012, Wang et al. found that genetic testing for first-degree relatives of Lynch Syndrome patients, who were at high risk of CRC, was a cost-effective strategy in Singapore [23]. Given the poor performance of *BRAF* and *MLH1* promoter methylation testing in excluding low-risk CRCs, it is reasonable and practical to carry out universal genetic testing for MLH1(−) CRCs among the Chinese population. Although in cost-effectiveness analysis, the *MLH1* methylation testing

screening approach had lower costs for the identification of MLH1(−) Lynch syndrome per case than universal genetic sequencing, it should be noted that the costs of genetic testing in our study cover 14 genes related to hereditary CRC syndromes. When this panel narrows down to five genes (*MLH1*, *MSH2*, *MSH6*, *PMS2*, and *EPCAM*), or even two (*MLH1* and *PMS2*), the gap between these two approaches may likely close.

Our study has some limitations. First, as a single-center study, it is subject to selection bias. Second, the sample of this study is relatively small, precluding it from drawing definitive conclusions. Third, family history of cancer is unavailable in some patients, which may weaken the performance of the Revised Bethesda Criteria. Last, somatic sequencing and germline *MLH1* methylation testing were not performed in this study, and therefore we were not able to assess other causes for MLH1 deficiency.

Conclusion

Taken together, our study demonstrates that *BRAF* and *MLH1* promoter methylation testing as prescreening approaches for Lynch Syndrome among patients with MLH1(−) CRC are less effective in the Chinese population than in the Western population. *MLH1* methylation testing as a prescreen helped to reduce the costs of identifying MLH1(−) Lynch syndrome per case by about 10% percent compared with universal genetic testing, but it ran the risk of omitting 4.3% of Lynch syndrome carriers. Universal genetic testing could be considered an up-front option for this population.

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Author contributions Conceptualization: P-RD and WJ; Methodology: JL, BX and LM; Formal analysis and investigation: JL, EX; Resources: EX, LK, QS, DL, WL, ZH and JT; Writing—original draft preparation: BX and JL; Writing—review and editing: BX and PD; Supervision: WJ and PD; Project administration: ZP and PD; Funding acquisition: ZP and PD.

Compliance with ethical standards

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

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