Contents lists available at ScienceDirect

Translational Oncology

Original Research

A modified screening strategy for Lynch syndrome among MLH1-deficient CRCs: Analysis from consecutive Chinese patients in a single center

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ARTICLE INFO

ABSTRACT

Background: The low prevalence of the *BRAF* V600E mutation in colorectal cancers (CRCs) in Chinese populations has stimulated concern about the efficacy of *BRAF* mutation analysis for Lynch syndrome (LS) screening.
Methods: In total, 169 of 4104 consecutive CRC patients with absent MLH1 staining were analyzed to compare the utility of the *BRAF* V600E mutation testing with *MLH1* promoter methylation analysis in the Chinese population. Germline genetic testing was performed in patients with wild-type *BRAF*/methylated *MLH*1.
Results: Compared with *BRAF* genotyping, the use of *MLH1* methylation testing alone to evaluate patients with MLH1 deficiency reduced referral rates for germline testing by 1.8-fold (82.8% vs. 47.1%). However, 6 patients harboring *MLH1* promoter methylation were verified to have LS through germline genetic testing. It is notable that all 6 patients had a family history of CRC in at least 1 first-degree relative (FDR) or second-degree relative (SDR). The combination of *MLH1* promoter methylation analysis and a family history of CRC could preclude significantly more patients from germline genetic testing than from *BRAF* mutation testing alone (45.5% vs. 17.2%, *p<0.001*) and decrease the number of misdiagnosed LS patients with *MLH1* promoter methylation.
Conclusion: The combination of a family history of CRC with *MLH1* promoter methylation analysis showed better performance than *BRAF* mutation testing in the selection of patients in the Chinese population for germline genetic testing.

Introduction

Lynch syndrome (LS) is the most common hereditary colorectal cancer (CRC) syndrome caused by germline pathogenic variants in any of four DNA mismatch repair (MMR) genes (*MSH2, MLH1, PMS2* or *MSH6*) or a deletion in the *EPCAM* gene, which leads to methylation of the adjacent *MSH2* promoter [1,2]. Mutations in MMR genes may lead to tumor DNA microsatellite instability (MSI) and deficient MMR (dMMR) [3–5].

Although LS accounts for only 2–3% of all CRC cases [6], pathogenic MMR gene mutation carriers have a high risk of developing multiple cancers, including colorectal, endometrial, ovarian, stomach, and urinary tract cancers. A recent study based on a prospective LS database reported that the calculated cumulative incidence of any cancer at age 70 years was 75% in females and 58% in males and that CRC was the most common [7]. More importantly, LS patients are susceptible to the development of subsequent cancers, including CRC (22% to 74%) [8,9]

after first onset, and periodic colonoscopic surveillance can reduce CRC-related mortality [10]. However, routine germline genetic testing for hereditary cancers in the clinic is rarely available and is expensive in many areas of China. Therefore, it is essential to increase the efficiency of screening and reduce unnecessary patient referral to germline genetic testing. Particularly for patients with MLH1 deficiency, a proportion of those with sporadic disease can be excluded before germling testing through *BRAF* mutation and/or *MLH1* promoter methylation testing.

Sporadic CRC with MLH1 deficiency commonly results from methylation of the cytosine-phosphate-guanine (CpG) island in the *MLH1* gene promoter, thus causing transcriptional silencing of the *MLH1* gene [11]. This pathologic phenotype is associated with the serrated neoplasia carcinogenetic pathway and highly correlated molecular characteristics, including the CpG island methylator phenotype (CIMP), the *BRAF* V600E mutation and methylation of the *MLH1* gene promoter [11,12]. Therefore, testing of the *BRAF* mutation and *MLH1* promoter methylation is

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now widely used to discriminate patients with sporadic dMMR among those with MLH1-deficient CRC in LS screening [13,14].

Previous reports showed evidence of somatic *MLH1* methylation, along with the recent proposal of a constitutional *MLH1* epimutation in a patient with suspected LS [15,16], being a second hit in some LS tumors and cast doubt on the performance of *MLH1* methylation as a definite negative predictor of pathogenic *MLH1* variants. In contrast, *BRAF* mutations are virtually absent in LS-associated tumors; approximately 1% of cancer patients with *BRAF* V600E mutations (and loss of MLH1) have LS [17]. The National Comprehensive Cancer Network (NCCN) guidelines recommend that for MLH1-deficient patients displaying a *BRAF* mutation, no further tests are required except in those with a young age of onset or a significant family history [18]. In addition, immunohistochemistry (IHC) has been proposed as an alternative method to detect the *BRAF* V600E mutation status, making this detection strategy efficient and effective in clinical practice [19,20]. Therefore, *BRAF* mutation testing is currently the most widely used test to exclude sporadic CRCs with MLH1 deficiency.

Nevertheless, the genetic variability among different ethnic groups must be seriously considered, especially when screening for hereditary diseases. Previous reports indicated that the *BRAF* mutation frequency varied among patients with different ethnicities, especially between Asians and individuals in most European and American studies. The latter studies reported that *BRAF* mutations accounted for approximately 15% of all CRCs, and 44.9 – 57.7% of patients with MLH1-deficient CRCs harbored a *BRAF* mutation [21–22]. In contrast, in Asian countries and some eastern European countries, such as Japan, Russia, Israel and China, the *BRAF* mutation frequencies in CRCs are much lower than those in Western countries, ranging from 3–6% [23–28]. This frequency increases slightly to 15.4%, even in CRCs with loss of the expression of MLH1/PMS2 [29]. Therefore, the low occurrence of the *BRAF* mutation in such populations has led to clinical concern regarding its superiority to *MLH1* promoter methylation as a negative predictor of germline *MLH1* mutations.

To our knowledge, however, the diagnostic role of *BRAF* mutation testing and *MLH1* methylation analysis in LS prescreening among patients with MLH1-deficient CRC in China is unclear. Therefore, the aim of this study was to compare the efficacy of these two diagnostic methods in a large consecutive cohort of Chinese patients with CRC and to investigate the feasibility and effectiveness of these methods for screening LS among Chinese patients.

Materials and methods

Patient cohort

We reviewed the pathology database for 4104 consecutive patients who had undergone radical surgery for CRC between June 2011 and December 2014 at the National Cancer Center/National Clinical Research Center for Cancer/Cancer Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. The immunohistochemical analysis of the 4 MMR proteins (MLH1, MSH2, MSH6 and PMS2) was performed routinely during the postoperative pathological examination at the Department of Pathology. CRC patients with a deficiency in at least one MMR protein were identified as being dMMR. Of these patients, those with MLH1-deficient CRC were enrolled in the study, and further tests were conducted as described below.

DNA extraction

Formalin-fixed and paraffin-embedded tumor tissues were used for *BRAF* V600E gene mutation and *MLH1* promoter methylation analyses. Normal colorectal mucosal tissues were collected for germline genetic testing of the *MLH1* gene. Genomic DNA was extracted by using a QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The quality and concentration of the DNA samples were assessed on a NanoDrop and Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA).

BRAF *V600E mutation analysis*

The *BRAF* V600E mutational status was assessed by real-time PCR using a multiplex allele-specific PCR-based kit (ACCB, Beijing, China) on a Stratagene Mx3000P system (Agilent Technologies Inc., Santa Clara, CA) in the Molecular Pathology Laboratory of the Department of Pathology, Cancer Hospital Chinese Academy of Medical Sciences, as previously reported [30].

MLH1 *promoter methylation analysis*

Methylation-specific PCR (MS-PCR) was used to distinguish unmethylated DNA from methylated DNA based on sequence alterations produced by chemical treatment with sodium bisulfite with the EZ DNA Methylation-Gold Kit (Zymo Research, CA, USA). In brief, the methylation status was identified by subsequent PCR using primers specific to the methylated or unmethylated DNA. The analyzed fragment was located in the 'C' region, as described previously [31,32]. The primer sequences for the amplification of unmethylated MLH1 were 5′-TGAATTAATAGGAAGAGTGGATAGT-3′ (sense) and 5′-TCCCTCCCTAAAACAACTACTACCCA-3′ (antisense); and for the amplification of methylated MLH1, 5′-AATTAATAGGAAGAGCGGATAGC-3′ (sense) and 5′-CCTCCCTAAAACGACTACTACCCG-3′ (antisense). The PCR product was directly loaded onto 2% agarose gels and visualized under UV illumination. The representative MS-PCR results are shown in Supplementary Figure S1.

Panel next-generation sequencing (NGS) germline genetic testing

In total, 200 ng of normal genomic DNA was fragmented by sonication using a Covaris M220 (Covaris Inc., Massachusetts, USA). After the fragmentation process, end repair, A-tailing, adapter ligation, PCR and target enrichment were performed following the manufacturer's recommended protocols for the SureSelect-XT Low Input Target Enrichment System and ClearSeq Inherited Disease panel, which included our genes of interest: *EPCAM, MLH1, PMS2, MSH2* and *MSH6* (Agilent Technologies, Santa Clara, CA, USA). Final libraries were quantified with a Qubit High Sensitivity Kit (Invitrogen, Carlsbad, CA, USA). Products were sequenced on a HiSeq 2500 to at least an average depth of 100-fold coverage with WuXi NextCODE. Reads were aligned to the reference human genome GRCh37. Germline variations, including point mutations and short indels (<30 base pairs), were called with GATK, SAMtools and Picard tools. Annotations were defined using ANNOVAR. Variants were reviewed as pathogenic, likely pathogenic, a variant of uncertain significance (VUS), likely benign, or benign based on the database of the International Society of Gastrointestinal Hereditary Tumors (InSiGHT) (http://insight-group.org/variants/classifications/). If no pathogenicity data were available, the significance of the variants was assessed by integrating the in silico prediction results from Align GVGD, SIFT, MutationTaster, PolyPhen-2, Human Splicing Finder, etc., with other criteria according to consensus guidelines of the American College of Medical Genetics and Genomics (ACMG) [33].

Statistical analyses

Statistical analyses were performed with SPSS 25.0 software (SPSS, Chicago, IL). To evaluate the statistical significance of differences observed between groups, the χ^2 test was applied. Specifically, Pearson's χ^2 test, Fisher's exact test and continuity correction were used as appropriate. All *P*-values represent two-sided statistical tests, and only those less than 0.05 were considered significant.
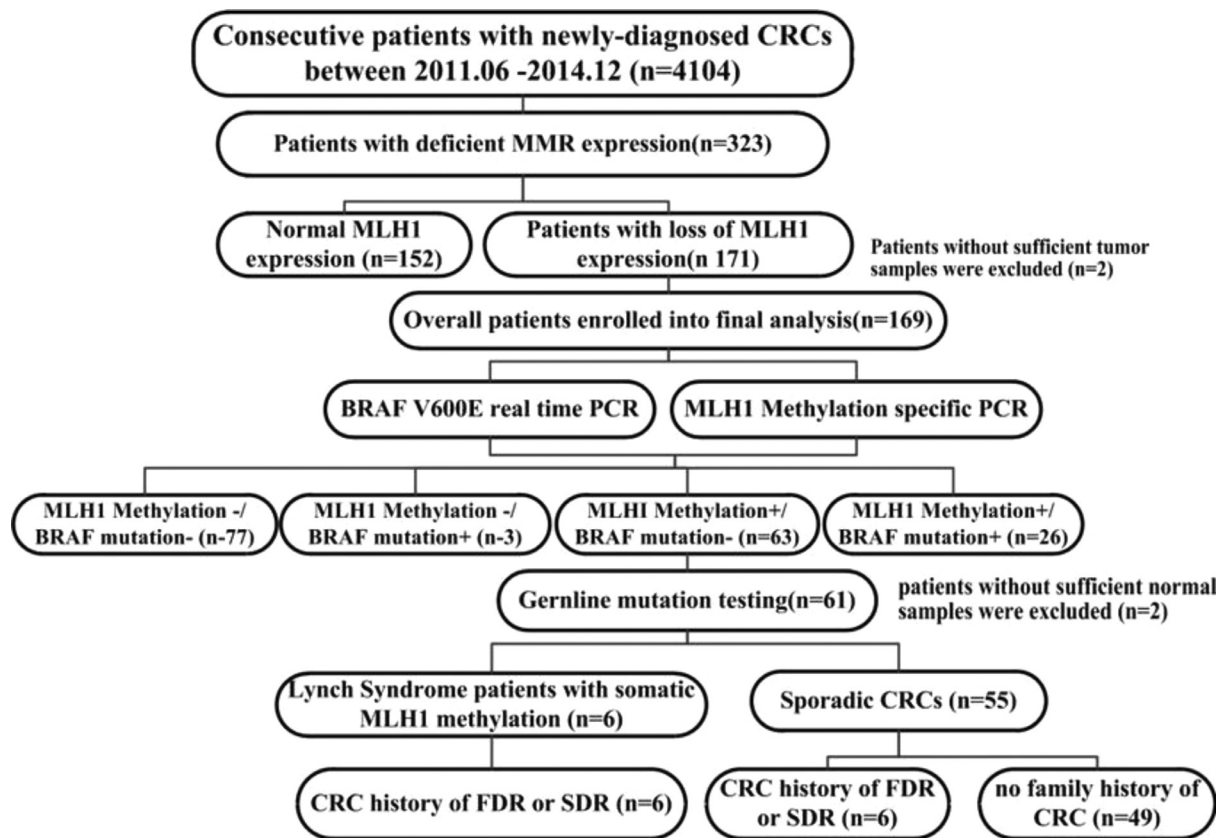
Fig. 1. Flow diagram of the analytic strategy and main results of the study.

Results

Patients

Of the 4104 CRC patients, 323 (7.9%) were identified as being dMMR. Of these patients, 171 (52.9%, 171/323) were MLH1 deficient. Two of these patients were excluded due to a lack of tumor samples. Therefore, a total of 169 patients were included in the final analysis (Fig. 1).

The clinicopathological features of the patients in this study are summarized in Supplementary Table S1. Tumors were characterized by certain clinicopathological features, consistent with those previously reported for dMMR CRC, such as a tendency to be proximal and poorly differentiated, to contain mucinous or signet ring cell components, and to exhibit few lymph node and distant metastases despite a late stage in the primary sites (T3, T4). Thirteen patients (7.7%, 13/169) had a personal history of CRC.

Family histories of cancer, namely, LS-related, possibly LS-related and other histories according to the revised Bethesda guidelines, are shown in Supplementary Table S2. Forty-four patients had a family history of CRC, which was top in the list of LS-related cancers. Stomach cancer was the second most commonly observed (11 patients), followed by biliary and urinary cancers, whereas endometrial cancer was less commonly observed.

MLH1 *promoter methylation and* BRAF *V600E mutation statuses*

MLH1 promoter methylation was observed in 89 (52.7%) of 169 MLH1-deficient patients. However, only 29 (17.2%, 29/169) patients harbored the *BRAF* V600E mutation. 63 patients (37.3%, 63/169) presented wild-type *BRAF* and *MLH1* promoter methylation. The comparisons of the *BRAF* mutation and *MLH1* promoter methylation statuses

are presented in Fig. 2. As expected, *MLH1* promoter methylation was found in most patients harboring the *BRAF* V600E mutation (26/29).
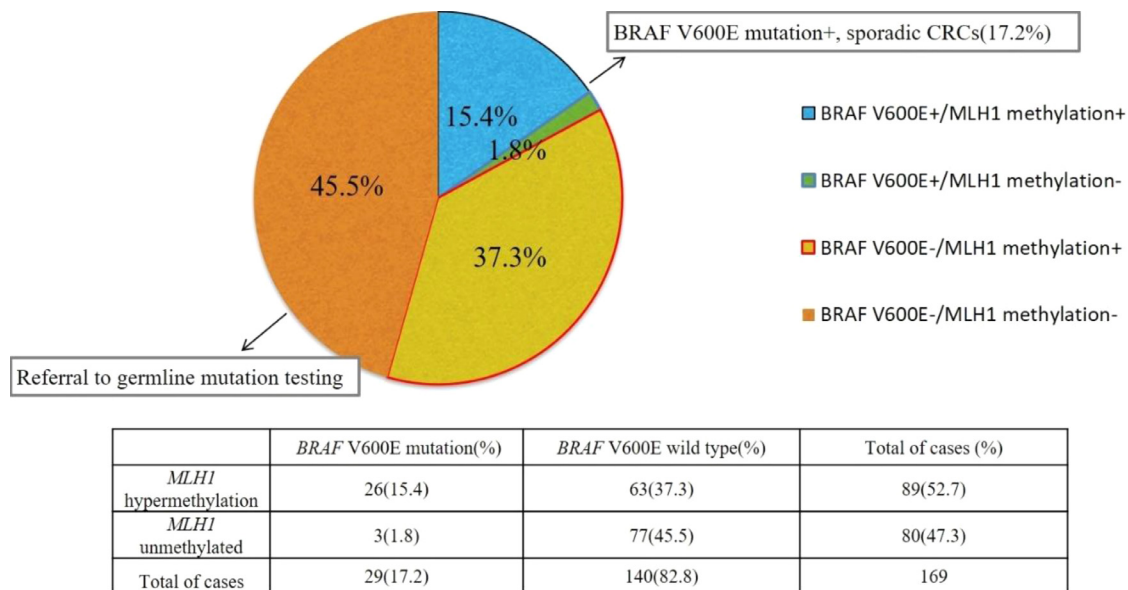
Clinicopathological characteristics of patients with the BRAF *V600E mutation or* MLH1 *promoter methylation*

Compared with *BRAF* V600E-mutated CRC patients, *BRAF* V600E wild-type CRC patients were younger (median age: 64 years vs 54 years, respectively). In addition, more patients with *BRAF* V600E-mutated CRC than patients with *BRAF* V600E wild-type CRC had primary tumors located in the proximal colon (93.1% vs 57.1%, respectively; $P<0.001$). A similar pattern was observed between patients with *MLH1* promoter methylation and patients without *MLH1* promoter methylation (70.8% vs. 55.0%; $p = 0.033$). There were no significant differences in the clinicopathological features, such as the differentiation grade, presence of mucinous or signet ring cell components and personal tumor history, between patients displaying different statuses of the *BRAF* V600E mutation or *MLH1* promoter methylation.

A previous study showed that a family history of CRC was a strong indicator of LS in patients with MLH1/MSH2 deficiency [4]. In our study, no patients with *BRAF* V600E-mutated CRC had a family history of CRC. Patients who had unmethylated *MLH1* CRCs had a notably stronger family history of CRC than those who had methylated *MLH1* CRCs. It is worth noting that 13.5% of the patients in the methylation group had a family history of CRC. The details are shown in Table 1.

Family history of CRC could help discriminate LS patients with MLH1 *promoter methylation*

According to the NCCN guidelines, LS is rarely observed in CRCs harboring a *BRAF* mutation [18]. Therefore, germline genetic testing was conducted in 61 of 63 patients with wild-type *BRAF* and *MLH1* promoter methylation (two patients were excluded because of poor DNA

	BRAF V600E mutation(%)	*BRAF* V600E wild type(%)	Total of cases (%)
MLH1 hypermethylation	26(15.4)	63(37.3)	89(52.7)
MLH1 unmethylated	3(1.8)	77(45.5)	80(47.3)
Total of cases	29(17.2)	140(82.8)	169

Fig. 2. Results of *BRAF* V600E mutation testing and *MLH1* promoter methylation analysis. According to the guidelines of LS screening, 17.2% of patients with the *BRAF* V600E mutation could be precluded from germline genetic testing, while 45.5% of patients who displayed negative *MLH1* promoter methylation were referred for germline genetic testing. The pathogenic variant status of the remaining 37.3% of patients needed to be clarified due to the possibility of coexisting *MLH1* promoter methylation and a germline mutation.

Table 1
Clinicopathological features of patients with different *MLH1* promoter methylation or *BRAF* mutation statuses.

Characteristic	Positive *MLH1* promoter methylation n = 89 (%)	Negative *MLH1* promoter methylation n = 80 (%)	p	Mutant *BRAF* n = 29 (%)	Wild-type *BRAF* n = 140 (%)	p
Age at onset (y)						
Median	63	52		64	54	
Mean	60.3	49.5	<0.001	66.2	52.9	<0.001
SD	13.2	11.8		9.8	13.2	
Proximal location	63 (70.8)	44 (55.0)	0.033	27 (93.1)	80 (57.1)	<0.001
Poor differentiation	39 (43.8)	27 (33.8)	0.180	16 (55.2)	50 (35.7)	0.051
Mucinous component	46 (51.7)	33 (41.3)	0.175	12 (41.4)	67 (47.9)	0.525
Signet ring component	2 (2.2)	1 (1.3)	0.624	1 (3.4)	2 (1.4)	1.000
Signet ring+ Mucinous component	5 (5.6)	8 (10.0)	0.286	2 (6.9)	11 (7.9)	1.000
Synchronic CRC	3 (3.4)	8 (10.0)	0.081	1 (3.4)	10 (7.1)	0.749
Lynch-related tumor	3 (3.4)	6 (7.5)	0.395	0	9 (6.4)	0.343
FDR or SDR with CRC	12 (13.5)	32 (40)	<0.001	0	44 (31.4)	<0.001
FDR or SDR with CRC aged <50 y	3 (3.4)	17 (21.3)	<0.001	0	19 (13.6)	0.075
FDR with CRC	9 (10.0)	27 (33.8)	<0.001	0	35 (25.0)	0.002
FDR with CRC aged <50 y	3 (3.4)	14 (17.5)	0.002	0	16 (11.4)	0.118
FDR or SDR with a LS-related tumor	17 (18.9)	14 (17.5)	0.788	6 (20.7)	25 (17.9)	0.720
FDR or SDR with a LS-related tumor aged <50 y	4 (4.5)	6 (7.5)	0.617	1 (3.4)	9 (6.4)	0.852
Fulfillment of revised Bethesda guidelines	42 (47.8)	69 (86.3)	<0.001	8 (27.6)	103 (73.6)	<0.001

CRC: colorectal cancer, FDR: first-degree relative, defined as a patient's parent (father or mother), full sibling (brother or sister) or child, SDR: second-degree relative, defined as a patient's uncle, aunt, nephew, niece, grandparent, grandchild, half-sibling, and double cousin who shared 25% of a person's genes.

quality) to determine whether any LS patients in the *MLH1* promoter methylation group failed to be identified. As expected, 6 patients were identified to have LS with germline pathogenic *MLH1* variants (Table 2).

To further refine the clinical criteria for selecting patients who should be subjected to germline genetic testing, variables associated with germline pathogenic *MLH1* variants were identified. Table 3 shows the parameters significantly associated with pathogenic variant carriers in the univariate analysis. A family history of CRC in at least 1 first- or second-degree relative (FDR/SDR) could discriminate all patients (6/6) with germline pathogenic variants. In other words, a family history of CRC in at least 1 FDR or SDR could increase the performance of *MLH1* promoter methylation analysis in LS screening.

Therefore, since no patient with *BRAF* mutations had a family history of CRCs, only 80 patients without *MLH1* promoter methylation,

along with 12 patients with *MLH1* promoter methylation whose FDR or SDR had a history of CRC, required further germline genetic testing (92/169), while the number would have increased to 140 patients when using the *BRAF* V600E mutation alone as a negative predictor of germline pathogenic variants. The total costs of LS screening for all patients (n = 169) in our study were calculated based on the two strategies (Supplementary Figure 2). Use of the *BRAF* status alone as a selection criterion would require a total of ¥ 941,400 ($145,258.02), while the introduction of a family history in addition to *MLH1* promoter methylation would reduce this cost by 30.6%.

Overall, when taking a family history of CRC into consideration, *MLH1* promoter methylation analysis significantly reduced the number of patients referred to germline genetic testing compared with *BRAF* genotype testing (54.5% vs 88.2%, p < 0.001).

Table 2

Characteristics of CRC patients with germline pathogenic *MLH1* variants.

No.	Sex	Age (y)	Germline *MLH1* mutation	Mutation significance	Tumor location	Family history	Fulfillment of the revised Bethesda criteria
1	F	62	*MLH1*:c.793C>T (p.Arg265Cys)	Pathogenic	Ascending colon	Colon	N
2	M	52	*MLH1*:c.677G>A (p.Arg226Gln)	Pathogenic	Rectum	Breast, colon, ovary, endometrium	Y
3	F	69	*MLH1*:c.2179_2182del (p.His727fs)	Pathogenic	Ascending colon	Colon, endometrium	Y
4	M	52	*MLH1*:c.883A>G (p.Ser295Gly)	Pathogenic	Ascending colon	Stomach, colon	Y
5	M	32	*MLH1*:c.1845_1847del (p.615_616del)	Pathogenic	Transverse colon	Colon	Y
6	F	34	*MLH1*:c.678–2del (splicing)	Likely pathogenic	Sigmoid colon	Colon	Y

CRC: colorectal cancer, F: female, M: male.

Table 3

Predictors of germline pathogenic variants in patients with *MLH1* hypermethylation/wild-type *BRAF* (univariate analysis).

Characteristic	Patients with a (likely) pathogenic variant [a] ($n = 6$)	Patients without a pathogenic variant ($n = 55$)	*p*
Colorectal cancer in ≥1 FDR	5	4	<0.001
Colorectal cancer in a FDR at age 50 y	3	0	0.001
Colorectal cancer in ≥1 FDR or SDR	6	6	<0.001
Synchronous, metachronous colorectal, or other LS-related cancer	2	5	0.136
Colorectal cancer with dMMR diagnosed at age 60 y	4	30	0.893
FDR or SDR with a LS-related tumor	2	11	0.816
Fulfillment of revised Bethesda guidelines	5	32	0.449

a. Patients with a pathogenic variant and a likely pathogenic variant were included; FDR, first-degree relative; SDR, second-degree relative; LS, Lynch syndrome; dMMR, mismatch repair deficiency.

Discussion

The calculated cumulative incidence of CRC by age 70 years is 46% in patients harboring germline pathogenic *MLH1* variants [7]; however, 70–80% of CRC patients displaying MLH1 loss are diagnosed with sporadic CRC. Therefore, a screening strategy with high sensitivity and specificity should be adapted to distinguish patients with possible LS from those with sporadic CRC for germline genetic testing.

According to the widely used NCCN guidelines, patients with MLH1 deficiency are recommended to undergo *BRAF* V600E and/or *MLH1* methylation testing to exclude patients from germline testing; however, both methods have certain flaws. For example, *BRAF* V600E testing in populations with a low *BRAF* mutation frequency has low specificity, and *MLH1* promoter testing can result in a missed diagnosis. Therefore, we aimed to find a screening flow that may increase the efficiency and accuracy of testing in the population with low incidence of *BRAF* V600E mutations.

BRAF mutation analysis has been well acknowledged as the strongest negative predictor of germline pathogenic *MLH1* variants in MLH1-deficient tumors. Of note, the *BRAF* mutation frequency varies among individuals with different ethnicities. Parsons et al. reviewed 35 studies assessing the *BRAF* V600E mutation status in CRC patients mostly from Western countries and found that *BRAF* V600E mutations occurred in 63.5% of CRC patients displaying *MLH1* promoter methylation or MLH1 protein loss (95% CI 46.98%–78.53%) [17]. However, in our study, *BRAF* mutations were found in only 17.2% of CRC patients with MLH1 expression loss, and only 29.2% (26/89) of CRC patients displaying *MLH1* promoter methylation harbored *BRAF* mutations, which is much lower than the percentage of patients in Western countries.

The variance in the *BRAF* mutation rate may be associated with the difference in serrated tumorigenic pathways according to ethnicity. A previous large cohort study showed that people of southern European origin had a lower risk of CRCs with CIMP and *BRAF* mutations than people of Anglo-Celtic origin, suggesting an ethnic difference in the development of *BRAF* mutation-related CRCs [34].

The low frequency of *BRAF* mutations among Chinese patients with CRC in this study raises concerns about the low specificity of *BRAF* V600E mutation testing as an exclusion strategy in LS screening. Under this clinical reality, more patients with MLH1 deficiency will be referred for germline genetic testing, which yields financial and emotional burdens on cancer patients and their relatives. Therefore, a modified screening strategy with increased specificity is urgently needed in Chinese patients with MLH1-deficient CRC.

In this study, the incidence of *MLH1* promoter methylation was almost threefold that of the *BRAF* V600E mutation (47.3% vs. 17.2%), indicating a strong potency to preclude more sporadic CRC patients from germline genetic testing. It is notable that some rare cases of somatic *MLH1* promoter methylation may be the second hit in some LS patients [15,35] and may lead to a missed diagnosis of LS when using *MLH*1 promoter methylation testing as an exclusive method. To tackle this problem, the NCCN guidelines recommend that for patients with wild-type *BRAF V600E/MLH1* promoter methylation, no additional tests are needed except for those with a young age of onset or a significant family history [19]; however, they fail to specify criteria such as family history, and therefore, their potency is difficult to assess.

In our study, we identified 6 (9.8%, 6/61) LS patients with pathogenic *MLH1* mutations among 61 patients displaying wild-type *BRAF* and *MLH1* promoter methylation, similar to the results reported by Moreira (7.5%, 3/40) [36]. Previous studies showed that a family history of CRC in at least 1 FDR was an independent predictor of germline pathogenic *MLH1* variants in the assessment of each clinicopathological parameter possibly related to LS [4]. Similarly, in our study, a family history of CRC in at least 1 FDR or SDR also showed outstanding performance for discriminating patients with possible LS from those with *MLH1* promoter methylation, and no LS patient was misdiagnosed using such selection criteria.

The strengths of this study are as follows. First, our results contribute to a better understanding of the role of these two methods in LS screening among CRC patients of some races with a low incidence rate of the *BRAF* V600E mutation. We comprehensively analyzed both the *BRAF* mutation status and *MLH1* promoter methylation status in MLH1-deficient patients from a large consecutive CRC cohort. Therefore, the different genetic profiles of CRC patients with *MLH1* protein expression loss in this study may help to modify the strategy of LS screening for Chinese patients with MLH1-deficient CRC. Second, to our knowledge, this study is the first to systematically evaluate the characteristic of family history associated with LS patients harboring somatic *MLH1* promoter methylation in Chinese population. Use of a history of CRC in at least 1 FDR or SDR as a selection criterion for the referral of patients displaying wild-type *BRAF* and *MLH1* promoter methylation for germline genetic testing could significantly increase the efficiency of LS screening. The number of patients who needed germline genetic testing decreased compared with that using the *BRAF V600E* mutation alone. Unlike when *MLH1* methylation analysis was used alone, no LS patient with MLH1 promoter methylation was missed.

In the past, despite guideline recommendations, MMR and germline testing were less prevalent in developing countries, including China, due to technical issues. Recently, the DNA sequencing technique has experienced rapid development, and immunotherapy has shown outstanding efficacy in cancer patients with dMMR, which means that more patients accept MSI/MMR testing to pursue effective treatment. As a result, the demand for LS screening is expected to increase in the next few years. Therefore, a more efficient and accurate screening algorithm would decrease the medical burden.

Nevertheless, our study has limitations. First, germline mutations were tested only in patients with wild-type *BRAF* V600E/*MLH1* promoter methylation and not in the whole study population; therefore, the sensitivity and specificity of the traditional methods (*BRAF* V600E or *MLH1* promoter testing alone) and the one we proposed (combined with a family history) could not be compared directly. In addition, the screening strategy suggested in this study awaits confirmation in another cohort of patients with CRC.

In conclusion, the *BRAF* V600E mutation rate in Chinese patients with MLH1-deficient CRC was significantly lower than that in patients from Western countries, thus leading to a decreased ability to rule out patients with sporadic disease from germline genetic testing.

A family history of CRC in at least 1 FDR or SDR should be considered a valuable clinical characteristic when referring MLH1-deficient patients with *MLH1* promoter methylation to germline MLH1 genetic testing. *MLH1* promoter methylation analysis combined with a family history of CRC in the LS screening algorithm is more efficient and cost effective than *BRAF* mutation testing alone in the Chinese population.

Author contributions

Ning Lyu: Conceptualization, Funding acquisition. **Shuangmei Zou**: Conceptualization, resources, Supervision. **Lin Dong**: Conceptualization, Methodology, Formal analysis. **Wenmiao Wang**:Data curation, Methodology, Formal analysis, Writing - original draft. **Jianming Ying**: Conceptualization, Supervision, Writing - review & editing. **Susheng Shi**: Supervision. **Qiurong Ye**: Methodology.

Ethics statement

This study was approved by the Ethics Committee of NCC/CICAMS (NCC1790). The need for individual informed consent was waived because of the retrospective nature of the study. Patients were informed if they were determined to have Lynch syndrome.

Declaration of Competing Interest

All authors declare no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tranon.2021.101049.

References

[1] P. Peltomaki, R.A. Lothe, L.A. Aaltonen, L. Pylkkänen, M. Nyström-Lahti, R. Seruca, et al., Microsatellite instability is associated with tumors that characterize the hereditary non-polyposis colorectal carcinoma syndrome, Cancer Res. 53 (1993) 5853–5855.

[2] M.J. Ligtenberg, R.P. Kuiper, T.L. Chan, M. Goossens, K.M. Hebeda, M. Voorendt, et al., Heritable somatic methylation and inactivation of MSH2 in families with Lynch syndrome due to deletion of the 3′ exons of TACSTD1, Nat. Genet. 41 (2009) 112–117, doi:10.1038/ng.283.

[3] M. Grilley, J. Holmes, B. Yashar, P. Modrich, Mechanisms of DNA-mismatch correction, Mutat. Res. 236 (1990) 253–267, doi:10.1016/0921-8777(90)90009-t.

[4] V. Piñol, A. Castells, M. Andreu, S. Castellví-Bel, C. Alenda, X. Llor, et al., Accuracy of revised Bethesda guidelines, microsatellite instability, and immunohistochemistry for the identification of patients with hereditary nonpolyposis colorectal cancer, JAMA 293 (2005) 1986–1994, doi:10.1001/jama.293.16.1986.

[5] H. Hampel, W.L. Frankel, E. Martin, M. Arnold, K. Khanduja, P. Kuebler, et al., Feasibility of screening for Lynch syndrome among patients with colorectal cancer, J. Clin. Oncol. 26 (2008) 5783–5788, doi:10.1159/000481413.

[6] S. Popat, R. Hubner, R.S. Houlston, Systematic review of microsatellite instability and colorectal cancer prognosis, J. Clin. Oncol. 23 (2005) 609–618, doi:10.1200/JCO.2005.01.086.

[7] P. Møller, T. Seppälä, I. Bernstein, E. Holinski-Feder, P. Sala, D.G. Evans, et al., Cancer incidence and survival in Lynch syndrome patients receiving colonoscopic and gynaecological surveillance: first report from the prospective Lynch syndrome database, Gut 66 (2017) 464–472, doi:10.1136/gutjnl-2015-309675.

[8] K. Newton, K. Green, F. Lalloo, D.G. Evans, J. Hill, Colonoscopy screening compliance and outcomes in patients with Lynch syndrome, Colorectal Dis. 17 (2015) 38–46, doi:10.1111/codi.12778.

[9] H.J. Järvinen, L. Renkonen-Sinisalo, K. Aktán-Collán, P. Peltomäki, L.A. Aaltonen, J.P. Mecklin, Ten years after mutation testing for Lynch syndrome: cancer incidence and outcome in mutation-positive and mutation-negative family members, J. Clin. Oncol. 27 (2009) 4793–4797, doi:10.1200/JCO.2009.23.7784.

[10] H.J. Järvinen, M. Aarnio, H. Mustonen, K. Aktan-Collan, L.A. Aaltonen, P. Peltomäki, et al., Controlled 15-year trial on screening for colorectal cancer in families with hereditary nonpolyposis colorectal cancer, Gastroenterology 118 (5) (2000) 829–834 May, doi:10.1016/s0016-5085(00)70168-5.

[11] D.J. Weisenberger, K.D. Siegmund, M. Campan, J. Young, T.I. Long, M.A. Faasse, et al., CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer, Nat. Genet. 38 (2006) 787–793, doi:10.1038/ng1834.

[12] N.J. Hawkins, C. Bariol, R.L. Ward, The serrated neoplasia pathway, Pathology 34 (2002) 548–555.

[13] H.F. Vasen, I. Blanco, K. Aktan-Collan, J.P. Gopie, A. Alonso, S. Aretz, et al., Revised guidelines for the clinical management of Lynch syndrome (HNPCC): recommendations by a group of European experts, Gut 62 (2013) 812–823, doi:10.1136/gutjnl-2012-304356.

[14] J. Gibson, J. Lacy, E. Matloff, M. Robert, Microsatellite Instability Testing in Colorectal Carcinoma: a Practical Guide, Clin. Gastroenterol. Hepatol. 12 (2014) 171–176, doi:10.1016/j.cgh.2013.11.001.

[15] N. Rahner, N. Friedrichs, V. Steinke, S. Aretz, W. Friedl, R. Buettner, et al., Coexisting somatic promoter hypermethylation and pathogenic MLH1 germline mutation in Lynch syndrome, J. Pathol. 214 (1) (2008) 10–16 Jan, doi:10.1002/path.2263.

[16] M.P. Hitchins, R.L. Ward, Constitutional (germline) MLH1 epimutation as an aetiological mechanism for hereditary non-polyposis colorectal cancer, J. Med. Genet. 46 (2009) 793–802, doi:10.1136/jmg.2009.068122.

[17] M.T. Parsons, D.D. Buchanan, B. Thompson, J.P. Young, A.B. Spurdle, Correlation of tumor BRAF mutations and MLH1 methylation with germline mismatch repair (MMR) gene mutation status: a literature review assessing utility of tumor features for MMR variant classification, J. Med. Genet. 49 (2012) 151–157, doi:10.1136/jmedgenet-2011-100714.

[18] National Comprehensive Cancer Network (NCCN) clinical practice guidelines in oncology: genetic/familial high-risk assessment: colorectal; version 3.2019. 25p.

[19] E. Vakiani, R. Yaeger, S. Brooke, Y. Zhou, D.S. Klimstra, J. Shia, Immunohistochemical detection of the BRAF V600E mutant protein in colorectal neoplasms, Appl. Immunohistochem. Mol. Morphol. 23 (6) (2015) 438–443, doi:10.1097/PAI.0000000000000116.

[20] D. Capper, A. Voigt, G. Bozukova, A. Ahadova, P. Kickingereder, A. von Deimling, et al., BRAF V600E-specific immunohistochemistry for the exclusion of Lynch syndrome in MSI-H colorectal cancer, Int. J. Cancer 133 (7) (2013) 1624–1630, doi:10.1002/ijc.28183.

[21] P. Lochhead, A. Kuchiba, Y. Imamura, X. Liao, M. Yamauchi, R. Nishihara, et al., Microsatellite instability and BRAF mutation testing in colorectal cancer prognostication, J. Natl. Cancer Inst. 105 (2013) 1151–1166, doi:10.1093/jnci/djt173.

[22] S. Ogino, K. Nosho, G.J. Kirkner, T. Kawasaki, J.A. Meyerhardt, M. Loda, et al., CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer, Gut 58 (1) (2009) 90–96, doi:10.1136/gut.2008.155473.

[23] T.T. Seppälä, J.P. Böhm, M. Friman, L. Lahtinen, V.M. Väyrynen, T.K. Liipo, et al., Combination of microsatellite instability and BRAF mutation status for subtyping colorectal cancer, Br. J. Cancer 112 (12) (2015) 1966–1975 Jun 9, doi:10.1038/bjc.2015.160.

[24] S. Natsume, T. Yamaguchi, M. Takao, T. Iijima, R. Wakaume, K. Takahashi, et al., Clinicopathological and molecular differences between right-sided and left-sided colorectal cancer in Japanese patients, Jpn. J. Clin. Oncol. 48 (7) (2018) 609–618 Jul 1, doi:10.1093/jjco/hyy069.

[25] M.C. Hanna, C. Go, C. Roden, R.T. Jones, P. Pochanard, A.Y. Javed, et al., Colorectal cancers from distinct ancestral populations show variations in BRAF mutation frequency, PLoS One 8 (2013) e74950, doi:10.1371/journal.pone.0074950.

[26] T. Qiu, H. Lu, L. Guo, W. Huang, Y. Ling, L. Shan, et al., Detection of BRAF mutation in Chinese tumor patients using a highly sensitive antibody immunohistochemistry assay, Sci. Rep. 5 (2015) 9211, doi:10.1038/srep09211.

[27] G.A. Yanus, A.V. Belyaeva, A.O. Ivantsov, Kuligina ESh, E.N. Suspitsin, N.V. Mitiushkina, et al., Pattern of clinically relevant mutations in consecutive series of Russian colorectal cancer patients, Med. Oncol. 30 (2013) 686, doi:10.1007/s12032-013-0686-5.

[28] L.S. Rozek, C.M. Herron, J.K. Greenson, V. Moreno, G. Capella, G. Rennert, et al., Smoking, gender, and ethnicity predict somatic BRAF mutations in colorectal cancer, Cancer Epidemiol. Biomarkers Prev. 19 (2010) 838–843, doi:10.1158/1055-9965.EPI-09-1112.

[29] J.X. Ye, Y. Liu, Y. Qin, H.H. Zhong, W.N. Yi, X.Y. Shi, KRAS and BRAF gene mutations and DNA mismatch repair status in Chinese colorectal carcinoma patients, World J. Gastroenterol. 21 (5) (2015) 1595–1605 Feb 7, doi:10.3748/wjg.v21.i5.1595.

[30] W. Li, T. Qiu, W. Zhi, S. Shi, S. Zou, Y. Ling, et al., Colorectal carcinomas with KRAS codon 12 mutation are associated with more advanced tumor stages, BMC Cancer 15 (2015) 340, doi:10.1186/s12885-015-1345-3.

[31] E. Capel, J.F. Fléjou, R. Hamelin, Assessment of MLH1 promoter methylation in relation to gene expression requires specific analysis, Oncogene 26 (54) (2007) 7596–7600 Nov 29, doi:10.1038/sj.onc.1210581.

[32] M.1. Gausachs, P. Mur, J. Corral, M. Pineda, S. González, L. Benito, et al., MLH1 promoter hypermethylation in the analytical algorithm of Lynch syndrome: a cost-effectiveness study, Eur. J. Hum. Genet. 20 (7) (2012) 762–768, doi:10.1038/ejhg.2011.277.

[33] S. Richards, N. Aziz, S. Bale, D. Bick, S. Das, J. Gastier-Foster, et al., Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology, Genet. Med. 17 (5) (2015) 405–424, doi:10.1038/gim.2015.30.

[34] D.R. English, J.P. Young, J.A. Simpson, M.A. Jenkins, M.C. Southey, M.D. Walsh, et al., Ethnicity and risk for colorectal cancers showing somatic BRAF V600E mutation or CpG Island Methylator Phenotype, Cancer Epidemiol. Biomarkers Prev. 17 (7) (2008) 1774–1780, doi:10.1158/1055-9965.EPI-08-0091.

[35] M. Hewish, C.J. Lord, S.A. Martin, D. Cunningham, A. Ashworth, Mismatch repair deficient colorectal cancer in the era of personalized treatment, Nat. Rev. Clin. Oncol. 7 (2010) 197–208, doi:10.1038/nrclinonc.2010.18.

[36] L. Moreira, J. Muñoz, M. Cuatrecasas, I. Quintanilla, M.L. Leoz, S. Carballal, et al., Prevalence of somatic MutL Homolog 1 Promoter Hypermethylation in Lynch Syndrome Colorectal Cancer, Cancer 121 (9) (2015) 1395–1404, doi:10.1002/cncr.29190.