**BRAF and MLH1 Analysis Algorithm for the Evaluation of Lynch Syndrome Risk in Colorectal Carcinoma Patients: Evidence-Based Data from the Analysis of 100 Consecutive Cases**

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**Abstract:** Several causes may lead to CRC, either extrinsic (sporadic forms) or genetic (hereditary forms), such as Lynch syndrome (LS). Most sporadic deficient mismatch repair (dMMR) CRC cases are characterized by the methylation of the MLH1 promoter gene and/or BRAF gene mutations. Usually, the first test performed is the mismatch repair deficiency analysis. If a tumor shows a dMMR, BRAF mutations and then the MLH1 promoter methylation status have to be assessed, according to the ACG/ASCO screening algorithm. In this study, 100 consecutive formalin-fixed and paraffin-embedded samples of dMMR CRC were analyzed for both BRAF mutations and MLH1 promoter methylation. A total of 47 (47%) samples were BRAF p.V600E mutated, while MLH1 promoter methylation was found in 77 cases (77.0%). The pipeline “BRAF-followed-by-MLH1-analysis” led to a total of 153 tests, while the sequence “MLH1-followed-by-BRAF-analysis” resulted in a total of 123 tests. This study highlights the importance of performing MLH1 analysis in LS screening of BRAF-WT specimens before addressing patients to genetic counseling. We show that MLH1 analysis performs better as a first-line test in the screening of patients with LS risk than first-line BRAF analysis. Our data indicate that analyzing MLH1 methylation as a first-line test is more cost-effective.

**Keywords:** MLH1; BRAF; colorectal cancer; Lynch syndrome; mismatch repair; microsatellite instability
1. Introduction

Colorectal cancer (CRC) is the third leading cause of cancer death in the world and its incidence is increasing in many countries [1–4] (Figure 1).

In the US, 149,500 new cases and 52,980 deaths were estimated in 2021 [5]. Several causes may lead to CRC, both extrinsic (sporadic forms) and genetic (hereditary forms), such as Lynch syndrome—previously called hereditary non-polyposis colorectal cancer (HNPCC). Lynch syndrome is one of the most common hereditary cancer predisposition syndromes and is defined by germline alterations in the DNA mismatch repair (MMR) system [6].

During the replication processes, mutations may occur frequently and some (but not all of them) are corrected by the proofreading function of the DNA polymerases. By decreasing the number of spontaneous mutations, the MMR system increases the overall fidelity of replication [7]. The MMR system is composed of several proteins, including MSH2, MSH6, MLH1, and PMS2.

A heterozygous alteration in one of these genes is insufficient to give a mismatch repair deficiency (dMMR). Inactivating mutations in one of the MMR genes or 3′-deletion of EPCAM (the epithelial-cell adhesion molecule gene) lead to the malfunction of repair mechanisms and then to genomic instability. MMRd is evident mainly in microsatellite regions, short tandem repeats of nucleotides particularly susceptible to DNA polymerase slippage [8–11].

The National Comprehensive Cancer Network (NCCN) 2019 guidelines recommended deficient mismatch repair (dMMR) analysis to be performed in patients with CRC, for clinical management and to identify Lynch syndrome [12]. In fact, dMMR/high-microsatellite instability (MSI-H) in somatic tumor cells is associated with better outcomes in CRC patients. Whether 5-FU adjuvant chemotherapy is appropriate to treat MSI-H/dMMR tumors is still controversial [13–15]. Cases with inactivated MLH1 gene due to promoter methylation represents approximately 90% of sporadic dMMR/MSI-H CRC [16,17]. BRAF mutations rarely occur in the presence of germline mutations in MMR genes (1.6%), while the mutation is present in approximately 50% of sporadic CRC [18,19]. It is then clear that the absence of oncogenic BRAF variants in CRC is not sufficient to suspect Lynch syndrome. Moreover, some patients with Lynch syndrome may have a sporadic form of CRC, thus BRAF mutation analysis alone is not sufficient to discriminate between hereditary (Lynch syndrome) and sporadic dMMR/MSI-H CRC [6,20].

A screening algorithm is recommended for all CRC specimens to select patients for germline testing to diagnose Lynch syndrome (American College of Gastroenterology, and

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**Figure 1.** Estimated age-standardized incidence rates (World) in 2020, colorectal carcinoma, both sexes, all ages. Available at: https://gco.iarc.fr/today (accessed on 17 December 2021) [2–4].
the American Society of Clinical Oncology (2015)) [21–23]. The first step consists of dMMR analysis, usually performed with immunohistochemistry (IHC) for MLH1, MSH2, MSH6, and PMS2 proteins. Positive immunostaining for all proteins is compatible with an intact MMR system, and no additional analyses are needed. Samples with immunohistochemical loss of MMR proteins must then be analyzed for the **BRAF** mutation. The ACG/ASCO screening algorithm suggests performing **BRAF** analysis in CRC with the absence of **MLH1** protein staining, followed by **MLH1** methylation analysis if **BRAF** p.V600E mutation is not detected [21] (Figure 2).

**Figure 2.** Algorithm for **MLH1** methylation and **BRAF** analyses in dMMR/MSI-H CRC. Modified from Chen W. et al. [24].

If an oncogenic **BRAF** mutation is identified, the tumor is considered sporadic. If no **BRAF** mutations are identified, **MLH1** promoter methylation analysis must be performed to infer whether the lack of **MLH1** protein expression is due to epigenetic causes (a somatic event) pointing to the sporadic origin of the tumor or putative inactivating mutations (a genetic event) pointing to Lynch syndrome [24].

If neither oncogenic **BRAF** mutations nor **MLH1** promoter methylation are found, genetic counseling and germline MMR gene analysis are recommended to diagnose Lynch syndrome.

While the ACG/ASCO screening algorithm is widely accepted, it is not clear whether the order in which **BRAF** and **MLH1** promoter methylation are tested makes any difference. The aim of this study was to define whether it is more cost-effective to analyze dMMR/MSI-H CRC first for **BRAF** and then for **MLH1** promoter methylation or vice versa, first for **MLH1** promoter methylation and then for **BRAF** [21].

2. Materials and Methods

2.1. Case Selection

A total of 100 consecutive formalin-fixed and paraffin-embedded (FFPE) samples of colorectal carcinoma (CRC) with a deficient mismatch repair (dMMR) system, with loss of MLH1/PMS2, were analyzed for **BRAF** and **MLH1** molecular status during the period 2018–2021 at the solid tumor molecular pathology laboratory of the University of Bologna.
medical center. The MMR status of all cases had been previously evaluated by IHC for MSH2, MSH6, MLH1, and PMS2 using an automatic immunostainer (BenchMark ULTRA System—Roche Diagnostics, Mannheim, Germany), following established guidelines [25].

2.2. DNA Extraction and Quantification

DNA was extracted from 2–4 unstained 10 µm thick sections. The area of interest was selected by a pathologist on a final hematoxylin and eosin (H&E) section. DNA extraction was performed using the QuickExtract FFPE DNA Extraction Kit (Epicentre, Madison, WI, USA), according to the manufacturer’s instructions. DNA concentration was calculated using the Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. BRAF Analysis

BRAF analysis was performed using a laboratory-developed next-generation sequencing (NGS) panel designed to analyze common hot-spot mutations in solid tumors, commercially available real-time PCR, or pyrosequencing kits for BRAF analysis, as follows.

NGS was performed using approximately 50 ng of input DNA, and libraries were set up using a laboratory-developed next-generation multi-gene panel as previously described [26]. The entire BRAF exons 11 and 15 were sequenced using this multi-gene panel. The analysis was performed with the Gene Studio S5 sequencer (Thermo Fisher Scientific). The sequences were evaluated using the Ion Reporter tools (v5.16, Thermo Fisher Scientific) and the Integrative Genome Viewer tool (v5.11.2, https://software.broadinstitute.org/software/igv/, accessed on 7 January 2021).

Real-time PCR was performed using the EasyPGX BRAF kit (Diatech pharmagenetics, Jesi, AN, Italy), according to the manufacturer’s protocol and results analyzed using the EasyPGX Analysis Software (v4.0.5, Diatech pharmagenetics, Jesi, Italy). Briefly, approximately 20 ng of input DNA was used in each one of the 4 reactions, allowing the detection of 4 different mutations in BRAF codon 600: p.V600E (c.1799T>A, c.1799_1800delinsAA), p.V600K (c.1798_1799delinsAA), p.V600D/p.V600R (c.1799_1800delinsAT, c.1798_1799delinsAG—not distinguishable between them).

Pyrosequencing was performed using a BRAF pyro kit, allowing the detection of mutations in codons 599, 600, and 601 of the BRAF gene. A minimum input of 10ng was used to perform the analysis. The results were analyzed by the PyroMark Q24 software. The pyrosequencing analysis was run on the PyroMark Q24 system. The analytical sensitivity was 2–11% of mutated DNA on wild-type (WT) genomic DNA, according to the detected mutation.

2.4. MLH1 Promoter Methylation Analysis

MLH1 promoter methylation was evaluated with the PyroMark® Q24 CpG MLH1 kit (Qiagen). DNA was converted using the EZ DNA Methylation-Lightning kit (Zymo Research Corporation). Pyrosequencing was run on the PyroMark Q24 system. Results were analyzed by the PyroMark Q24 software. The PyroMark® Q24 CpG MLH1 kit allows the detection of the methylation status of 5 CpG islands in the MLH1 promoter region, from nucleotide c.1-209 to c.1-181. A sample was considered methylated when the percentage of methylation exceeded 10%, according to the manufacturer’s protocol.

3. Results

All 100 samples were successfully analyzed for BRAF and MLH1 promoter methylation.

3.1. BRAF Analysis

BRAF mutations were found in 47 of 100 cases (47.0%), all were BRAF p.V600E substitutions (Table 1). No BRAF mutations other than p.V600E were identified. Interestingly, all BRAF-mutated specimens (47/47, 100%) were also MLH1 methylated (MET), while no samples were BRAF mutated (MUT)/MLH1 unmethylated (UMET) (Table 1, Figure 3). Of the 53 cases without the BRAF mutation, 30 of them (56.6%) were MLH1 promoter
methylated, while the remaining 23 cases (43.4%) were \textit{BRAF WT/MLH1 UMET} (Table 1, Figure 3).

\textbf{Table 1.} Molecular profile according to \textit{BRAF} mutational status.

<table>
<thead>
<tr>
<th>dMMR/MSI-H Samples</th>
<th>N° (Frequency)</th>
</tr>
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<tbody>
<tr>
<td>\textit{BRAF MUT}</td>
<td>47 (47.0%)</td>
</tr>
<tr>
<td>\textit{BRAF MUT &amp; MLH1 MET}</td>
<td>47 (100.0%)</td>
</tr>
<tr>
<td>\textit{BRAF MUT &amp; MLH1 UMET}</td>
<td>0 (/)</td>
</tr>
<tr>
<td>\textit{BRAF WT}</td>
<td>53 (53.0%)</td>
</tr>
<tr>
<td>\textit{BRAF WT &amp; MLH1 MET}</td>
<td>30 (56.6%)</td>
</tr>
<tr>
<td>\textit{BRAF WT &amp; MLH1 UMET}</td>
<td>23 (43.4)</td>
</tr>
<tr>
<td>\textbf{TOTAL}</td>
<td>100</td>
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</tbody>
</table>

MUT: Mutated; WT: Wild-type; MET: Methylated; UMET: Unmethylated; \textit{BRAF MUT}: \textit{BRAF} mutated—all these cases were \textit{BRAF p.V600E}.

\textbf{Figure 3.} Number of samples according to \textit{BRAF} and \textit{MLH1} molecular status. MET: Methylated; UMET: Unmethylated; WT: Wild-type; MUT: Mutated.

\textbf{3.2. MLH1 Methylation Analysis}

The \textit{MLH1} promoter region was found methylated in 77 of 100 samples (77.0%) (Table 2). Of these 77 methylated specimens, 30 (39.0%) also harbored a \textit{BRAF p.V600E} mutation (\textit{MLH1 MET/BRAF MUT}), while the remaining 47 specimens (61.0%) were \textit{MLH1 MET/BRAF WT} (Table 2). All the 23 \textit{MLH1} unmethylated specimens were also \textit{BRAF WT} (\textit{MLH1 UMET/BRAF WT}), thus no \textit{MLH1 UMET/BRAF MUT} specimens were identified (Table 2, Figure 3). Of these 23 patients, 9 were tested for germline mutations, and 5 harbored a pathogenic alteration suggestive of Lynch syndrome.

\textbf{Table 2.} Molecular profile according to \textit{MLH1} methylation status.

<table>
<thead>
<tr>
<th>dMMR/MSI-H Samples</th>
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<tr>
<td>\textit{MLH1 MET}</td>
<td>77 (77.0%)</td>
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</tr>
<tr>
<td>\textit{MLH1 MET &amp; BRAF MUT}</td>
<td>47 (61%)</td>
</tr>
<tr>
<td>\textit{MLH1 UMET}</td>
<td>23 (23.0%)</td>
</tr>
<tr>
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<tr>
<td>\textbf{TOTAL}</td>
<td>100</td>
</tr>
</tbody>
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MUT: Mutated; WT: Wild-type; MET: Methylated; UMET: Unmethylated; \textit{BRAF MUT}: \textit{BRAF} mutated—all these cases were \textit{BRAF p.V600E}.
Following the ACG/ASCO algorithm, currently used to screen for Lynch syndrome dMMR/MSI-H CRC patients, 53 of 100 (53.0%) specimens (i.e., samples without the \( \textit{BRAF} \) mutation) would have needed a “second-level” analysis for \( \textit{MLH1} \) promoter methylation, for a total of 153 molecular tests (100 samples analyzed for \( \textit{BRAF} \) and 53 analyzed also for \( \textit{MLH1} \) methylation status) (Figure 4). On the contrary, starting the dMMR/MSI-H CRC analysis with an evaluation of the \( \textit{MLH1} \) methylation status would lead to performing a “second-level” analysis for the \( \textit{BRAF} \) alteration in only 23 of 100 (23.0%) samples (i.e., those samples without \( \textit{MLH1} \) promoter methylation), for a total of 123 molecular tests (100 samples analyzed for \( \textit{MLH1} \) methylation status and 23 specimens analyzed also for \( \textit{BRAF} \) mutational status) (Figure 4).

Table 2. Molecular profile according to \( \textit{MLH1} \) methylation status.

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<th>dMMR/MSI-H samples</th>
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<tr>
<td>( \textit{MLH1} ) MET</td>
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<td>( \textit{MLH1} ) MET &amp; ( \textit{BRAF} ) WT</td>
<td>30 (39.0%)</td>
</tr>
<tr>
<td>( \textit{MLH1} ) MET &amp; ( \textit{BRAF} ) MUT</td>
<td>47 (61%)</td>
</tr>
<tr>
<td>( \textit{MLH1} ) UMET</td>
<td>23 (23.0%)</td>
</tr>
<tr>
<td>( \textit{MLH1} ) UMET &amp; ( \textit{BRAF} ) WT</td>
<td>23 (100%)</td>
</tr>
<tr>
<td>( \textit{MLH1} ) UMET &amp; ( \textit{BRAF} ) MUT</td>
<td>0 (/)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>100</strong></td>
</tr>
</tbody>
</table>

MUT: Mutated; WT: Wild-type; MET: Methylated; UMET: Unmethylated; BRAF: \( \textit{BRAF} \) analyses.

**4. Discussion**

Considering the clinical relevance of Lynch syndrome diagnosis, routine CRC screening for the disease is of absolute importance [21].

To date, recommendations for the evaluation of Lynch syndrome’s risk mandate that the loss of \( \textit{MLH1} \) IHC in patients with colorectal cancer should be followed by \( \textit{BRAF} \)-mutation analysis. If no \( \textit{BRAF} \) alteration is found, promoter methylation analysis of the \( \textit{MLH1} \) promoter region is then performed to discriminate sporadic cases from those potentially associated with Lynch syndrome [21,27].

In patients with \( \textit{BRAF} \) wild-type and \( \textit{MLH1} \) unmethylated CRC, genetic counseling is recommended to identify Lynch syndrome patients [28]. In the screening algorithm proposed by Chen et al. [24], the first step for dMMR/MSI-H CRC evaluation is \( \textit{BRAF} \) p.V600E mutational analysis, and when no \( \textit{BRAF} \) mutation is identified \( \textit{MLH1} \) promoter methylation is tested.

In our cohort of CRC specimens following the classical scheme—\( \textit{BRAF} \) followed by \( \textit{MLH1} \) analysis—we performed a total of 153 tests (100 \( \textit{BRAF} \) and 53 \( \textit{MLH1} \)).

![Figure 4](image-url). Comparison of the total number of molecular tests for dMMR/MSI-H CRC: (a) first \( \textit{BRAF} \) and then \( \textit{MLH1} \) methylation analysis (Algorithm 1) vs. (b) performing first \( \textit{MLH1} \) methylation and then \( \textit{BRAF} \) analysis (Algorithm 2); (c) total number of tests following Algorithm 1 and Algorithm 2. MET: Methylated; MUT: Mutated; WT: Wild-type; UMET: Unmethylated; MLH1: \( \textit{MLH1} \) analyses; BRAF: \( \textit{BRAF} \) analyses.
Starting the evaluation with MLH1 methylation analysis would have allowed us to perform a total of only 123 tests (100 MLH1 and 23 BRAF), saving the laboratory 30 unnecessary tests for MLH1 out of a total of 53 dMMR/MSI-H BRAF-WT CRCs that needed MLH1 evaluation according to current guidelines. The frequency of BRAF mutation found in our cohort is in accordance with previously published data [24,29].

In a previous paper, Xiao and colleagues [30] compared four types of different screening approaches: (i) only BRAF testing; (ii) only MLH1 promoter region methylation testing; (iii) MLH1 testing and BRAF; and (iv) MLH1 methylation testing and revised Bethesda criteria. From their data, the best approach for Lynch syndrome pre-screening is the combination of MLH1 methylation and BRAF testing, reducing the rate of unnecessary referral to genetic counseling.

Newton and colleagues demonstrated that performing only BRAF testing has a lower specificity (66%) in detecting possible Lynch syndrome patients than performing only MLH1 analyses (88%) [31]. These data are also in accordance with those by Adar and colleagues: in CRC with loss of MLH1 staining, using BRAF as the only test has a 31% rate of sending to genetic counseling, compared with MLH1 methylation testing alone, which has a rate of 13.5% (2.3-fold lower) [32]. Moreover, Adar and colleagues stated that a hybrid approach further reduces the referral rate for genetic tests [32].

Our data confirm that performing MLH1 methylation analysis as the first step, followed by BRAF mutation analysis would decrease by 56.6% ([(23 MLH1 UMET—53 BRAF WT)/53 BRAF WT] × 100) the number of “second-level” tests performed by the laboratory (Figure 4). As we have not observed MLH1-UMET/BRAF-MUT specimens in a series of 100 consecutive dMMR/H-MSI CRCs consideration should be given to the possibility of using MLH1 methylation analysis as a stand-alone test for Lynch syndrome risk screening, as is the case for endometrial carcinoma [33]. However, it should be considered that some very rare cases of CRC with the BRAF mutation but without MLH1 methylation have been reported in the literature [31,32]. From the point of view of molecular epidemiology, the relationship between BRAF status (BRAF WT vs. BRAF p.V600E) and MLH1 promoter methylation status (MLH1 MET vs. MLH1 UMET) in the dMMR/MSI-H CRC population of a given geographic region is currently unclear. A reasonable approach would be to first define the proportion of dMMR/MSI-H CRC cases that are: (i) BRAF MUT and MLH1 MET, (ii) BRAF MUT and MLH1 UMET, (iii) BRAF WT and MLH1 MET, and (iv) BRAF WT and MLH1 UMET, in a given medical center. On the basis of these data, the molecular pathology laboratory can then adopt the most cost-effective protocol: either to test first for BRAF and then perform MLH1 methylation analysis (Algorithm 1 of Figures 2 and 4) or to perform first MLH1 methylation evaluation and then BRAF analysis (Algorithm 2 of Figures 4 and 5).

It should be further considered that even larger studies underline the superiority of MLH1 promoter hypermethylation over BRAF testing; somatic analysis of mismatch repair genes helps to identify patients with MLH1 IHC loss but without germline alterations [34]. Even if the MLH1 methylation test by pyrosequencing is more technically labor-intensive than BRAF analysis, in our medical center, it is clearly more convenient to use the second approach (Algorithm 2 of Figures 4 and 5). In a medical center where BRAF is the “first-level” analysis for Lynch syndrome risk screening of CRC patients it is crucial to perform MLH1 analysis of all BRAF-WT specimens, without directly addressing patients with BRAF-WT tumors to genetic counseling, as stated by current guidelines (https://www.nccn.org/professionals/physician_gls/pdf/genetics_colon.pdf, accessed date 21 January 2022).
Figure 5. Cost-effective algorithm for MLH1 promoter methylation and BRAF analyses in dMMR/MSI-H CRC at the University of Bologna medical center. The dashed arrow indicates the possible algorithm if MLH1 promoter methylation analysis is used as a stand-alone test for Lynch syndrome risk screening.

Considering that testing for BRAF p.V600E mutation using IHC is not sufficiently sensitive [25], one alternative approach could be testing the BRAF mutation status by IHC with BRAF p.V600E specific antibodies at the time of pathologic evaluation and immunohistochemical definition of the dMMR status, and then performing MLH1 promoter methylation analysis in all immunohistochemically BRAF p.V600E negative specimens.

In patients with metastatic CRCs, BRAF testing of CRC metastases can be integrated with the other biomarkers important for the clinical management of the patients, i.e., KRAS and NRAS mutational status, by analyzing all three genes using a next-generation-sequencing multi-gene panel [35].

5. Conclusions

Our study highlights the importance of not being limited to BRAF analysis in the selection of CRC patients to be addressed to genetic counseling for Lynch syndrome. The best approach should be chosen after the definition of the specific BRAF (BRAF WT vs. BRAF p.V600E) and MLH1 promoter methylation status (MLH1 MET vs. MLH1 UMET) in the dMMR/MSI-H CRC population referred to the medical center. MLH1 analysis as the first-line test followed by BRAF analysis may be more cost-effective than the current protocol that recommends BRAF analysis as the first-line test.

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Institutional Review Board Statement: All the experimental procedures were carried out in accordance with the general authorization to process personal data for scientific research purposes from “The Italian Data Protection Authority” (http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/2485392/, accessed on 7 January 2021). All information regarding the human material was managed using anonymous numerical codes and the study was carried out in accordance with the ethical principles of the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/, accessed on 27 August 2021). The study did not affect the clinical management of the involved patients’ samples. Follow-up information was not used for this study.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data are contained within the article.

Conflicts of Interest: Dario de Biase has received personal fees (as speaker bureau) from Boehringer Ingelheim, and Eli Lilly, unrelated to the current work.

References


