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Selection of patients with germline *MLH1* mutated Lynch syndrome by determination of *MLH1* methylation and *BRAF* mutation

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Abstract Lynch syndrome is one of the most common hereditary colorectal cancer (CRC) syndrome and is caused by germline mutations of *MLH1*, *MSH2* and more rarely *MSH6*, *PMS2*, *MLH3* genes. Whereas the absence of MSH2 protein is predictive of Lynch syndrome, it is not the case for the absence of MLH1 protein. The purpose of this study was to develop a sensitive and cost effective algorithm to select Lynch syndrome cases among patients with MLH1 immunohistochemical silencing. Eleven sporadic CRC and 16 Lynch syndrome cases with MLH1 protein abnormalities were selected. The *BRAF* c.1799T> A mutation (p.Val600Glu) was analyzed by direct sequencing after PCR amplification of exon 15. Methylation of *MLH1* promoter was determined by Methylation-Sensitive Single-Strand Conformation Analysis. In patients with Lynch syndrome, there was no *BRAF* mutation and only one case showed *MLH1* methylation (6%). In sporadic CRC, all cases were *MLH1* methylated (100%) and 8 out of 11 cases carried the above *BRAF* mutation (73%) whereas only 3 cases were *BRAF* wild type (27%). We propose the following algorithm: (1) no further molecular analysis should be performed for CRC exhibiting *MLH1* methylation and *BRAF* mutation, and these cases should be considered as sporadic CRC; (2) CRC with unmethylated *MLH1* and negative for *BRAF* mutation should be considered as Lynch syndrome; and (3) only a small fraction of CRC with *MLH1* promoter methylation but negative for *BRAF* mutation should be true Lynch syndrome patients. These potentially Lynch syndrome patients should be offered genetic counselling before searching for *MLH1* gene mutations.

Keywords *BRAF* gene · *BRAF* mutation · Germline mutations · Hereditary colorectal cancer · Lynch syndrome · *MLH1* gene · *MLH1* methylation

Abbreviations

CRC	Colorectal cancer
HNPCC	Hereditary nonpolyposis colorectal cancer
MMR	Mismatch repair
MSI	Microsatellite instability
MSI-H	MSI-high
IHC	Immunohistochemistry

Introduction

Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC) syndrome is one of the most common hereditary colon cancer syndrome, accounting for 3–6% of the total colorectal cancer burden [1], and is caused by germline mutations of mismatch repair (MMR) genes [2, 3]. *MLH1* and *MSH2* are the most commonly mutated MMR genes in HNPCC, with mutations in *MSH6* and *PMS2* being significantly less common and *MLH3* mutations very rare [3, 4]. Complete inactivation in any of the MMR genes result in genomic instability, most evident within repetitive mononucleotide or dinucleotide microsatellite DNA

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sequences which are particularly prone to replication errors [5]. The resulting microsatellite instability (MSI) is widely used as a prescreen in patients candidates for HNPCC according to standardised methodology, and tumours with a significant instability are referred to as MSI-high (MSI-H) [6]. Nevertheless, approximately 10–15% of all sporadic CRCs are also MSI-H, although these show no association with HNPCC [7]. These common MSI-H sporadic tumours do not result from a predisposing constitutive MMR gene mutation, and typically show loss of expression of MLH1 protein consecutive to mono- or bi-allelic hypermethylation of the gene promoter [8].

Sporadic MSI-H colorectal cancers and Lynch syndrome superficially resemble each other in that they are frequently located in the proximal colon and share morphological features such as mucin production and tumour-infiltrating lymphocytes [9]. However, they have been shown to differ in terms of demographics, molecular alterations and natural history [10–12]. These two subsets of MSI-H colorectal cancer need to be distinguished and investigated separately since the identification of Lynch syndrome warrants specific management policies with respect to genetic screening and surveillance measures in both the patients and their first-degree relatives.

The most effective strategy for the diagnosis of Lynch syndrome is the compilation of a thorough family history of colorectal cancer and fulfilment of the Amsterdam criteria [13, 14]. However, this information is often not available and about 20% of Lynch syndrome families with germline mutations do not meet these criteria [15]. Current laboratory testing algorithms for patients with suspected Lynch syndrome typically include a prescreen step, in which MSI testing and/or MMR protein immunohistochemistry (IHC) are performed on tumour tissue prior to more laborious and costly efforts aimed at identifying mutations in the responsible MMR genes. Patients whose tumours show loss of MSH2 expression almost invariably have an underlying *MSH2* or *MSH6* germline mutation [16, 17]. As mentioned above MSI-H CRC cancers with loss of MLH1 protein expression are more difficult to classify into familial versus sporadic CRC categories because the underlying germline *MLH1* mutation or methylation of the *MLH1* gene promoter cannot be distinguished a priori. Therefore, analysis of *MLH1* promoter methylation status can be a valuable mean to spare unnecessary genetic testing in patients with sporadic MSI-H tumours [18, 19]. However, as our results strongly suggest, this strategy is not sufficient because *MLH1* promoter methylation is not exclusive to sporadic CRC [20–24].

Recently, activating mutations in the *BRAF* protooncogene have been associated with MSI-H tumours in general [25], and subsequently with the sporadic subset in particular, providing a further avenue for distinction between familial and sporadic MSI-H tumours [26–29].

Here, we present the results of a comparative study where we determined the methylation status of *MLH1*, and the presence of *BRAF* mutations in both sporadic MSI-H cancers with loss of expression of MLH1 protein, and *MLH1* mutated HNPCC patients. The aim of the study was (1) to determine whether *MLH1* methylation and *BRAF* mutations might be used as negative predictors for HNPCC in patients with MSI-H tumours, and (2) to design a cost effective algorithm for the detection of *MLH1* mutated HNPCC patients.

Methods

Patients

Sixteen patients with Lynch syndrome harbouring an *MLH1* constitutive mutation were selected from an anonymised series investigated at the Institut Central des Hôpitaux Valaisans in Sion, Switzerland. In addition, eleven patients with sporadic MSI-H CRC and lacking of MLH1 protein expression were selected from a previously studied series after clinical genetic screening [19].

Immunohistochemistry

Immunohistochemical expression of MLH1 was investigated as follows for each slide, dewaxed paraffin sections were immunostained using the streptavidin–biotin peroxidase complex method. Four micrometre thick tissue sections were mounted on aminopropylmethoxysilane-coated glass slides, deparaffinized in xylol, taken through to absolute alcohol, and blocked for endogenous peroxidase with 1% hydrogen peroxide in methanol for 45 min. Slides were then heated in a microwave oven for 15 min in 10 mM citrate buffer pH 6.0. To reduce nonspecific binding, they were incubated in normal goat serum (Pel-Freez Biologicals, Rogers, Arkansas) diluted 1:30 in TBS for 10 min. Sections were then incubated for 30 min with the primary monoclonal antibodies for the gene products of *MLH1* (1:100, Pharmingen, Basel). Following the primary antibody incubation, the sections were incubated for 30 min with goat anti-mouse IgG (Sternberger, Baltimore, MD, USA) diluted 1:100 in NFDM/TBS, and with PAP-complex diluted 1:600 in NFDM/TBS. Peroxidase activity was revealed with 3,3′-diaminobenzidine as the chromogen, and the sections were counterstained with Mayer's acid-free hematoxylin. As a negative control, the monoclonal primary antibody was replaced by hybridoma supernatant of a similar isotype but without reactivity in the tissue examined.

MSI analysis

For MSI analysis, microdissection was performed after selection of tumour tissue by a pathologist (H.B.). DNA was extracted from tumour cells and from normal mucosa from two different blocks to avoid contamination. Purified DNA was amplified by PCR, using the reference panel of microsatellite primers recommended for colorectal cancer by the National Cancer Institute which includes the markers BAT25, BAT26, D5S346 (*APC*), D2S123 (-*MSH2*), and D17S250 (*p53*). The presence of additional bands in the PCR products from tumour DNA that were not observed in DNA from corresponding normal tissue was scored as unstable at that particular locus. Tumour samples were classified as reflecting high-frequency microsatellite instability (MSI-H) when instability was observed for 2 or more of the loci screened, low-frequency microsatellite instability when less than 2 of the loci screened were unstable, or microsatellite stability, when stability was present at all the loci tested.

MLH1 methylation analysis

Methylation was by Methylation-Sensitive Single-Strand Conformation Analysis [30]. After deparaffinization and staining in 0.1% toluidine blue, histologically selected areas in tissue sections were manually microdissected. Only the tumour cells were retained and final histological control before collection of the tumour cells confirmed that contamination with other cells was negligible. Extracted DNA was modified with sodium bisulfite. A 178-bp fragment of the *MLH1* gene promoter was amplified by nested PCR using the following primers: FW 5′-GATTTTTTAAG GTTAAGAG-3′ and RV 5′-ATAAAACCCTATACCTA ATC-3′ for the outer PCR and FW 5′-TTTTTAGGAGTGA AGGAG-3′ and RV 5′-AAACCCTATACCTAATCTAT C-3′ for the inner PCR amplification. The outer PCR amplification was performed with 2 µl of modified DNA in a total volume of 20 µl for 40 cycles. Twenty cycles were performed for the inner PCR. Amplification products were confirmed by visualization on a 2% agarose gel. Single-strand conformation analysis was performed as previously described [30]. The percentage of methylated alleles was semiquantitatively estimated by comparing the intensity of the methylated and unmethylated bands.

Detection of *BRAF* V600E mutation

Genomic DNA was extracted from fixed materials using the DNeasy Tissue Kit (Qiagen, Hilden, Germany). DNA was eluted with 60 µL of elution buffer and kept at −20°C. The most common T1799A transversion mutation (*BRAF* V600E) was studied by direct sequencing after PCR amplification of exon 15 of the *BRAF* gene. DNA was amplified using the following primers: forward 5′-TCTTC ATAATGCTTGCTCTGATAG-3′; reverse 5′-TGGAAAA ATAGCCTCAATTCTTAC-3′. Sequencing with the internal primer 5′-TCTACTGTTTTCCTTTACTTACT-3′ was performed colorimetrically using the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3770 genetic analyzer (AppliedBiosystems, Foster City, CA, USA).

Germline mutation analysis

Complete screening for both point mutations and gross deletions in the *MLH1*gene (NM 251.2) was performed on genomic DNA from each proband. Exons and adjacent splice junctions were amplified by PCR and both DNA strands were sequenced. To standardize bidirectional sequencing, M13-21 and M13REV primers were added as tails at either primer of the pair corresponding to a specific exon. Genomic rearrangements, mostly as large deletions, were searched for using multiplex ligation-dependent probe amplification (MLPA, MRC-Holland). Mutation nomenclature follows HGVS guidelines (www.hgvs.org) with number one corresponding to "A" of the initiating translation codon. The presence of a pathogenic germline mutation in the proband was confirmed from a second sample of peripheral blood.

Results

The results are summarised in Table 1. All cases (100%) of sporadic MSI-H MLH1 silenced CRC were *MLH1* methylated. Eight out of 11 cases carried the *BRAF* mutation (73%) whereas 3 cases were *BRAF* wild type (27%). In *MLH1* mutated HNPCC patients, one case showed *MLH1* methylation (6%) and none of the cases was *BRAF* mutated (0%). Sporadic MLH1 silenced CRC and *MLH1* mutated HNPCC were subdivided into 4 groups according to their *MLH1* et *BRAF* status: Group 1 of sporadic CRC: 8/11 (73%) presented with *BRAF* mutation and *MLH1* methylation; Group 2 of sporadic CRC: 3/11 (27%) presented with *BRAF* wild type and *MLH1* methylation; Group 3 of HNPCC: 15/16 (94%) harboured a *BRAF* wild type and were *MLH1* unmethylated; Goup 4 of HNPCC: 1/16 (6%) were *BRAF* wild type and *MLH1* methylated.

When we added all sporadic MLH1 silenced CRC and *MLH1* mutated Lynch syndrome we found that 4 cases (15%) out of 27 MSI-H CRC presented with both BRAF wild type and *MLH1* methylation gene.

Table 1 *MLH1* methylation and *BRAF* mutation in sporadic *MLH1* negative CRC and *MLH1* mutated HNPCC cases

	MLH metlylated	*MLH* unmethylated	*BRAF* mutated	*BRAF* wild type
Sporadic CRC	11/11 (100%)	0/11 (0%)	8/11 (73%)	3/11 (27%)
HNPCC	1/16 (6%)	15/16 (94%)	0/16 (0%)	16/16 (100%)

Discussion

Germline mutations in mismatch repair (MMR) genes affecting mainly *MSH2* and *MLH1* cause susceptibility to Lynch syndrome or HNPCC, a dominant inherited disorder accounting for approximately 2–5% of all cases of CRC [2, 31–33]. The identification of patients with a pathogenic MMR mutation is a major issue because morbidity and mortality from CRC can be reduced by early and intensive screening [1, 18, 34–36]. However, the diagnosis of Lynch syndrome is hampered by the lack of simple and specific diagnostic criteria. Because MSI resulting from defective MMR is a hallmark of tumours arising in the Lynch syndrome [1, 3], international criteria have been developed to combine clinical and molecular features to help identify patients at high risk of a diagnosis of Lynch syndrome [15]. Current recommendations include a prescreen phase by analysis of tumours for the presence of MSI and the absence of at least one MMR protein expression by immunohistochemistry (IHC) [6, 37, 38]. Results from IHC allow to target the relevant gene which needs to be extensively analyzed. In this context, available data show that an abnormal immunostaining of MSH2 and MSH6 proteins is most likely the result of germline mutations [16, 17]. MSI-H CRC with loss of MLH1 protein are more difficult to interpret and classify as MLH1 extinction is also observed in approximately 10–15% of sporadic CRC, as a result of epigenetic silencing of the *MLH1* gene by methylation of the promoter region [8]. The aim of this study was to evaluate strategy that would select more effectively *MLH1* mutated HNPCC patients. This is of major importance as the search for a causative germline mutation remains the most time-consuming and expensive step of the entire approach.

In a previously published study of a series of MSI-H CRC patients, we proposed a cost effective and time saving procedure for *MLH1* mutated Lynch syndrome cases detection algorithm [19]. Selection of MSI and IHC analysis represented the first step in Lynch syndrome detection and in cases of abnormal expression of MLH1 protein, the search for a *MLH1* methylation represented the second step. In the present study, we wished to improve the selection of *MLH1* mutated HNPCC patients by adding the search for *BRAF* mutation, and this was evaluated in a series of sporadic CRC negative for an *MLH1* mutation and familial HNPCC positive for an MLH1 mutation. In this report, we examined the prevalence of *MLH1* methylation in *MLH1* mutated HNPCC and sought to determine whether the presence of a *MLH1* methylator phenotype is informative to improve selection of true HNPCC patients. Overall, there was significantly less *MLH1* gene-promoter methylation in HNPCC patients when compared with sporadic cancers that were MSI-H. Only 6% of HNPCC cases were methylated in the *MLH1* promoter region, compared to all *MLH1* negative sporadic MSI-H cases. At 100%, the level of *MLH1* methylation in sporadic tumours was consistent with previous findings [8, 21, 22, 39]. It has been suggested that HNPCC cancers, although characterised by an MSI-H phenotype, show significantly less methylation than their sporadic counterparts, and by inference, are driven by an alternative mechanism acting as the 'second hit' required to inactivate the wild-type allele in HNPCC such as allelic loss [29, 40]. The important variability of methylation levels reported to be associated with *MLH1* in Lynch syndrome is likely to reflect selection of different CpG-rich regions of the promoter examined between studies [24, 41].

Recently, an oncogenic V600E (previously known as V599E) hotspot mutation in *BRAF*, a kinase encoding gene from the RAS/RAF/MAPK pathway, has been found in colorectal tumours that show MMR deficiency [25, 42, 43]. Moreover, it has been shown that these mutations occur almost exclusively in tumours located in the proximal colon and with hypermethylation of *MLH1*, the gene involved in the initial steps of development of these tumours [26, 28]. However, in more detailed analyses, *BRAF* mutations were not detected in those cases with or presumed to have a germline mutation in either *MLH1* or *MSH2* [28, 44]. In a recent study, Loughrey et al. [45], demonstrated the clinical validity and utility of V600E mutation testing in a familial cancer clinic setting. Since mutation in *BRAF* is present in the majority of tumours with hypermethylation of the *MLH1* promoter but not in cases with germline *MLH1* mutations, the combination of microsatellite instability testing, *MLH1* hypermethylation, and *BRAF* (V600E) mutation analysis best distinguishes sporadic CRC from Lynch syndrome. In our series reported in the present report, we have shown that *BRAF*-V600E mutation was absent in 100% of *MLH1* mutated HNPCC, and was detected in 73% of MSI-H *MLH1* negative sporadic colorectal tumours, thus absent in 27% of this group. In addition, our data indicated a significant correlation

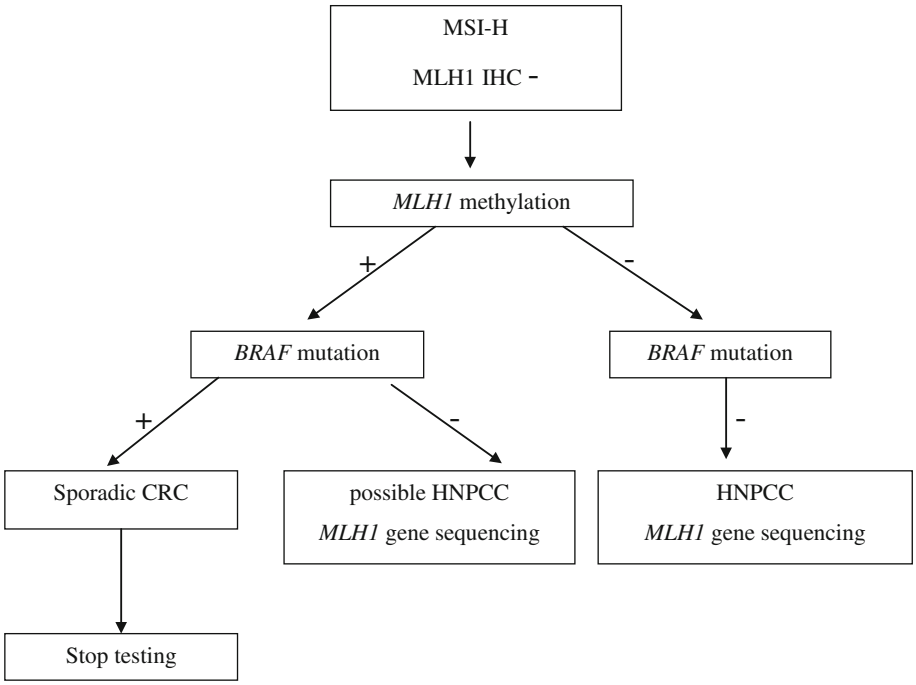

Fig. 1 Algorithm for investigating HNPCC patients

between a *BRAF* mutation and *MLH1* methylation in sporadic MSI-H MLH1 negative CRC and a significant correlation of a *BRAF* wild type and *MLH1* unmethylation in Lynch syndrome patients, as observed by others [29, 43, 46]. Overall, tumours that have the *BRAF* V600E mutation and exhibit *MLH1* promoter hypermethylation are almost certainly sporadic, whereas tumours that show neither are most likely inherited.

Although the above strategy allows to classify 85% of MSI-H CRC into HNPCC and sporadic variants, HNPCC cases with *MLH1* methylation and sporadic CRC cases without mutation of *BRAF* gene remain more difficult to classify. Nevertheless, this group represented only 15% of all MSI-H CRCs studied. This strategy has helped tightening the analysis on a small group of cancers with unclear genetic profile and for which a genetic counselling and sequencing *MLH1* mutation should be offered.

On the basis of our results we recommend the incorporation of *BRAF* V600E mutation and *MLH1* methylation testing into the laboratory algorithm for pre-screening patients with suspected HNPCC, whose CRCs show loss of expression of MLH1 protein. In summary, our data suggest the following algorithm (Fig. 1): (1) no further molecular analysis should be performed in cases of colorectal tumours with *MLH1* methylation and *BRAF* mutation and these cases should be considered as sporadic CRC; (2) colorectal tumours with unmethylated *MLH1* and negative for *BRAF* mutation should be considered as Lynch syndrome; (3) among CRC with *MLH1* promoter methylation but negative for *BRAF* mutation, only a subset of patients are expected to be true Lynch syndrome. Genetic counselling should be offered before searching for *MLH1* gene mutations. The proposed algorithm allows to spare time-consuming and costly efforts associated with unnecessary whole gene analysis.

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