

Incorporation of somatic *BRAF* mutation testing into an algorithm for the investigation of hereditary non-polyposis colorectal cancer

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Abstract Patients suspected on clinical grounds to have hereditary non-polyposis colorectal cancer (HNPCC) may be offered laboratory testing in order to confirm the diagnosis and to facilitate screening of pre-symptomatic family

members. Tumours from an affected family member are usually pre-screened for microsatellite instability (MSI) and/or loss of immunohistochemical expression of mismatch repair (MMR) genes prior to germline MMR gene mutation testing. The efficiency of this triage process is compromised by the more frequent occurrence of sporadic colorectal cancer (CRC) showing high levels of MSI (MSI-H) due to epigenetic loss of *MLH1* expression. Somatic *BRAF* mutations, most frequently V600E, have been described in a significant proportion of sporadic MSI-H CRC but not in HNPCC-associated cancers. *BRAF* mutation testing has therefore been proposed as a means to more definitively identify and exclude sporadic MSI-H CRC cases from germline MMR gene testing. However, the clinical validity and utility of this approach have not been previously evaluated in a familial cancer clinic setting. Testing for the V600E mutation was performed on MSI-H CRC samples from 68 individuals referred for laboratory investigation of suspected HNPCC. The V600E mutation was identified in 17 of 40 (42%) tumours showing loss of *MLH1* protein expression by immunohistochemistry but in none of the 28 tumours that exhibited loss of *MSH2* expression ($P < 0.001$). The assay was negative in all patients with an identified germline MMR gene mutation. Although biased by the fact that germline testing was not pursued beyond direct sequencing in many cases lacking a high clinical index of suspicion of HNPCC, *BRAF* V600E detection was therefore considered to be 100% specific and 48% sensitive in detecting sporadic MSI-H CRC amongst those cases showing loss of *MLH1* protein expression, in a population of patients with MSI-H CRC and clinical features suggestive of HNPCC. Accordingly, we recommend the incorporation of *BRAF* V600E mutation testing into the laboratory algorithm for pre-screening patients with suspected HNPCC, whose CRCs show loss of expression of

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MLH1. In such tumours, the presence of a *BRAF* V600E mutation indicates the tumour is not related to HNPCC and that germline testing of *MLH1* in that individual is not warranted. We also recommend that in families where the clinical suspicion of HNPCC is high, germline testing should not be performed on an individual whose CRC harbours a somatic *BRAF* mutation, as this may compromise identification of the familial mutation.

Keywords Hereditary non-polyposis colorectal cancer · *BRAF* · Allele-specific PCR · Microsatellite instability

Abbreviations

HNPCC Hereditary non-polyposis colorectal cancer
 MSI Microsatellite instability
 MMR Mismatch repair
 CRC Colorectal cancer
 AS-PCR Allele-specific polymerase chain reaction

Introduction

Hereditary non-polyposis colorectal cancer (HNPCC), the commonest cause of familial colorectal cancer (CRC), is caused by germline mutations of mismatch repair (MMR) genes [1]. These MMR genes encode proteins that correct random errors, or mismatches, which occur during the process of normal DNA replication. *MLH1* and *MSH2* are the most commonly mutated MMR genes in HNPCC, with mutations in *MSH6* and *PMS2* significantly less common and *PMS1* and *MLH3* mutations very rare [2–4]. Germline mutations in any of the MMR genes result in genomic instability, most evident within repetitive mononucleotide or dinucleotide microsatellite DNA sequences which are particularly prone to replication errors [5, 6]. The resulting microsatellite instability (MSI) is assessed according to standardised methodology and tumours with a significant degree of instability are referred to as MSI-high (MSI-H) [7].

Approximately 15% of all sporadic CRCs are MSI-H and have no association with HNPCC [8]. The more common sporadic cases lack a predisposing germline MMR gene mutation and typically show loss of expression of MLH1 protein due to hypermethylation of the gene promoter [9]. Evidence suggests that sporadic MSI-H CRCs arise within a class of polyps with serrated architecture, known as sessile serrated polyps or adenomas, in contrast to HNPCC-related CRCs which arise within conventional adenomas [10–13].

In the absence of a proven germline MMR gene mutation, a clinical diagnosis of HNPCC requires fulfilment of

the Amsterdam II criteria, which include demonstration of a family history, early onset or the development of multiple colorectal or extra-colonic HNPCC-associated neoplasms [14]. Current laboratory testing algorithms for patients with suspected HNPCC typically include a triage step, in which MSI testing and/or MMR protein immunohistochemistry (IHC) are performed on tumour tissue prior to more laborious and expensive germline testing for mutations in the MMR genes. Patients with tumours that are microsatellite stable and/or demonstrate intact MMR protein expression by IHC generally do not proceed to germline MMR testing. Patients whose tumours show loss of MSH2 expression almost invariably have an underlying *MSH2* or *MSH6* germline mutation [15, 16].

Eligibility for laboratory investigation usually requires fulfilment of the revised Bethesda criteria, which also captures individuals under 60 years with tumours showing morphological features suggestive of a MSI-H phenotype [17]. Under these criteria, more individuals with sporadic MSI-H CRC showing loss of MLH1 protein expression will be identified and triaged to subsequent but fruitless germline screening as increased sensitivity has been traded for reduced specificity. Although some studies have identified subtle differences in tumour morphology, the features of HNPCC-associated CRC are largely shared with sporadic MSI-H CRC [15, 18–20]. A clinical need exists, therefore, to definitively identify these sporadic MSI-H cases to avoid unnecessary germline screening. Methods to detect *MLH1* promoter hypermethylation have been widely used in a research setting, however the relative technical difficulty of the methods employed has so far prevented the incorporation of this technique into routine clinical practice [21]. Moreover, some patients with a germline *MLH1* mutation inactivate the second, wild-type allele in the tumour by hypermethylation and thus the detection of methylation does not necessarily indicate that the tumour is sporadic [20, 22, 23].

BRAF, a member of the *RAF* gene family, encodes a cytoplasmic serine/threonine kinase. Following the identification of somatic *BRAF* mutations in a subset of CRC, it was recognised that these mutations were mostly associated with tumours displaying MSI [24, 25]. Subsequent studies demonstrated that the *BRAF* V600E mutation (formerly reported as V599E), caused by a T > A transversion at nucleotide position 1799 (c.1799T > A), accounts for over 90% of *BRAF* mutations in CRC and is found in 31–83% of sporadic MSI-H CRC cases, but is extremely rare in tumours associated with HNPCC [23, 25–31]. No study has, as yet, reported any somatic *BRAF* mutation in an individual with a proven pathogenic germline MMR gene mutation.

Testing for the *BRAF* V600E mutation has been postulated, by several groups, as a potentially effective means of

excluding a diagnosis of HNPCC, thereby obviating the need for germline MMR testing [28, 29]. The clinical application of this approach, however, has not been evaluated in the intended target population of patients referred for clinical laboratory testing from a familial cancer clinic. Accordingly, the aim of this study was to determine the clinical validity and utility of incorporating *BRAF* mutation testing into the laboratory testing algorithm for patients with suspected HNPCC.

Materials and methods

Patients

All cases were referred to the Department of Pathology at the Peter MacCallum Cancer Centre, Melbourne, Australia for laboratory investigation of suspected HNPCC. From a consecutive series of over 500 cases referred between 1998 and 2004, CRC tumours from 68 patients were identified that were MSI-H or displayed abnormal MMR protein IHC, and in which tissue was available for DNA extraction. Where possible, each case was classified by fulfilment of Amsterdam II or revised Bethesda criteria by examination of clinical data and all had pathology review, MLH1, MSH2 and MSH6 IHC and MSI testing performed as part of the routine laboratory investigation of suspected HNPCC. All work related to this study was approved by the ethics committee of the Peter MacCallum Cancer Centre. The two-sided Fisher’s exact test was used for all statistical analysis.

Immunohistochemistry

IHC for MLH1, MSH2 and MSH6 was performed using standard laboratory protocols and reagents (Table 1) [16, 32–34]. Non-neoplastic colonic epithelium and lymphoid cells served as internal positive controls. Due to the often variable staining intensity, especially with MLH1, loss of protein expression was only reported when the entire tumour lacked nuclear staining and when there was satisfactory control staining.

MSI testing

DNA was extracted separately from normal and tumour paraffin-embedded tissue using the DNeasy® tissue DNA extraction kit (QIAGEN, Hilden, Germany), following microdissection by a pathologist of sections counterstained with methyl green. MSI status was assessed using 5–10 microsatellite markers, including the five NCI consensus markers, according to consensus guidelines [7, 35].

Germline DNA analysis

Germline mutation screening of the appropriate MMR gene was performed on those patients whose tumours were MSI-H or exhibited loss of MMR protein expression. As indicated by the results of MMR IHC, the coding regions of one or more of the *MLH1*, *MSH2* and *MSH6* genes were amplified by PCR from genomic DNA [35]. Pathogenicity was established if the identified genetic change was a small deletion, a frameshift mutation, a nonsense mutation or a missense mutation which had been previously reported as pathogenic [36]. Some patients negative by germline sequencing underwent investigation to look for large genomic rearrangements of *MLH1* and *MSH2* using a multiplex ligation-dependent probe amplification (MLPA) kit according to the manufacturer’s instructions (MRC-Holland, Amsterdam, The Netherlands) [37].

***BRAF* allele-specific PCR (AS-PCR)**

BRAF AS-PCR was performed in a duplex reaction with control primers amplifying a segment of the *GAPDH* gene, with PCR conditions as per Pollack et al. [38]. Table 2 shows the primers used. A colon tumour cell line, SW48, which is heterozygous for the c.1799T > A transversion, was used as a positive PCR control. To determine the analytical sensitivity of the AS-PCR assay, DNA from the SW48 cell line was mixed with normal human genomic DNA at a range of dilutions, to produce mutant allele frequencies of 50%, 25%, 12.5%, 5%, 2.5%, 1%, 0.1% and 0.01% and these DNA dilutions were used as templates in further duplex PCRs. All tumours positive by AS-PCR underwent confirmatory testing by direct automated

Table 1 Antibody clones, sources and antigen retrieval methods used in MMR protein immunohistochemistry

Antibody	Clone	Suppliers	Dilution	Ag retrieval buffer	Ag retrieval method
MLH1	G168-5	BD Biosciences, Franklin Lakes, NJ	1:100	Dako hipH solution (Carpinteria, CA) pH 9.9	20 min at 95–99°C in waterbath
MSH2	Fe11	Oncogene, Boston, MA	1:1000	10 mM sodium citrate pH 6.0	2 min in pressure cooker
MSH6	44	BD Transduction Laboratories, Franklin Lakes, NJ	1:2000	10 mM sodium citrate pH 6.0	2 min in pressure cooker

Table 2 Sequences and product sizes for the *BRAF* V600E allele-specific PCR (Fmutant) primers and control *GAPDH* primers (from Pollack et al. [38] and the *BRAF* exon 15 sequencing primers)

Primer	Sequence	Product size (bp)
<i>BRAF</i> exon 15 Fmutant For	5'-ACAGTAAAAATAGGTGAT TTTGGTCTAGCTACAGA-3'	198
<i>BRAF</i> exon 15 Fmutant Rev	5'-CTATGAAAATACTATAGTTG AGACCTCAATGACTTTC-3'	
<i>GAPDH</i> For	5'-GAAATGTGCTT TGGGGAGGCA-3'	247
<i>GAPDH</i> Rev	5'-TTTGCAGGGCT GAGTCAGCTTC-3'	
<i>BRAF</i> exon 15 Seq For	5'-TGCTTGCTCTGAT AGGAAAATGAGA-3'	219
<i>BRAF</i> exon 15 Seq Rev	5'-GGGCCAAAAAT TTAATCAGTGGA-3'	

sequencing, along with an equal number of AS-PCR negative cases for control purposes. Primers spanning *BRAF* exon 15 (Table 2 and Fig. 1) were used to amplify tumour DNA for sequencing.

Results

The experiment to determine the sensitivity of the *BRAF* AS-PCR assay demonstrated a weak PCR band at a mutant allele frequency of 1% and strong bands at higher mutant allele frequencies (Fig. 2). With the employment of microdissection to minimise tumour contamination by non-neoplastic tissue, this was considered more than adequate sensitivity to avoid false negative results. All positive patients showed strong mutant *BRAF* PCR bands.

Due to the referral nature of cases in this study, we were only able to collect sufficient clinical information to assess

fulfilment of the Amsterdam II or revised Bethesda criteria in 52 (76%) and 42 (62%) cases respectively. Of these, 25 (48%) and 40 (95%) met the respective criteria. Somatic tumour *BRAF* mutation was significantly less common in individuals from families that fulfilled the Amsterdam II criteria for HNPCC ($P = 0.029$). There was no significant association between *BRAF* mutation status and fulfilment of the revised Bethesda criteria in the assessable group of subjects. Interestingly, this study included three *BRAF* mutation-positive tumours from individuals who belong to Amsterdam II positive families. All of these individuals were aged 60 years or over, their tumours were all immunonegative for MLH1 and no germline mutation was revealed after direct sequencing of *MLH1* in any of these cases. *MLPA* was performed in one of these three cases and was negative. One of the other two patients, a 62-year-old female, had two metachronous CRCs in her proximal colon along with over 60 serrated-family polyps throughout her

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70250 ATAGAAATTAGATCTCTTACCTAAACTCTTCATAATGCTTGCTCTGATAGGAAAAATGAGA 70191
70190 TCTACTGTTTTTCCTTTACTTACTTACACCTCAGATATATTTTCTTCATGAAGACCTCAGT 70131
70130 AAAAATAGGTGATTTTGGTCTAGCTACAGTGAAATCTCGATGGAGTGGGTCCCATCAGTT 70071
                                     ↑
70070 TGAACAGTTGTCTGGATCCATTTTGTGGATGGTAAGAATTGAGGCTATTTTTCCTACTGAT 70011
70010 TAAATTTTTGGCCCTGAGATGCTGCTGAGTTACTAGAAAGTCATTGAAGGTCTCAACTAT 69951
69950 AGTATTTTCATAGTTCCCAGTATTCACAAAAATCAGTGTCTTATTTTTT 69901

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Fig. 1 *BRAF* primer-binding sites for allele-specific PCR (AS-PCR) and confirmatory direct sequencing. A segment of genomic DNA spanning *BRAF* exon 15 (in bold) demonstrating the binding sites of the AS-PCR primers (underlined) and the sequencing primers (in italics) used for confirmation of all AS-PCR positive samples. The

nucleotide of interest (c.1799 in cDNA sequence), T > A transversion of which underlies the oncogenic V600E mutation, is indicated with an arrow. The reverse AS-PCR primer is intronic in location, as are both sequencing primers. Complementary binding sequences are indicated for both reverse primers

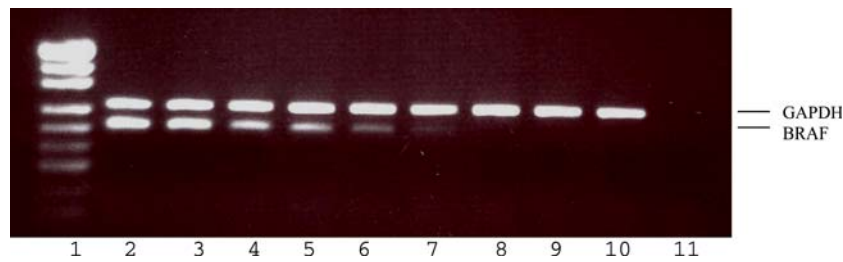


Fig. 2 *BRAF* allele-specific PCR sensitivity experiment. A 2% agarose gel showing SW48 cell line *BRAF* mutation positive control DNA mixed with “normal” human genomic DNA at a range of dilutions, to produce allele frequencies of 50%, 25%, 12.5%, 5%, 2.5%, 1%, 0.1% and 0.01% (lanes 2–9 respectively). These DNA cocktails were used as templates in a series of *BRAF* AS-PCRs (lower

band, 198 bp product) in duplex with *GAPDH* (upper band, 247 bp product) as a positive control, to determine the analytical sensitivity of the assay in detecting the target mutation. The target mutation was weakly visible at a concentration of 1% (lane 7) and strongly visible at higher frequencies (lanes 2–6). Lane 1 pUC19 HpaII DNA ladder; lane 10 normal DNA control; lane 11 no DNA control

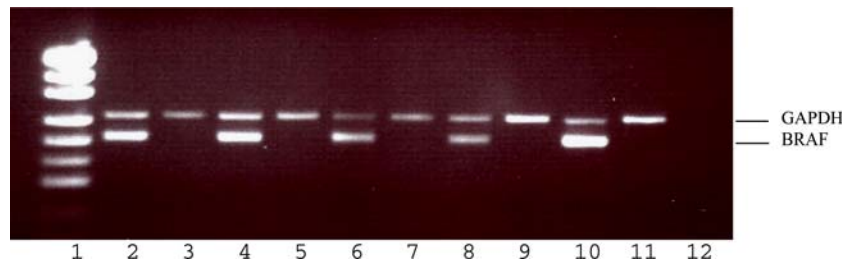


Fig. 3 Duplex AS-PCR to detect the somatic V600E *BRAF* mutation. A sample 2% agarose gel to demonstrate positive and negative *BRAF* AS-PCR results. Lane 1 pUC19 HpaII DNA ladder; lanes 2–11 upper band (247 bp) control *GAPDH* product, lower band mutant (198 bp)

BRAF product; lanes 2,4,6,8 positive for mutant *BRAF*; lanes 3,5,7,9 negative for mutant *BRAF*; lane 10 SW48 *BRAF* mutation positive control; lane 11 normal DNA control; lane 12 no DNA control

colon, highly suggestive of a diagnosis of hyperplastic polyposis.

Of the 68 CRCs in the study, 17 tumours (25%) possessed the somatic *BRAF* V600E mutation (Fig. 3). There was 100% concordance between the results of *BRAF* mutation testing by AS-PCR and by automated sequencing (Fig. 4). A univariate analysis comparing *BRAF* positive and negative tumours demonstrated statistically significant ($P < 0.05$) differences between the two groups for age, sex, germline MMR gene mutation status and the presence or absence of immunohistochemical staining for MMR proteins (Table 3). Somatic *BRAF* mutation was more common in patients over 50 years ($P < 0.001$) and in females ($P = 0.011$).

There was complete concordance between the presence of MSI-H and loss of expression of a MMR protein by IHC, with 40 cases demonstrating loss of MLH1 protein and 28 cases lacking MSH2 protein expression. All tumours showing loss of MSH2 expression were also immunonegative for MSH6. Germline mutation testing was not requested in 12 of these patients, for a combination of clinical reasons including patient unavailability. Seven of these patients had tumours immunonegative for MLH1 (mean age 67, range 48–94 years) and five immunoneg-

ative for MSH2 (mean age 55, range 38–73 years). Somatic *BRAF* mutation was identified in six of these tumours, all within the group of seven MLH1 immunonegative patients.

Of the 56 patients who underwent screening for germline MMR gene mutations by direct automated sequencing, pathogenic mutations were detected in 23 (41%) cases, with 10 mutations found in *MLH1*, 13 in *MSH2* and none in *MSH6*. Genetic changes of uncertain pathogenesis, so-called unclassified variants, were encountered in six cases (four in *MLH1* and two in *MSH2*). None of the six cases with identified unclassified variants had a somatic *BRAF* mutation. Of those germline negative cases (by automated sequencing), ten were tested by MLPA, revealing whole exonic deletions in *MSH2* in two further cases. The other eight cases (six *MLH1* immunonegative and two *MSH2* immunonegative) remained germline mutation negative after MLPA. Of the six *MLH1* immunonegative cases in which no germline mutation was found after thorough testing, three (50%) had somatic *BRAF* mutations (Table 3). Testing by MLPA was not pursued in the majority of cases for a range of clinical reasons including low index of suspicion of HNPCC or unavailability at the time of investigation of further screening tests such as MLPA.

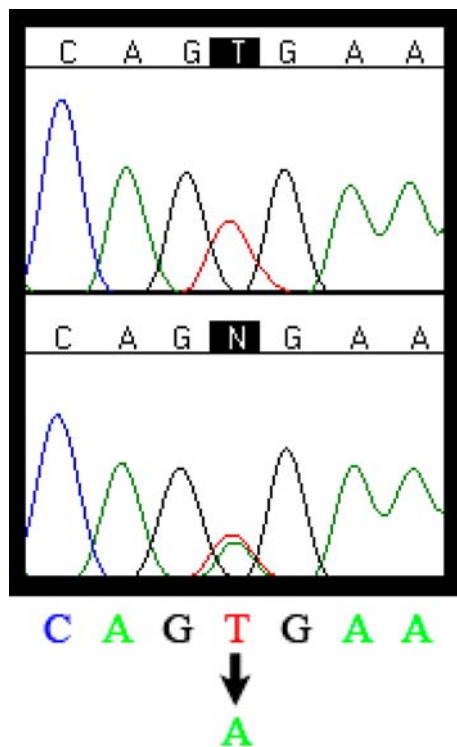


Fig. 4 Electrophoretogram from direct sequencing of *BRAF* exon 15 demonstrating the V600E mutation. The tumour DNA (lower panel) shows a heterozygous T > A transversion at nucleotide position 1799, confirming the presence of a V600E mutation. A negative tumour sample (upper panel) is shown for comparison, demonstrating only the wild-type thymidine at nucleotide position 1799

Based on the IHC findings of those cases with available germline testing results, 15 of 23 (65%) cases exhibiting loss of MSH2/MSH6 protein expression possessed an underlying germline *MSH2* gene mutation. Eight individuals (mean age 38 years, range 16–51 years) with tumours immunonegative for MSH2 and MSH6 lacked identifiable germline *MSH2* or *MSH6* mutations, although MLPA had only been performed in two of these eight cases. Only 10 of 33 (30%) cases showing loss of MLH1 protein expression had *MLH1* gene mutations identifiable by direct automated sequencing. Testing by MLPA was only performed in six of the remaining 23 cases, revealing no further mutations. The *BRAF* V600E mutation was not detected in any of the tumours from the 25 individuals with germline mutations, but was detected in 35% of tumours from individuals without identified germline mutations ($n = 31$) ($P < 0.001$).

BRAF mutation was also found in 50% of tumours from individuals who did not undergo germline testing ($n = 12$). The *BRAF* V600E mutation was identified in 17 of 40 (42%) tumours showing loss of MLH1 protein expression by IHC but in none of the 28 (0%) tumours that had intact MLH1 expression ($P < 0.001$). Of the 33 MLH1 immu-

Table 3 Univariate analysis of clinicopathological associations of *BRAF* mutation in MSI-H colorectal cancer

Factor	Level	BRAF	BRAF	BRAF	<i>P</i> value ^a
		+ve ($n = 17$)	-ve ($n = 51$)	+ve (%)	
Age (years)	≤ 50	0	35	0	<0.001
	>50	17	16	52	
Sex	Male	3	28	10	0.011
	Female	14	23	38	
Location ^b	Distal	0	6	0	0.155
	Proximal	10	23	30	
	Unknown	7	22		
Amsterdam II positive	No	11	16	41	0.029
	Yes	3	22	12	
	Unknown	3	13		
Revised Bethesda positive	No	2	0	100	0.106
	Yes	12	28	30	
	Unknown	3	23		
Germline MMR mutation ^c	Positive	0	25	0	<0.001
	Negative	11	20	35	
	Unknown	6	6		
MLH1 IHC	Present	0	28	0	<0.001
	Absent	17	23	42	
MSH2 IHC	Present	17	23	42	<0.001
	Absent	0	28	0	
Germline <i>MLH1</i> mutation ^c (MLH1 IHC -ve, $n = 40$)	Positive	0	10	0	0.013
	Negative	11	12	48	
	Unknown	6	1		
Germline <i>MLH1</i> mutation ^d (MLH1 IHC -ve, $n = 40$)	Positive	0	10	0	0.036
	Negative	3	3	50	
	Unknown	14	10		
Germline <i>MLH1</i> mutation	Positive	0	10	0	0.002
	Negative	17 ^e	12	59	
Germline <i>MSH2</i> mutation ^c (MSH2 IHC -ve, $n = 28$)	Positive	0	15	0	1
	Negative	0	8 ^f	0	
	Unknown	0	5		

^a *P*-values were derived from the two-sided Fisher's exact test, including only tumours for which the variable in question was known or assessed

^b Tumours situated at or beyond the splenic flexure were classified as distal

^c After testing by at least direct automated sequencing

^d After testing by direct automated sequencing and multiplex ligation-dependent probe amplification (MLPA)

^e Assumes the *BRAF* positive cases with unknown germline mutation status are germline negative

^f Two cases were also analysed by MLPA.

MMR, mismatch repair; IHC, immunohistochemistry

nonnegative cases with germline testing results, 11 of 23 (48%) cases germline mutation negative by sequencing were *BRAF* positive, compared to none of the ten cases

with germline *MLH1* mutations. Amongst this group of *MLH1* immunonegative tumours investigated for germline status by direct automated sequencing alone, this equates to 48% sensitivity and 100% specificity of a positive *BRAF* assay result in predicting the *absence* of a germline *MLH1* mutation. Restricting this analysis to those few cases which were also analysed by MLPA, gives a similar sensitivity of 50% and specificity of 100%, with the three *BRAF* positive cases and three *BRAF* negative cases analysed remaining germline negative after MLPA (Table 3).

Seventeen of 40 (42%) tumours showing intact MSH2 protein expression by IHC contained the *BRAF* V600E mutation but none of the 28 (0%) tumours that exhibited loss of MSH2 expression possessed the mutation ($P < 0.001$). The presence of somatic *BRAF* V600E mutations in MSI-H CRC is, therefore, restricted to those tumours showing loss of *MLH1* protein expression that arise in individuals without detectable germline *MLH1* gene mutations. Such tumours also share the same clinicopathological features, such as proximal colonic location and more frequent occurrence in elderly females, as sporadic MSI-H CRC [18].

Discussion

The results of this study confirm previous studies which showed that the presence of the *BRAF* V600E mutation is strongly associated with sporadic CRCs that exhibit MSI due to somatic inactivation of *MLH1* protein expression [23, 25–31]. These earlier studies, however, mainly investigated CRCs in the routine clinical setting or in a research setting, with some enrichment for MSI-H tumours [23, 26–29]. Our study extends these observations to the population of patients referred from a familial cancer clinic setting for laboratory investigation of suspected HNPCC and serves the purpose of establishing the clinical validity and utility of the assay in the patient population and clinical setting where the assay is intended for use.

This study population appears to be representative of patients with suspected HNPCC referred from familial cancer clinics for laboratory investigation. Although only 48% of evaluable cases met the Amsterdam II criteria, such low rates have been well documented previously which is, in part, why the criteria for selecting patients for MSI and IHC testing is usually based on the more inclusive revised Bethesda criteria [39]. Indeed, 95% of our evaluable subjects fulfilled the revised Bethesda criteria.

MMR gene mutations were identified in 25 of 56 (45%) individuals in whom germline testing by exon sequencing and, in some cases, MLPA of the relevant genes was performed. There were 12 cases in which mutation screening was not requested at all and in most cases mutation testing

was stopped after direct sequencing, without recourse to MLPA. The decision not to proceed with MMR gene mutation screening on certain individuals reflects the realities of clinical practice. Such decisions are based on a clinical evaluation that there is a low likelihood of HNPCC and on the need to make efficient use of limited laboratory resources. Some such cases in this series were encountered before MLPA or similar assays were available and others may have refused testing or been screened by another laboratory.

The 12 cases in this series in which germline testing was not requested comprised a more elderly group ($n = 7$, mean age 67 years, range 48–94 years) with tumours immunonegative for *MLH1* and a younger group ($n = 5$, mean age 55 years, range 38–73 years) with tumours immunonegative for MSH2. It was judged likely that the latter group would comprise mostly individuals with HNPCC caused by *MSH2* or *MSH6* mutations which, for various reasons, were not sought, whereas the former group would comprise mainly sporadic cases with *MLH1* inactivation due to gene promoter hypermethylation. This was supported by the detection of the *BRAF* mutation in six of the seven tumours immunonegative for *MLH1* but none of the five cases immunonegative for MSH2. If we assume that the six *BRAF* mutation-positive cases with loss of *MLH1* and unknown *MLH1* germline status indeed represent sporadic tumours, then the estimated sensitivity of the *BRAF* assay in predicting the *absence* of a germline MMR mutation in the *MLH1* immunonegative group would increase from 48% to 59% (Table 3).

The three individuals from Amsterdam II positive families with MSI-H CRC exhibiting loss of *MLH1* expression and somatic *BRAF* mutation but lacking an identified germline mutation (after direct sequencing) could be sporadic phenocopies of HNPCC. An alternative explanation is that they represent examples of a recently described syndrome characterised by a family history of CRC, CRC onset in the fifth to eighth decades, serrated morphology, widespread DNA methylation, frequent somatic *BRAF* mutation, variable MSI tumour status and the absence of germline MMR gene mutation [39, 40]. Although methylation analysis was not performed in this study, these cases appear to have the other characteristics of this syndrome, including serrated morphology which was observed in all three tumours. One patient fulfilled the WHO personal diagnostic criteria for hyperplastic polyposis, encompassed within this syndrome which perhaps represents the true hereditary counterpart of sporadic MSI-H CRC [40, 41]. The genetic basis of this ‘serrated pathway’ syndrome most likely relates to an inherited tendency to increased DNA methylation [39, 40]. This would help explain the surprisingly high overall *BRAF* mutation frequency in this study of 25%, given that all 68 cases had

clinical features or family histories sufficiently suggestive of HNPCC to warrant laboratory investigation and previous studies have shown that *BRAF* mutations are uniformly absent in HNPCC [23, 26–29]. Regardless of the underlying pathogenesis, these three cases highlight the fact that MSI-H CRC may arise in families that fulfil the clinical criteria of HNPCC but lack germline MMR gene mutations.

In all families with high clinical suspicion of HNPCC, germline testing should not be performed on an individual whose tumour harbours a somatic *BRAF* mutation, suggestive of involvement in that tumour of the serrated pathway to cancer, but rather on an individual with a *BRAF* negative tumour. The *BRAF* assay can therefore be utilised in this clinical scenario to select the most appropriate individual for germline testing in the investigation of HNPCC.

A suggested algorithm incorporating somatic *BRAF* V600E mutation testing of tumour tissue into the investigation of suspected HNPCC, is shown in Fig. 5. IHC directs germline screening towards the gene involved. Our results indicate that *BRAF* mutation testing has a clinically valid and useful role in triaging *MLH1* germline testing in those patients whose tumours show loss of MLH1 expression. Cases possessing a *BRAF* V600E mutation do not require germline *MLH1* testing unless the clinical suspicion of HNPCC is high, say in the event of Amsterdam criteria fulfilment, in which case another individual in the family should ideally be selected for testing. *BRAF* mutation testing, however, plays no role in triaging patients whose tumours show loss of MSH2 or MSH6 expression.

The current inability to definitively distinguish, at the pre-screening stage, sporadic MSI-H CRC with somatic

loss of MLH1 expression from HNPCC cases due to germline *MLH1* mutation results in the performance of a significant number of unnecessary *MLH1* germline mutation tests. Furthermore, in some families with HNPCC, germline testing may inappropriately and wastefully be performed on an individual who does not carry the germline mutation, but has a coincidental sporadic MSI-H tumour. Compilation of a thorough family history remains the most important tool in detecting HNPCC through fulfilment of the Amsterdam II criteria. However, up to 20% of HNPCC families with germline MMR gene mutations do not meet Amsterdam criteria [42]. Global demographic changes, such as decreasing family size and geographic dispersion of families, are leading to less reliable family histories, compounding the difficulty of making an accurate clinical diagnosis of HNPCC. MSI testing of tumours from individuals meeting Bethesda criteria, allied with MMR protein IHC, greatly facilitates the direction and efficiency of germline screening. Distinction of some HNPCC-related CRCs from sporadic MSI-H CRCs, however, remains problematic, as both are caused by inactivation of the *MLH1* gene, albeit by different mechanisms. The situation is further complicated by the recognition of familial CRC distinct from HNPCC with tumours showing variable MSI status and frequent somatic *BRAF* mutation [39, 40].

Given the time and expense involved in proband germline screening and surveillance of high risk family members, any assay that makes the detection of HNPCC more efficient is to be welcomed. The high specificity of a positive *BRAF* V600E assay in predicting the absence of a MMR gene germline mutation indicates that this relatively easy and inexpensive assay should be employed in the

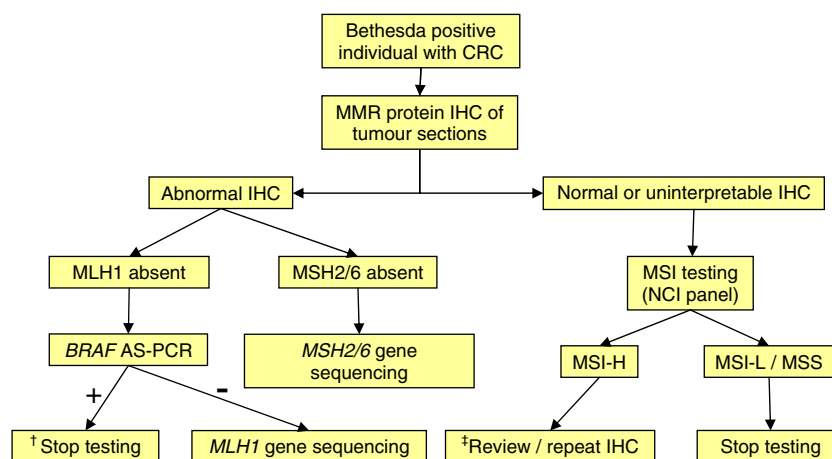


Fig. 5 Algorithm for investigating possible HNPCC, incorporating *BRAF* V600E mutation testing. CRC, colorectal cancer; MMR, mismatch repair; IHC, immunohistochemistry; MSI, microsatellite instability; NCI, National Cancer Institute; +, somatic *BRAF*

heterozygous V600E mutation present; –, *BRAF* wild-type; † if there is a strong clinical suspicion of HNPCC, another family member may be selected for testing; ‡ if IHC remains normal or uninterpretable upon review and repeat, germline sequencing is advised

routine laboratory investigation of suspected HNPCC. If employed judiciously and with consideration of the individual and family in question, it can be a safe, rapid and cost-saving aid to the clinical and laboratory investigation of familial colorectal cancer.

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