A Modified Lynch Syndrome Screening Algorithm in Colon Cancer

BRAF Immunohistochemistry Is Efficacious and Cost Beneficial

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ABSTRACT

Objectives: Somatic BRAF mutation in colon cancer essentially excludes Lynch syndrome. We compared BRAF V600E immunohistochemistry (IHC) with BRAF mutation in core, biopsy, and whole-section slides to determine whether IHC is similar and to assess the cost-benefit of IHC.

Methods: Resection cases (2009-2013) with absent MLH1 and PMS2 and prior BRAF mutation polymerase chain reaction results were chosen (n = 57). To mimic biopsy specimens, tissue microarrays (TMAs) were constructed. In addition, available biopsies performed prior to the resection were available in 15 cases. BRAF V600E IHC was performed and graded on TMAs, available biopsy specimens, and whole-section slides. Mutation status was compared with IHC, and cost-benefit analysis was performed.

Results: BRAF V600E IHC was similar in TMAs, biopsy specimens, and whole-section slides, with only four (7%) showing discordance between IHC and mutation status. Using BRAF V600E IHC in our Lynch syndrome screening algorithm, we found a 10% cost savings compared with mutational analysis.

Conclusions: BRAF V600E IHC was concordant between TMAs, biopsy specimens, and whole-section slides, suggesting biopsy specimens are as useful as whole sections. IHC remained cost beneficial compared with mutational analysis, even though more patients needed additional molecular testing to exclude Lynch syndrome.

Colorectal cancer (CRC) is one of the most common malignancies affecting patients in industrialized countries and is the third most commonly diagnosed cancer in the United States, regardless of sex.1 Approximately 10% to 25% of cases are due to inheritable germline mutations, and 2% to 3% have Lynch syndrome.1,3 Lynch syndrome is the most common inheritable syndrome predisposing to CRC. It is defined by a germline mutation in one of the DNA mismatch repair (MMR) genes.2

At The Ohio State University Wexner Medical Center (OSUWMC) and many other institutions, all patients with CRC are screened for microsatellite instability and Lynch syndrome using immunohistochemical (IHC) stains for the MMR proteins MSH2, MSH6, PMS2, and MLH1.4 Protein loss results in a loss of function in the MMR cellular mechanism, which consequently leads to expansion or contraction of microsatellite DNA elements. Since loss of one or more MMR proteins can be seen in either Lynch syndrome or
sporadic CRC, further testing is necessary to identify those with syndromic CRC.5,6

BRAF mutation analysis is helpful to distinguish Lynch syndrome from sporadic CRC. It is a proto-oncogene, and activating mutations most commonly involve substitution of valine by glutamic acid at position 600 (V600E) on codon 15.7 Whereas somatic BRAF mutations are almost never found in Lynch syndrome, they are frequently associated with sporadic microsatellite-unstable CRCs, usually occurring due to epigenetic inactivation of the MLH1 MMR gene.8-10 As a result, BRAF analysis is a commonly used tool to distinguish Lynch syndrome (BRAF wild-type) from sporadic CRC (BRAF mutated). The presumed sporadic CRC group requires no further testing or genetic counseling.4,11

Efficient and reliable assessment of BRAF mutation status is becoming more important in patient care for several tumors, including CRC. BRAF testing is indicated in those CRC patients with absence of MLH1 and PMS2 IHC. The most commonly used method to assess BRAF is polymerase chain reaction (PCR), followed by sequencing of exon 15.11 We previously showed that BRAF mutation analysis using PCR is a cost-effective subsequent reflexive test in patients with loss of MLH1 and PMS2 immunostaining.4 More recently, researchers have shown that BRAF V600E IHC is an efficacious screening tool since BRAF protein is present in the tumors of patients with activating BRAF mutations and absent in the tumors of patients without BRAF mutations (BRAF wild-type).12-16

In this study, we evaluated the utility of BRAF V600E IHC compared with BRAF mutational studies and performed a detailed cost-benefit analysis. Moreover, since some clinicians request BRAF analysis on biopsy specimens to help guide surgical management, we compared BRAF V600E IHC in available biopsy specimens, tissue microarrays (TMAs), and whole-section slides. TMAs and whole-section slides were from the resection specimens.

Materials and Methods

Case Selection

BRAF mutation analysis has been routinely included in the OSUWMC Lynch syndrome screening algorithm since January 1, 2009. Patients with potential Lynch syndrome were identified by absent IHC for one or more of the MMR proteins: MSH2, MSH6, PMS2, and MLH1. All patients with CRC and small intestinal cancer showing absence of MLH1 and PMS2 IHC are reflexively tested for the BRAF mutation using multiplex PCR and sequencing analysis. Patients with BRAF mutation are not referred to genetics for additional molecular testing since BRAF mutation essentially excludes Lynch syndrome.8-11 Patients without BRAF mutation are referred to genetics and offered informed consent and molecular testing, most commonly for the MLH1 gene. The study group includes consecutive and available primary small intestinal and CRC cases diagnosed between 2009 and 2013 with absence of MLH1 and PMS2 IHC and known BRAF mutation status at our institution. In addition, 15 of these patients had available resection biopsy specimens that were included. All human experiments were conducted in accord with the Helsinki Declaration of 1975.

BRAF Immunohistochemistry

To simulate biopsy specimens, we prepared TMAs with two 1-mm cores per case from resection cases with known absence of MLH1 and PMS2 by IHC. TMAs, pre-resection biopsy specimens, and whole-section slides were stained with BRAF V600E IHC. IHC was performed on formalin-fixed, paraffin-embedded tissue using the VE1 antibody (1:900, incubation 15 minutes; Spring Bioscience, Pleasanton, CA) with an automated staining system (Bond Autostainer; Leica Microsystems, Buffalo Grove, IL). The antigen retrieval was online in the Bond (EDTA-based solution with a pH of 8.0 for 20 minutes at 99°C).

BRAF V600E IHC was graded as positive when there was diffuse cytoplasmic staining in tumor cells (presumed BRAF mutation present) and negative when there was no definitive cytoplasmic staining in tumor cells (presumed no BRAF mutation). Staining was scored by two pathologists (R.M.R. and W.L.F.) with an interest in gastrointestinal pathology who were not aware of clinical or mutation data. BRAF V600E IHC results from TMAs, biopsy specimens, and whole-section slides were then compared with BRAF mutation status by PCR and sequencing analysis.

BRAF Molecular Analysis

Formalin-fixed, paraffin-embedded tumor tissue extracted for each case was used to perform BRAF V600E molecular mutation analysis using standard multiplex PCR and sequencing analysis. Sequencing of exon 15 of the BRAF gene, which includes the V600E mutation, was performed to identify any activating mutations.8-11 Approximately 25 to 50 ng of tumor DNA was amplified in a 15-µL PCR reaction using Promega’s GoTaq master mix (Promega, Madison, WI) and the following primer pair: forward, 5’-CTTCCATAAT-GCTTGCTCTGATAGGA-3’ and reverse, 5’-TTTCTAGTA-ACTCAGCAGCATCTCA-3’. PCR products were analyzed using an ABI3700 sequencer (Life Technologies, Grand Island, NY) following suitable amplification.
Results

Patient Demographics

From 2009 to 2013, we identified 57 patients (of 557 total tested cases) who had small and/or large intestinal resections with tumors lacking MLH1 and PMS2 protein expression as assessed by IHC. Two cases were excluded from the study due to insufficient tumor for TMA construction. Thirty-two tumors (58%) were positive for BRAF mutation by multiplex PCR, while the others (23 cases [42%]) did not have a BRAF mutation by PCR. The mean patient age was 69.8 years, with an age range of 31 to 95 years. Twenty-nine were women (53%), and 26 (47%) were men. Tumor locations were as follows: two in the small intestine, 14 in the cecum, 20 in the right colon, six in the transverse colon, seven in the left colon, four in the rectosigmoid colon, and two at an unspecified colonic location.

Comparison of TMA, Biopsy Specimen, and Whole-Section Immunostaining

All cases showed similar IHC staining patterns between the biopsy specimens, TAMs, and whole-section slides. Most cases that scored positive for BRAF V600E demonstrated strong, diffuse, and uniform cytoplasmic staining Image 1. A few cases that scored positive for BRAF V600E showed weaker but diffuse and convincing cytoplasmic immunostaining. Cases with negative BRAF V600E staining showed lack of any convincing cytoplasmic immunostaining in all tumor cells. Weak background staining was seen in some cases in smooth muscle cells and nonneoplastic colonic or small intestinal mucosa Image 2.

Comparison of Immunostaining and Molecular Analysis

Twenty-eight (51%) of 55 cases showed positive BRAF V600E expression by IHC, while the remaining 27 cases were negative. Fifty-one (93%) of 55 cases showed concordant BRAF V600E IHC results compared with the molecular analysis Table 1. The four (7%) discordant cases included two women (mean age 69 years) and two men (mean age 67 years) and were tumors from the right or transverse colon. All discordant cases were negative for BRAF V600E based on IHC with a positive BRAF mutation by molecular analysis Table 2. When the mutational analysis with PCR was used as the gold standard, the sensitivity and specificity of BRAF V600E IHC were 88% and 100%, respectively.

Cost-Benefit Analysis

A cost-benefit analysis was performed using the 2014 US Medicare reimbursement rates for the Current Procedural Terminology code. The current Medicare reimbursement rates are as follows: BRAF molecular testing = $198.33, MLH1 gene testing = $665.32, and BRAF V600E IHC stain = $83.53. All rates include the technical and the professional component, and the IHC rate is the amount per slide interpreted. All patients with negative BRAF V600E IHC required genetic counseling and consent prior to additional molecular testing ($174 per genetic counseling encounter).

The cost for using BRAF mutational testing in all 55 patients with absent MLH1 and PMS2 IHC is $10,908.15 (55 × $198.33 per BRAF gene test). Twenty-three of these cases were negative and, therefore, needed subsequent genetic counseling and molecular testing for MLH1 gene mutations. The additional cost is $19,304.36 (23 × $665.32 per MLH1 gene test plus 23 × $174 per genetic counseling encounter). As a result, the total cost for using PCR molecular testing for BRAF status is $10,908.15 + $19,304.36 = $30,212.51.

The cost of using BRAF V600E IHC in all 55 patients with absent MLH1 and PMS2 IHC is $4,594.15 (55 × $83.53 per BRAF immunostained slide). Twenty-eight of 55 patients had positive BRAF V600E IHC and did not need further testing. These patients were considered to have sporadic CRC characterized by somatic hypermethylation of MLH1; no further gene testing or genetic counseling was necessary. The remaining 27 patients who were BRAF V600E negative via IHC would likely need to undergo genetic counseling and, subsequently, MLH1 gene testing. The additional cost is $22,661.64 (27 × $665.32 per MLH1 gene test plus 27 × $174 per genetic counseling encounter). The total cost when using BRAF V600E IHC stain is $4,594.15 + $22,661.64 = $27,255.79. The comparison between the total costs with BRAF mutation testing vs BRAF V600E IHC testing ($30,212.51 vs $27,255.79) shows a savings of 10% at our institution.

Discussion

It is becoming increasingly imperative to identify patients with cancer who have specific familial syndromes, not just for prognostic significance and specific treatment protocols but also for the patient’s and family members’ well-being and peace of mind. BRAF mutation analysis is important in the workup of patients with CRC to help identify patients likely to have Lynch syndrome. The identification of a BRAF V600E mutation virtually excludes Lynch syndrome, and these patients do not require additional molecular workup or genetic testing. BRAF mutation analysis has been performed using various PCR-based methods, all of which require additional time, technical acumen, and expense. As such, BRAF mutation testing is not universally available in all centers, and send-out testing adds further delays and cost.
Image II Tissue microarray cores. Presence (A, B, D, and E) and absence (C and F) of BRAF expression by immunohistochemistry (×4).
Image 2. BRAF V600E immunohistochemistry. BRAF expression (×20) (A and B), BRAF expression in tumor adjacent to uninvolved colonic mucosa (×20) (C), no BRAF expression (×20) (D), no BRAF expression in cytoplasm with nonspecific nuclear staining (×20) (E), and BRAF background nuclear staining in uninvolved colonic mucosa (×40) (F).
BRAF V600E IHC has been shown to be efficacious in melanoma, papillary thyroid carcinoma, and hairy cell leukemia.\(^{18-20}\) Recently, the BRAF antibody has been studied in CRC.\(^{12-16,21,22}\) We found that BRAF V600E IHC showed a sensitivity of 88% and a specificity of 100% using the gold standard of BRAF PCR mutation testing and sequencing analysis. We report a concordance rate of 93% (51 of 55 cases) between BRAF V600E IHC and mutational analysis, similar to other investigators.\(^{12-16}\) A possible explanation for some of the four discordant cases in our study with negative BRAF V600E IHC and positive BRAF mutation by molecular analysis could have been sampling error in the tissue stained with IHC. Another potential explanation could be false-positive results from the molecular testing, a phenomenon that has been described previously using multiplex PCR as opposed to real-time PCR.\(^{16,21}\) Additional possibilities include decreased immunoreactivity due to a decreased level of mutant BRAF protein expression or less than ideal conservation of BRAF antigen during processing.\(^{22}\)

Staining process optimization in individual laboratories is necessary for ideal immunostaining performance. There have been many different methods described for BRAF V600E IHC. Multiple other studies have used the VE1 Spring Bioscience monoclonal antibody on a Ventana Medical Systems (Tucson, AZ) or a Leica Bond automated slide stainer.\(^{12-16,21}\) Problematic staining patterns have been reported when using the same antibody with a DAKO (Carpinteria, CA) automated slide stainer or manual staining methods.\(^{22}\) We used the VE1 Spring Bioscience antibody with a Leica Bond automated slide stainer, comparable to Toon et al\(^{16}\) with similar results. It is clear that careful antibody optimization is necessary, and automated staining methods are helpful.

Retrieval solutions with a basic pH could be an important step in the staining process. The study by Kuan et al\(^{15}\) compared several methods and differences between previous studies, and they noted that most previous studies used a retrieval solution with a pH of either 8.0 or 9.0 with sensitivities and specificities greater than 90%,\(^{12-14,16}\) A previous study using a citrate solution with a pH of 6.0 reported the lowest staining sensitivity.\(^{22}\) Kuan et al\(^{15}\) concluded that basic solutions may improve the nuclear staining of the BRAF V600E antibody by revealing the nuclear antigen. In contrast to the majority of recent studies, Lasota et al\(^{21}\) reported an extremely low specificity of 51% when including weak as well as strong positive staining with the BRAF VE1 clone. It is difficult to directly compare studies since not all staining conditions are specified in each. Lasota et al\(^{21}\) did not specify the pH level of the solution used for IHC. It is unclear why this study showed a lower specificity than most of the others. In our study, a few cases showed weak cytoplasmic staining. However, the staining was diffuse and convincing, and the cases were subsequently considered positive for BRAF V600E IHC. We found that all these cases were positive for BRAF mutation by molecular analysis. As in our study, occasional false-negative immunostaining for BRAF V600E has been reported; however, false-positive staining is extremely rare and has been seen in tumors with KRAS mutations.\(^{21}\)

Most BRAF V600E IHC studies have used whole-section slides from resection specimens.\(^{12-15,22}\) However, the diagnosis of Lynch syndrome is sometimes important to establish before resection to guide surgical management. For example, the risk of developing a metachronous CRC in Lynch syndrome following partial colectomy ranges from 11% to 45% over an 8- to 14-year period,\(^{23}\) and the cumulative risk of metachronous CRC is 16% at 10 years, 41% at 20 years, and 62% at 30 years after segmental resection.\(^{24}\) The risk for development of metachronous CRC has been shown to decrease as more intestine is resected.\(^{24}\) On the basis of these risks, patients with Lynch syndrome sometimes benefit from subtotal colectomy.\(^{23}\) As such, accurate and timely diagnosis of Lynch syndrome at the time of biopsy can be critical to guide surgical management. For this reason, testing for MMR proteins as well as BRAF V600E IHC may be helpful in the initial diagnostic biopsy of patients with CRC. Previous studies have shown that biopsy specimens are as effective as resection specimens for the evaluation of MMR proteins.\(^{25}\) We found that BRAF V600E IHC analysis results are equivalent in TMAs (simulating biopsy specimens) and preresection biopsy specimens compared with whole-section slides from resection specimens, similar to Toon et al.\(^{16}\) Interestingly, they found that five of 201 cases showed negative staining on

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<tr>
<th>Location</th>
<th>TMA IHC</th>
<th>WS IHC</th>
<th>PCR</th>
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<tr>
<td>Transverse colon</td>
<td>Negative</td>
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<td>Positive</td>
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<td>Right colon</td>
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IHC, immunohistochemistry; PCR, polymerase chain reaction and sequencing analysis.
TMAs with positive staining on whole-section slides, and they attributed this discordance to heterogeneous BRAF V600E IHC. In our study, all TMA and whole-section slide BRAF V600E IHC results were similar. We also found uniform staining in all TMAs, biopsy specimens, and whole-section slides. These findings suggest biopsy specimens are as useful as whole-section slides for assessing BRAF V600E by IHC.

We encountered important diagnostic challenges in the course of this study. Since BRAF V600E IHC is cytoplasmic, particular care should be taken when interpreting positive staining in CRC cells with little cytoplasm (ie, signet ring cell or mucinous carcinomas). Nuclear staining can represent another diagnostic pitfall. In our series, nuclear reactivity was seen occasionally, but none of these cases showed a mutation by PCR, supporting that only BRAF cytoplasmic staining should be interpreted as positive (since BRAF protein is cytoplasmic). In cases of equivocal BRAF V600E IHC, we recommend additional molecular testing. Furthermore, molecular testing should be considered regardless of BRAF V600E IHC or molecular results in those patients who are at high risk for Lynch syndrome (including multiple family members with CRC diagnosed at a young age, patient diagnosis of CRC at a young age, and synchronous or metachronous primary CRCs and other Lynch syndrome–-associated cancers). Some centers use MLH1 promoter hypermethylation analysis, as opposed to BRAF molecular testing, in their screening algorithm for Lynch syndrome in those patients with absent MLH1 and PMS2. The addition of either test decreases the number of patients needing additional genetic testing, but it remains unclear which test is most useful. Finally, our results are limited to protein products obtained from the V600E mutant because other BRAF mutations are extremely rare, although they do exist.

In conclusion, we report that BRAF V600E IHC shows similar results in whole-section slides, biopsy specimens, and TMA cores, suggesting its usefulness in biopsy material. We found that BRAF V600E IHC is as specific as molecular analysis and can be a useful ancillary test for cancer cases with absence of the MLH1 and PMS2 proteins. Moreover, we found a cost benefit in using BRAF V600E IHC in MLH1- and PMS2-negative patients as opposed to BRAF molecular studies in the screening algorithm for Lynch syndrome. BRAF V600E IHC is much more widely available, faster, and easier to execute in most laboratories, and from our study, we concluded it is efficacious and cost beneficial in the algorithm for Lynch syndrome screening in CRC.

References


