Assessment of BRAF V600E Status in Colorectal Carcinoma: Tissue-Specific Discordances between Immunohistochemistry and Sequencing


Abstract

Although sequencing provides the gold standard for identifying colorectal carcinoma with BRAF V600E mutation, immunohistochemistry (IHC) with the recently developed mouse monoclonal antibody VE1 for BRAF V600E protein has shown promise as a more widely available and rapid method. However, we identified anecdotal discordance between VE1 IHC and sequencing results and therefore analyzed VE1 staining by two different IHC methods (Leica Bond and Ventana BenchMark) in whole tissue sections from 480 colorectal carcinomas (323 BRAF wild-type, 142 BRAF V600E mutation, and 15 BRAF non-V600E mutation). We also compared the results with melanomas and papillary thyroid carcinomas (PTC). With the Bond method, among 142 BRAF V600E-mutated colorectal carcinomas, 77 (54%) had diffuse VE1 staining and 48 (33%) had heterogeneous staining. In contrast with colorectal carcinoma, Bond and Ventana VE1 IHC in melanoma and PTC were highly concordant with sequencing results. We conclude that VE1 IHC produces suboptimal results in colorectal carcinoma and should not be used to guide patient management. Mol Cancer Ther; 14(12); 1–9. ©2015 AACR.

Introduction

The BRAF proto-oncogene encodes a serine/threonine kinase belonging to the raf/mil family. The BRAF protein resides at the apex of, and thus provides a critical regulatory function for, the MAPK signaling cascade. The most frequent somatic alteration in the BRAF gene is a point mutation in codon 600 that replaces valine with glutamate (NM_004333.4[BRAF]:c.1799T>A p.V600E, referred to as BRAF V600E hereon) and results in constitutive activation of the MAPK pathway (1). Using in vivo mouse models, Rad and colleagues demonstrated that the BRAF V600E-associated pathway of intestinal tumorigenesis occurs through a hyperplasia/adenoma/carcinoma sequence with subsequent acquisition of high levels of microsatellite instability, then activation of the Wnt pathway and intensification of MAPK signaling, and, finally, late-stage inactivation of p16 and p53 (2).

Colorectal carcinomas that harbor the BRAF V600E mutation are a distinct subset of tumors. They are frequently associated with poor differentiation, mucinous histology, and advanced tumor–node–metastasis stage (3). In patients with microsatellite-stable colorectal carcinoma (4, 5) and those with advanced colorectal carcinoma (6), BRAF V600E mutation confers worse survival compared with their wild-type counterpart. Furthermore, Lynch syndrome (hereditary nonpolyposis colorectal cancer syndrome) is virtually excluded when a colorectal carcinoma that exhibits loss of the MLH1 and PMS2 proteins by immunohistochemistry (IHC) also harbors BRAF V600E mutation (7).

BRAF V600E mutation confers important predictive value in the treatment of patients with colorectal carcinomas. Some clinical studies, supported by in vitro results (8), have reported a detrimental effect of BRAF V600E mutation in patients with colorectal carcinoma treated with the anti-EGF receptor therapy cetuximab or panitumumab (9–12), although data are conflicting. In a subsequent analysis of pooled data from the randomized phase III trial, Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer (CRYSTAL), and the randomized phase II trial, Oxaliplatin and Cetuximab in...
First-Line Treatment of Metastatic Colorectal Cancer (OPUS), no significant differences in outcome (overall survival, progression-free survival, and best overall response rate) were found between patients with colorectal carcinoma with BRAF mutations and BRAF wild-type (13). In vitro and in vivo xenograft models have shown that colorectal carcinoma that harbor BRAF V600E may be sensitive to proteasome inhibitors (14). Although colorectal carcinomas have very limited response to BRAF V600E inhibitor monotherapy (15, 16), combinatorial strategies are currently under investigation to improve response, and outcome for these patients (17, 18).

Given that BRAF V600E has these important hereditary, prognostic, and therapeutic implications, there is a critical need to ensure accurate identification of patients whose colorectal carcinoma has this mutation. PCR-based sequencing assays that detect the BRAF V600E mutation are considered to be the gold standard for assessing the mutational status of this gene for patient management decisions. More recently, a mouse monoclonal antibody for the BRAF V600E protein (clone VE1, Spring Bioscience) has become commercially available for use in IHC. This method has the advantages of being relatively fast, inexpensive, and widely available for use in routine formalin-fixed, paraffin-embedded tissue. In addition, IHC overcomes numerous challenges associated with sequence analysis that include limited availability of the technology, requirement for larger tumor sample, and effects of dilution with non-neoplastic tissue.

Previous studies on the suitability of VE1 IHC for the detection of BRAF V600E mutation in colorectal carcinoma found sensitivity ranging from 59% to 100% and specificity ranging from 51% to 100% with use of different IHC techniques, including a wide spectrum of antibody conditions, and different sequencing techniques as the comparator (19–31). In the largest such series to date, investigators used whole tissue sections to analyze 113 BRAF wild-type and 52 BRAF V600E-mutated colorectal carcinomas in the validation cohort (21) and reported sensitivity of 96% and specificity of 99%. Despite these reportedly high concordances between results of VE1 IHC and sequence analysis, in our clinical practice we had noted discordances between VE1 IHC and sequencing results when both tests were performed on colorectal carcinoma samples. In contrast, concordance was high between results of the two tests in melanoma and papillary thyroid carcinoma samples. Because VE1 IHC has been proposed for use as a surrogate marker for BRAF V600E mutation—even replacing molecular studies altogether—we believed that more rigorous validation of VE1 IHC with a large cohort of patients was necessary to establish analytical and clinical validity. Therefore analyzed whole tissue sections from 480 colorectal carcinoma cases, including 323 BRAF wild-type tumors, 142 tumors with BRAF V600E mutation, and 15 tumors with BRAF mutation other than V600E, to compare two different VE1 IHC methods and three different BRAF sequencing methods. We also compared the results in colorectal carcinoma with those in melanomas and papillary thyroid carcinomas analyzed in our laboratory using the same sequencing and IHC techniques.

Materials and Methods

Study population

The pathology files of the University of Texas MD Anderson Cancer Center were searched for all resection specimens of colorectal carcinoma (primary tumors and metastases) from 2008 through 2013 for which BRAF mutation analysis had been performed. As previously published studies have reported sensitivity and specificity of up to 100% for VE1 IHC, we planned to analyze a sample size of at least 300 cases each of BRAF wild-type colorectal carcinoma and BRAF V600E colorectal carcinoma so that 100% concordance between sequencing and IHC would give us an upper 95% confidence boundary of less than 1% discordance. We therefore randomly selected for inclusion in this study a total of 323 colorectal carcinoma cases to include equal numbers of cases each year from over 1,500 colorectal carcinoma cases that had no mutation detected in the BRAF gene (wild-type) by sequencing and that had available material for IHC testing. Our search of the pathology files revealed only 142 colorectal carcinoma cases with BRAF V600E mutation and 15 additional cases with BRAF mutation other than V600E on sequencing that had residual material available to perform IHC, all of these cases were included in this study. Thus, a total of 480 colorectal carcinomas were analyzed with VE1 IHC, as described below.

For comparison, we also searched our pathology files to identify cases of melanoma and papillary thyroid carcinoma that had already been analyzed for BRAF V600E mutation by both sequence analysis and VE1 IHC in 2013 and 2014. For these cases, we abstracted the previously recorded findings on sequence analysis and VE1 IHC from patient records.

The study was approved by the MD Anderson Institutional Review Board.

Sequence analysis

DNA had been extracted from microdissected unstained, formalin-fixed, paraffin-embedded whole tissue sections, and sequencing had been performed in the College of American Pathologists (CAP)-accredited and Clinical Laboratory Improvements Amendments (CLIA)-certified Molecular Diagnostics Laboratory, as previously described (32–34), as a component of routine patient care or eligibility for integral-marker clinical trials. Several different sequencing assays had been employed during the years covered by our study: (1) DNA pyrosequencing (PSQ96 HS System; Biotage AB; n = 202; ref. 34); (2) sequenom matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Sequenom MassARRAY; Sequenom; n = 148; ref. 32); and (3) next-generation sequencing (NGS) with the Ion Torrent Personal Genome Machine (Life Technologies; n = 130; ref. 33). For all three sequencing techniques, a minimum cellularity of 20% (i.e., tumor nuclei represent 20% of total nuclei in the tested sample) was required to avoid false negative results.

VE1 IHC

IHC was performed for this study on formalin-fixed, paraffin-embedded whole tissue sections of the 480 colorectal carcinomas in the CAP-accredited and CLIA-certified clinical IHC Laboratory with the VE1 mouse monoclonal antibody to BRAF V600E protein (clone VE1, 1:50; Spring Bioscience). For the Leica Bond method (n = 480 colorectal carcinoma), parameters were as follows: antigen retrieval, Tris-EDTA buffer, pH 9.0 (20 minutes); ADV-060 antibody diluent (Spring Bioscience); and Leica Bond automated system with Bond Polymer Refine Detection kit (Leica Biosystems). For the Ventana BenchMark system subset (n = 92 colorectal carcinoma), parameters were as follows: antigen retrieval, Tris-EDTA buffer, pH 9.0 (20 minutes); ADV-060 antibody diluent (Spring Bioscience); and Ventana BenchMark ultra-automated system and standard reagents provided by
Ventana (OptiView DAB IHC Detection Kit; Ventana Medical Systems).

VE1 cytoplasmic staining was scored microscopically at the time of the study by semiquantitative analysis by one pathologist (J.S. Estrella) according to the following scoring system: 0, negative; 1, weak staining in <20% of tumor cells (Fig. 1A); 2, moderate to strong staining in <20% of tumor cells (Fig. 1B); 3, weak staining in 20% to 70% of tumor cells; 4, moderate to strong staining in 20% to 70% of tumor cells; 5, weak staining in >70% of tumor cells (Fig. 1C); and 6, moderate to strong staining in >70% of tumor cells (Fig. 1D). Cases that had weakly positive staining (scores 1, 3, and 5), cases that exhibited moderately to strongly positive staining in <20% of tumor cells (score 2), and cases discordant with the sequencing results were reviewed by two other pathologists (M.T. Tetzlaff and R.R. Broaddus) to evaluate the initial interpretation. Difference in classification was resolved by a consensus agreement. None of the pathologists (J.S. Estrella, M.T. Tetzlaff, and R.R. Broaddus) had access to the sequencing results at the time of IHC scoring.

Statistical analysis
Sensitivity was defined as the ratio of true positives on VE1 IHC to positives identified by \(BRAF\) sequencing, and specificity was defined as the ratio of true negatives on VE1 IHC to negatives on sequencing, both expressed as percentage. Spearman rho (\(\rho\)) was calculated to assess the nonparametric correlation between VE1 IHC results and results of the different sequencing methods.

Results
Study population
The characteristics of patients included in the study are summarized in Table 1. The 15 \(BRAF\) mutations other than \(V600E\) identified by sequencing were as follows: \(D594G\) (6), \(K601E\) (2), \(G464V\) (1), \(G466V\) (1), \(G466E\) (1), \(G469R\) (1), \(D594N\) (1), \(L597R\) (1), and \(V600K\) (1).

Approximately equal numbers of \(BRAF\) wild-type cases had been analyzed with one of the three sequencing techniques, because \(BRAF\) wild-type cases were randomly selected to include an equivalent number of cases from each year of the study. In contrast, most (63%) of the tumors with a \(BRAF\) mutation, including both \(V600E\) and mutations other than \(V600E\), had been identified using pyrosequencing. The percentage of \(BRAF\) mutation by pyrosequencing was 49% (99/202); by Sequenom, 28% (41/148); and by NGS, 13% (17/130). These data highlight an important limitation in that the relative frequency of mutation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>(BRAF) wild-type ((n = 323))</th>
<th>(BRAF) V600E mutation ((n = 142))</th>
<th>(BRAF) non-V600E mutation ((n = 15))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age, years (range)</td>
<td>59 (14–91)</td>
<td>65 (27–89)</td>
<td>69 (30–81)</td>
</tr>
<tr>
<td>Sex, female: male</td>
<td>150:173</td>
<td>77:65</td>
<td>9:6</td>
</tr>
<tr>
<td>Sequencing method, no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>103 (32)</td>
<td>91 (64)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Sequenom</td>
<td>107 (33)</td>
<td>37 (26)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Next-generation sequencing</td>
<td>113 (35)</td>
<td>34 (10)</td>
<td>3 (20)</td>
</tr>
</tbody>
</table>

Table 1. Colorectal carcinoma patient characteristics
may be dependent upon the methodology employed. Additional studies are warranted to determine the most appropriate gold standard for comparison to IHC, although, these additional studies are beyond the scope of the current study. As a result of the findings, however, the Bond IHC method was evaluated in relation to the individual sequencing methods, as well as to all three sequencing methods combined.

VE1 IHC using the Bond method in all colorectal carcinoma

Results of VE1 IHC using the Bond method are tabulated in Supplementary Table S1. Among the 142 colorectal carcinoma cases that had a BRAF V600E mutation by sequence analysis, 77 cases (54%) had cytoplasmic staining in >70% of tumor cells (scores 5–6) by VE1 IHC, 29 cases (20%) had cytoplasmic staining in 20% to 70% of tumor cells (scores 3–4), and 19 cases (13%) had cytoplasmic staining in <20% of tumor cells (score 1). However, 17 cases (12%) known to have a BRAF V600E mutation by sequencing did not stain with the VE1 antibody. None of the colorectal carcinoma cases with BRAF V600E mutation had moderate to strong cytoplasmic staining in <20% of tumor cells (score 2). Thus, the sensitivity of VE1 IHC was low, as only 54% of the colorectal carcinoma cases with BRAF V600E mutation by sequence analysis had unambiguously positive VE1 IHC.

Among the 323 colorectal carcinoma cases with wild-type BRAF by sequence analysis, 196 cases (61%) did not stain with the VE1 antibody. However, 104 cases (32%) exhibited cytoplasmic staining in <20% of tumor cells (scores 1–2), and 23 cases (7%) had cytoplasmic staining in ≥20% of tumor cells (scores 3–6), including 7 cases with diffuse staining. Thus, the specificity of VE1 IHC was low, as 39% of BRAF wild-type colorectal carcinomas exhibited VE1 IHC staining.

Among the 15 colorectal carcinoma cases with BRAF mutation other than V600E by sequence analysis, 12 cases (80%) did not stain with the VE1 antibody, although 3 cases (20%) had cytoplasmic staining in <20% of tumor cells.

The sensitivity and specificity using different definitions for positive VE1 staining are summarized in Table 2. The definition yielding the highest sensitivity and specificity was staining in ≥20% of tumor cells regardless of intensity, which we applied in the subanalysis below.

Non-specific staining was frequently seen in both BRAF wild-type and BRAF-mutated cases (Supplementary Fig. S1) and included the following patterns: weak to strong nuclear and cytoplasmic staining of histopathologically normal colonic mucosa and inflammatory cells; weak to strong granular cytoplasmic staining of smooth muscle in muscularis mucosae, muscularis propria, and thick-walled blood vessels; strong staining of luminal mucin; and strong staining of brush border of bronchial epithelium accompanying lung metastasis.

VE1 IHC using the Bond method in comparison with sequencing method

The sensitivity and specificity of VE1 IHC using the Bond method and applying the definition of positive staining as cytoplasmic staining in ≥20% of tumor cells are summarized in Table 3 according to the BRAF sequencing technique that was used. For all three sequencing techniques, the majority of colorectal carcinoma cases with BRAF V600E identified by sequencing was positive by VE1 IHC in ≥20% of tumor cells; sensitivities ranged from 89% to 95%. However, for each sequencing technique, some cases identified as positive for BRAF V600E were negative by VE1 IHC; sensitivities ranged from 70% to 84%. The relatively low sensitivity of VE1 IHC to detect BRAF V600E has great potential clinical impact, as this finding indicates that some colorectal carcinoma cases with BRAF V600E mutation would be missed if only IHC were used as the method of detection.

Among 36 cases with BRAF V600E mutation by sequence analysis but negative cytoplasmic staining (scores 0 and 1) on VE1 IHC (Supplementary Table S1), BRAF mutation status in the

Table 2. Sensitivity and specificity of VE1 IHC in colorectal carcinoma using the Bond method

<table>
<thead>
<tr>
<th>BRAF mutation status by sequence analysis</th>
<th>VE1 IHC result</th>
<th>Definition of positive VE1 IHC result*</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT or non-V600E mutation</td>
<td></td>
<td>Scores 1–6</td>
</tr>
<tr>
<td>Negative</td>
<td>208</td>
<td>315</td>
</tr>
<tr>
<td>Positive</td>
<td>130</td>
<td>23</td>
</tr>
</tbody>
</table>

V600E mutation

| Negative                                 | 17             | 36         | 36         | 97         |
| Positive                                 | 125            | 106        | 106        | 45         |

Negative

| Sensitivity                               | 88%            | 75%        | 75%        | 32%        |
| Specificity                               | 62%            | 93%        | 88%        | 91%        |

Abbreviation: WT, wild-type.

*A positive VE1 IHC result was defined as staining in ≥20% of tumor cells.

Table 3. Sensitivity and specificity of VE1 IHC in colorectal carcinoma using the Bond method according to sequencing method used as the criterion standard

<table>
<thead>
<tr>
<th>VE1 IHC result*</th>
<th>Pyrosequencing result</th>
<th>Illumina sequencing result</th>
<th>Next-generation sequencing result</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BRAF WT or non-V600E mutation</td>
<td>BRAF V600E mutation</td>
<td>BRAF WT or non-V600E mutation</td>
</tr>
<tr>
<td>Negative</td>
<td>99</td>
<td>27</td>
<td>106</td>
</tr>
<tr>
<td>Positive</td>
<td>12</td>
<td>64</td>
<td>5</td>
</tr>
</tbody>
</table>

Sensitivity

| 70%            | 84%        | 95%        | 79%        |
| Specificity    | 89%        | 95%        | 95%        |
| Spearman’s rho | 0.487      | 0.707      | 0.576      |

Abbreviation: WT, wild-type.

*A positive VE1 IHC result was defined as staining in ≥20% of tumor cells.
The majority of cases was determined by pyrosequencing (27 cases, 75%). One possible explanation for the discordance was that pyrosequencing may have erroneously detected the \(\text{BRAF} \ V600E \) mutation. Indeed, comparison of VE1 IHC results against pyrosequencing results yielded the lowest correlation coefficient (\(\rho = 0.487\), Table 3) and the lowest sensitivity (70%) and specificity (89%). We attempted to repeat \(\text{BRAF} \ V600E \) mutation analysis by pyrosequencing, results of additional molecular analyses performed on the same tumor block were as follows: 21 of 22 were \(\text{KRAS} \ \text{wild-type} \) in codons 12, 13, and 61, as expected because \(\text{KRAS} \) and \(\text{BRAF} \) mutations are usually mutually exclusive; 5 cases exhibited high levels of microsatellite instability by PCR and methylation of the \(\text{MLH-1} \) promoter region, again expected because MSI-H secondary to somatic \(\text{MLH-1} \) hypermethylation or mutation is commonly associated with \(\text{BRAF} \) mutation; 2 cases exhibited methylation of the \(\text{MLH-1} \) promoter region but did not have microsatellite instability analyzed by PCR; 2 cases exhibited high levels of microsatellite instability by PCR but did not have \(\text{MLH-1} \) promoter methylation analyzed. Only two cases did not have additional molecular analyses.

We reviewed the sequencing result of 18 cases with \(\text{BRAF} \ V600E \) mutation but negative (score 0) VE1 IHC by the Bond method to determine the mutation signal of these cases. Among 18 cases, 17 (94%) were definitive for the \(\text{BRAF} \ V600E \) mutation with allele burden ranging from 10% to 60% (median = 30%). In one case, there is a low level mutation (allele burden = 6%); however, repeat testing yielded the same result.

Among the 13 cases that were \(\text{BRAF} \) wild-type by sequence analysis but had moderate to strong cytoplasmic staining in \(\geq20\% \) of tumor cells (scores 4 and 6) on VE1 IHC (Supplementary Table S1), \(\text{BRAF} \) mutation status had been determined by pyrosequencing in five cases (38%, 5/13) and by Sequenom and NGS in four cases each (31%, 4/13). One possible explanation for the discordance was that the tissue was amenable to IHC studies, whereas DNA isolated from the tumor was suboptimal for molecular testing. However, in 9 of the 13 cases, molecular testing performed on the same tumor block generated informative results for other genes that were tested; in 4 cases other sequence analyses yielded a negative result. Additional molecular analyses for all the 13 cases are tabulated in Supplementary Table S3.

VE1 IHC using the Ventana method in colorectal carcinoma

Because the Ventana platform was the method recommended by the VE1 antibody vendor and the majority of previously published reports used the Ventana method ([22–24, 26, 27, 29, 30]), we also performed VE1 IHC on selected colorectal carcinoma cases, which by the Bond method were concordant or discordant with the sequencing result. A total of 92 cases was re-evaluated in this manner: 57 cases harboring the \(\text{BRAF} \ V600E \) mutation, 33 \(\text{BRAF} \) wild-type cases, and 2 cases harboring \(\text{BRAF} \) mutations other than \(\text{V600E} \). Results are summarized in Supplementary Table S2. With the Ventana method, among the 57 colorectal carcinoma cases that harbored \(\text{BRAF} \ V600E \) mutation by sequencing, 36 cases (63%) exhibited diffuse cytoplasmic staining, whereas 6 cases (11%) had no staining or weak staining in <20% of tumor cells. Among the \(\text{BRAF} \) wild-type colorectal carcinomas, 16 (48%) had no staining or weak staining in <20% of tumor cells, whereas 15 (45%) had positive cytoplasmic staining, including 5 cases with moderate to strong cytoplasmic staining in >20% of tumor cells.

To compare our Bond and Ventana VE1 IHC findings in these 92 cases, we plotted the Bond score (x-axis) versus the Ventana score (y-axis) for each individual case (Fig. 2). As depicted in the figure, there is a random distribution of individual cases, both \(\text{BRAF} \ V600E \) and \(\text{BRAF} \) wild-type/non-\(\text{V600E} \), across VE1 IHC scores using the two different IHC staining platforms.

**Figure 2.**

Scatter plot of VE1 IHC scoring results with the Bond method (x-axis) and Ventana method (y-axis) for 92 colorectal carcinoma cases. Blue represents \(\text{BRAF} \ V600E \), and red represents \(\text{BRAF} \) wild-type or \(\text{BRAF} \) mutations other than \(\text{V600E} \). There is a random distribution of individual cases, both \(\text{BRAF} \ V600E \) and \(\text{BRAF} \) wild-type/non-\(\text{V600E} \), across VE1 IHC scores using the two different IHC staining platforms.

**VE1 IHC with Bond method in melanoma sequenced by NGS**

VE1 IHC with the Bond method had been performed at our laboratory on 144 melanomas with known \(\text{BRAF} \) mutation status by NGS (49 with \(\text{BRAF} \ V600E \), 74 \(\text{BRAF} \) wild-type, and 21 with \(\text{BRAF} \) non-\(\text{V600E} \) mutations). Among the 49 melanomas harboring \(\text{BRAF} \ V600E \) mutation, 48 stained with the VE1 antibody, representing 98% sensitivity. Among the 95 \(\text{BRAF} \) wild-type and non-\(\text{V600E} \) melanomas, 91 were negative with the VE1 antibody, representing 96% specificity [data presented in part in Tetzlaff and colleagues (35)].

**VE1 IHC with Ventana method in melanoma sequenced by NGS**

VE1 IHC with the Ventana method had been performed at our laboratory on 90 melanomas with known \(\text{BRAF} \) mutation status by NGS (25 with \(\text{BRAF} \ V600E \), 48 \(\text{BRAF} \) wild-type, and 17 with \(\text{BRAF} \) non-\(\text{V600E} \) mutations). Among the 25 melanomas with \(\text{BRAF} \ V600E \) mutation, 23 were positive for the VE1 antibody, representing sensitivity of 92%. Among the 65 \(\text{BRAF} \) wild-type and non-\(\text{V600E} \) melanomas, 63 were negative for the VE1 antibody, representing specificity of 97%.
VE1 IHC in papillary thyroid carcinoma

Our sample size of papillary thyroid carcinoma is limited. Only 36 cases were previously tested by Sequenom or NGS. Of these, 16 harbored BRAF V600E mutation, and 20 were BRAF wild-type. VE1 IHC by the Bond method was performed in 17 cases (9 BRAF V600E and 8 wild-type). All BRAF V600E cases were positive by VE1 IHC, and all BRAF wild-type cases were negative by VE1 IHC, yielding sensitivity and specificity of 100%. VE1 IHC by the Ventana method was performed in 19 cases (7 BRAF V600E and 12 wild-type). All BRAF V600E cases were positive by VE1 IHC, and 11 of 12 BRAF wild-type cases were negative by VE1 IHC, for a sensitivity of 100% and a specificity of 92%. The discordant case was a metastatic papillary thyroid carcinoma involving a lymph node that showed strong VE1 expression by IHC but no mutation by NGS. Given the small amount of tumor present in the tissue tested, it is likely that tumor DNA was diluted with lymphocyte DNA during DNA extraction, resulting in a false-negative finding on sequencing.

Discussion

In our study, we examined VE1 IHC findings as a surrogate for BRAF V600E mutation status in colorectal carcinoma and compared the results with melanoma and papillary thyroid carcinoma. We believe this is the largest study of its type to date in colorectal carcinoma. In colorectal carcinoma, VE1 IHC using the Bond method and a definition of positivity of cytoplasmic staining in ≥20% of tumor cells yielded a sensitivity of 75% and a specificity of 93%. When selected cases were re-evaluated using the Ventana method, a disturbing proportion of cases again showed IHC-sequencing discordance: 11% of colorectal carcinomas with BRAF V600E were negative with the VE1 antibody, and 18% of colorectal carcinomas with BRAF wild-type exhibited diffuse cytoplasmic staining. Our findings show that in colorectal carcinoma, VE1 IHC is not a reliable method for determining the BRAF V600E mutational status. In contrast, our results in melanoma and papillary thyroid carcinoma are in keeping with previously published data showing excellent concordance between molecular analysis and VE1 IHC (36–41).

Other published studies examining the concordance between IHC and sequencing for assessing BRAF V600E in colorectal carcinoma are summarized in Table 4. Our results are similar to those of three prior studies (19, 25, 30). Adackapara and colleagues evaluated 52 colorectal carcinoma whole tissue sections and reported a sensitivity of 71% and a specificity of 74% using a manual staining technique (19). Lasota and colleagues used the Leica Bond platform to evaluate 113 colorectal carcinoma whole tissue sections and reported a sensitivity of 89% and specificity of 51% when weak staining was considered positive, and a sensitivity of 85% and specificity of 68% when only moderate to strong staining was considered positive. Finally, Loes and colleagues evaluated 99 colorectal carcinoma tissue microarray cores using the Ventana BenchMark XT platform and reported a sensitivity of 59% and a specificity of 84%. In contrast, multiple other studies have reported sensitivities of 96% to 100% and specificities of 94% to 100% in colorectal carcinoma using the Leica Bond and Ventana platforms (20–24, 29).

As multiple studies have highlighted, a proper scoring system is necessary to reduce false-positive and false-negative cases; however, VE1 IHC scoring is controversial. The studies by Lasota and colleagues (25) and Kuan and colleagues (29) suggested that weak staining (even diffuse) was an unreliable result, although the study by Bledsoe and colleagues (21) concluded that diffuse (or near-uniform) weak positive staining should be regarded as true-positive staining. In our study, we found that defining positive staining as cytoplasmic staining in ≥20% of tumor cells, regardless of intensity, yielded the best combination of sensitivity and specificity.

The discrepancies between these different studies have been attributed to methodology and equipment (21, 23, 24, 26). Multiple factors have been shown to affect the performance of the VE1 antibody. Staining was reported to differ significantly between different antibody lots from the vendor and different batches of reagents used (29). In addition, certain pre-analytical variables, including fixation for <6 or >72 hours and a delay in fixation of >6 hours, negatively impact the staining pattern and signal intensity (24). Testing of these variables was beyond the scope of this study using retrospective specimens, and thus we cannot determine whether these issues contributed to our observed low sensitivity and specificity. In addition, heat-induced antigen retrieval using an acidic solution was reported to be suboptimal compared with the use of a basic solution (29, 31). In our laboratory, we used an acidic solution was reported to be suboptimal compared with the use of a basic solution (29, 31).

Table 4. Studies of concordance between BRAF V600E IHC (VE1 IHC) and sequencing results in colorectal carcinoma

<table>
<thead>
<tr>
<th>First author</th>
<th>Sequencing method</th>
<th>Tissue source</th>
<th>Detection method for IHC</th>
<th>VE1 IHC/BRAF V600E by sequencing</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adackapara et al. (11)</td>
<td>Pyrosequencing</td>
<td>WS</td>
<td>Manual</td>
<td>12/17</td>
<td>71</td>
<td>74</td>
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<td>Affolter et al. (12)</td>
<td>Pyrosequencing</td>
<td>WS</td>
<td>Ventana Ultraview</td>
<td>14/14</td>
<td>100</td>
<td>100</td>
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<td>Bledsoe et al. (13)</td>
<td>Multiplex PCR</td>
<td>TMA + WS</td>
<td>Leica Bond-III</td>
<td>57/59</td>
<td>96</td>
<td>99</td>
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<tr>
<td>Capper et al. (14)</td>
<td>Sanger and pyrosequencing</td>
<td>WS</td>
<td>Ventana OptiView</td>
<td>10/11</td>
<td>100</td>
<td>99</td>
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<tr>
<td>Day et al. (15)</td>
<td>Sanger and SNPShot</td>
<td>TMA</td>
<td>Ventana OptiView</td>
<td>59/59</td>
<td>100</td>
<td>100</td>
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<tr>
<td>Dvorak et al. (16)</td>
<td>Sanger, SNPShot, and NGS</td>
<td>TMA</td>
<td>Ventana OptiView</td>
<td>86/86</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Kuan et al. (19)</td>
<td>Multiplex allele-specific PCR</td>
<td>WS</td>
<td>Ventana BenchMark Ultra</td>
<td>74/74</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>Lasota et al. (17)</td>
<td>Multiples analyses</td>
<td>WS</td>
<td>Leica Bond-Max</td>
<td>24/27</td>
<td>89</td>
<td>51</td>
</tr>
<tr>
<td>Loes et al. (22)</td>
<td>Sanger and LightMix</td>
<td>TMA</td>
<td>Ventana BenchMark XT</td>
<td>13/22</td>
<td>59</td>
<td>84</td>
</tr>
<tr>
<td>Rossie et al. (18)</td>
<td>Sanger and ultra-deep sequencing</td>
<td>WS</td>
<td>Ventana OptiView</td>
<td>37/37</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Roth et al. (23)</td>
<td>Multiplex PCR</td>
<td>WS</td>
<td>Leica Bond</td>
<td>28/32</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Sincrope et al. (19)</td>
<td>Multiplex PCR and RT-PCR</td>
<td>WS</td>
<td>Leica Bond-III</td>
<td>43/44</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: RT, reverse transcription; TMA, tissue microarray; WS, whole tissue sections; WT, wild-type.

a Only colorectal cases were included.

b Combined retrospective and prospective.
c Results using whole tissue sections only were included.

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broad solution for antigen retrieval (Tris-EDTA, pH 9.0) with the Bond method and used ultra cell conditioning 1 (recommended by the vendor) with the Ventana method. Studies also showed that the use of VE1 antibody with the Ventana detection kit and Ventana automated stainer yielded optimal results (24). In our study, VE1 IHC using the Ventana method on selected cases again provided results with substantial IHC-sequencing discordance.

Kuan and colleagues performed rigorous optimization of VE1 IHC staining conditions and concluded that this step is necessary to achieve reliable results (29). Our laboratory similarly tested numerous protocols and employed two different automated stainers to optimize staining conditions for the VE1 antibody to attempt to improve sensitivity and specificity, albeit the VE1 antibody optimization relied heavily upon BRAF V600E mutant melanomas with fewer colorectal carcinoma and papillary thyroid carcinomas as positive controls. Despite these steps, in our experience, VE1 IHC was a suboptimal technique for detecting BRAF V600E mutation in colorectal carcinoma. In contrast, we found that VE1 IHC reliably detected BRAF V600E mutation in both melanoma and papillary thyroid carcinomas. Similarly, Loes and colleagues demonstrated high concordance between molecular and VE1 IHC results in melanoma (sensitivity, 89%; specificity, 100%) but suboptimal VE1 IHC results in colorectal carcinoma (sensitivity, 59%; specificity, 84%; ref. 30). Together, these findings suggest that the IHC-sequencing discordance observed in colorectal carcinoma may not be secondary to the IHC protocols and platforms used.

Multiple studies have suggested that cases in which VE1 IHC results are positive but sequencing results are negative might be attributable to the lower sensitivity of molecular analysis (24, 26, 27). Specifically, the contention is that low tumor volumes and dilution with nontumoral tissue can produce a false-negative attributable to the lower sensitivity of molecular analysis IHC protocols and platforms used.

Multiple studies have suggested that in cases where VE1 IHC results are positive but sequencing results are negative, it may be attributable to the lower sensitivity of molecular analysis (24, 26, 27). Specifically, the contention is that low tumor volumes and dilution with nontumoral tissue can produce a false-negative attributable to the lower sensitivity of molecular analysis IHC protocols and platforms used.

In summary, we performed the largest study to date to evaluate the reliability of VE1 IHC as a surrogate marker for BRAF V600E mutation in colorectal carcinoma. We found that, in contrast with what has been reported in melanoma and papillary thyroid carcinoma, the sensitivity and specificity of VE1 IHC in colorectal carcinoma are suboptimal, indicating that this technique should not be used to guide therapy and clinical management of patients with colorectal carcinoma. In the era of personalized medicine, more rigorous validation of tests with prognostic and predictive importance is necessary to optimize care for cancer patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J.S. Estrella, A. Rashid, R.R. Broaddus


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Estrella, M.T. Tezlafl, K.P. Patel, M.D. Williams, A. Rashid, R.R. Broaddus

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.S. Estrella, M.T. Tezlafl, R.I. Bassett Jr, M.D. Williams, S.R. Hamilton, R.R. Broaddus
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.S. Estrella, J.L. Curry, S.R. Hamilton

Study supervision: R.R. Broaddus

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References


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