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

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Abstract

Although sequencing provides the gold standard for identifying colorectal carcinomas 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 V600E-mutated colorectal carcinomas, 36 BRAF non-V600E-mutated colorectal carcinomas, 77 with diffuse VE1 staining and 48 with BRAF wild-type colorectal carcinomas, 16 (48%) had no or weak staining, whereas 15 (45%) had heterogeneous staining. In contrast 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, but 17 (12%) were negative. Among 323 BRAF wild-type colorectal carcinomas, 196 (61%) were negative, but 127 (39%) had staining, including 7 with diffuse staining. When positivity was defined as staining in ≥20% of tumor cells, VE1 IHC had sensitivity of 75% and specificity of 93% for BRAF V600E mutation. With the Ventana method, among 57 BRAF V600E-mutated colorectal carcinomas, 36 (63%) had diffuse VE1 staining, whereas 6 (11%) had no or weak (<20% of tumor cells) staining. Among 33 BRAF wild-type colorectal carcinomas, 16 (48%) had no or weak staining, whereas 15 (45%) 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.

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Introduction

The BRAF proto-oncogene encodes a serine/threonine kinase belonging to the raf/mrk 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 panitumunab (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
carcinomas have very limited response to BRAF V600E inhibitor monotherapy (13).

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 man-
agement 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 associ-
ated 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 sensi-
tivity 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 sequenc-
ing 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. 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. We 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 col-
rectal carcinoma (primary tumors and metastases) from 2008 through 2013 for which BRAF mutation analysis had been performed. As previously published studies have reported sen-
sitivity 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
carcinomas 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 iden-
tify 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, for-
malin-fixed, paraffin-embedded whole tissue sections, and sequencing had been performed in the College of American Pathologists (CAP)-accredited and Clinical Laboratory Improve-
mants Amendments (CLIA)-certified Molecular Diagnostics Lab-
atory, 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, ultra cell conditioning 1 (64 minutes), and Ventana Bench-
Mark 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 cytoplasmic staining in <20% of tumor cells (Fig. 1B); 3, weak cytoplasmic staining in 20% to 70% of tumor cells (score = 5); D, moderate to strong cytoplasmic staining in >70% of tumor cells (score = 6). A-D, magnification, ×100.

Figure 1.
Examples of VE1 IHC in colorectal carcinomas. A, weak cytoplasmic staining in <20% of tumor cells (score = 1); B, moderate to strong cytoplasmic staining in <20% of tumor cells (score = 2); C, weak cytoplasmic staining in 20% to 70% of tumor cells (score = 5); D, moderate to strong cytoplasmic staining in >70% of tumor cells (score = 6). A-D, magnification, ×100.

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 (ρ) 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...
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 these 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 VE IHC was low, as only 54% of the colorectal carcinoma cases with BRAF V600E mutation by sequence analysis had unambiguous 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, muscular 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 were 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 was 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></td>
</tr>
<tr>
<td>Negative</td>
<td>208</td>
<td>70%</td>
</tr>
<tr>
<td>Positive</td>
<td>130</td>
<td>84%</td>
</tr>
<tr>
<td>V600E mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>677</td>
<td>89%</td>
</tr>
<tr>
<td>Positive</td>
<td>106</td>
<td>95%</td>
</tr>
</tbody>
</table>

Abbreviation: WT, wild-type.

*0, negative; 1, weak staining in <20% of tumor cells; 2, moderate to strong staining in <20% of tumor cells; 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; and 6, moderate to strong staining in >70% 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>Sequenom 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>
<tr>
<td>Sensitivity</td>
<td>70%</td>
<td>84%</td>
<td>95%</td>
</tr>
<tr>
<td>Specificity</td>
<td>89%</td>
<td></td>
<td>95%</td>
</tr>
</tbody>
</table>

Abbreviation: WT, wild-type.

*A positive VE1 IHC result was defined as staining in ≥20% of tumor cells.
majority of cases was determined by pyrosequencing (27 cases, 75%). One possible explanation for the discordance was that pyrosequencing may have erroneously detected the 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 BRAF mutation analysis by NGS to address this issue, but insufficient DNA remained in our laboratory. Among the 27 cases with BRAF V600E mutation by pyrosequencing, results of additional molecular analyses performed on the same tumor block were as follows: 21 of 22 were KRAS wild-type in codons 12, 13, and 61, as expected because KRAS and BRAF mutations are usually mutually exclusive; 5 cases exhibited high levels of microsatellite instability by PCR and methylation of the MLH-1 promoter region, again expected because MSI-H secondary to somatic MLH-1 hypermethylation or mutation is commonly associated with BRAF mutation; 2 cases exhibited methylation of the 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 MLH-1 promoter methylation analyzed. Only two cases did not have additional molecular analysis.

We reviewed the sequencing result of 18 cases with 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 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 BRAF wild-type by sequence analysis but had moderate to strong cytoplasmic staining in $\geq$20% of tumor cells (scores 4 and 6) on VE1 IHC (Supplementary Table S1), 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 BRAF V600E mutation, 33 BRAF wild-type cases, and 2 cases harboring BRAF mutations other than V600E. Results are summarized in Supplementary Table S2. With the Ventana method, among the 57 colorectal carcinoma cases that harbored 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 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 $\geq$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 BRAF V600E and BRAF wild-type/non-V600E across VE1 IHC scores using the two different IHC staining platforms. Neither BRAF V600E nor wild-type/non-V600E cases demonstrated concordance with respect to their BRAF status by VE1 IHC.

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 BRAF mutation status by NGS (49 with BRAF V600E, 74 BRAF wild-type, and 21 with BRAF non-V600E mutations). Among the 49 melanomas harboring BRAF V600E mutation, 48 stained with the VE1 antibody, representing 98% sensitivity. Among the 95 BRAF wild-type and non-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 had been performed with the Ventana method in our laboratory on 90 melanomas with known BRAF mutation status by NGS (23 with BRAF V600E, 48 BRAF wild-type, and 17 with BRAF non-V600E mutations). Among the 25 melanomas with BRAF V600E mutation, 23 were positive for the VE1 antibody, representing sensitivity of 92%. Among the 65 BRAF wild-type and non-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 a

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**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>+VE1 IHC/BRAF WT by sequencing</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
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<td>Adackapara et al. (11)</td>
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<td>Manual</td>
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<td>WS</td>
<td>Ventana UltraView</td>
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<td>0/17</td>
<td>100</td>
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<td>TMA + WS</td>
<td>Leica Bond-Max</td>
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<td>2/45</td>
<td>96</td>
<td>99</td>
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<td>Sanger and pyrosequencing</td>
<td>WS</td>
<td>Ventana OptiView</td>
<td>11/11</td>
<td>1/80</td>
<td>100</td>
<td>99</td>
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<td>Sanger and SNapShot</td>
<td>TMA</td>
<td>Ventana OptiView</td>
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<td>0/416</td>
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<td>TMA</td>
<td>Ventana OptiView</td>
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<td>2/193</td>
<td>100</td>
<td>99</td>
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<td>PCR</td>
<td>WS</td>
<td>Ventana BenchMark Ultra</td>
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<td>3/54</td>
<td>100</td>
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<td>Lasota et al. (18)</td>
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<td>Ventana BenchMark XT</td>
<td>24/27</td>
<td>22/86</td>
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<tr>
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<td>TMA</td>
<td>Ventana BenchMark XT</td>
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<td>12/77</td>
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<td>Sanger and ultra-deep sequencing</td>
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<td>Ventana OptiView</td>
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<td>1/21</td>
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<td>95</td>
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<tr>
<td>Roth et al. (21)</td>
<td>Multiplex PCR</td>
<td>WS</td>
<td>Leica Bond</td>
<td>28/32</td>
<td>0/23</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Sinicrope et al. (22)</td>
<td>Multiplex PCR and RT-PCR</td>
<td>WS</td>
<td>Leica Bond</td>
<td>49/49</td>
<td>0/25</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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

*Only colorectal cases were included.*

*Combined retrospective and prospective.*

*Results using whole tissue sections only were included.*
These CLIA-certified, CAP-accredited laboratories reliably detect the mutation of interest (ref. 42), our lab has shown that 10 ng of formalin-fixed, paraffin-embedded DNA is sufficient to amplify mutation hotspots in 46 genes (including BRAF) using Ion Torrent Personal Genome Machine (NGS; ref. 33). In fact, we routinely perform DNA sequencing on biopsy specimens, including fine-needle aspiration specimens, in our laboratory. 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 molecular result. Although this may be the case in studies using Sanger sequencing in which the limit of detection is approximately 20% (i.e., at least 20% of tumor cells must be present in order to reliably detect the mutation of interest; ref. 42), our CAP-accredited, CLIA-certified molecular laboratory has shown the analytical sensitivity of pyrosequencing to be 5% (34) and the analytical sensitivity of both Sequenom (32) and NGS (33) to be approximately 10%. Moreover, in our study, all the 13 cases in which sequence analysis indicated wild-type BRAF and VE1 IHC had either moderate to strong staining in ≥20% of tumor cells. In four of these cases, we and others have reported non-specific VE1 staining on normal cells adjacent to colorectal carcinoma (20, 24, 25, 28) and in normal anterior pituitary and adrenal cortex (47). Recently, Jones and colleagues showed sequence homology between the VE1 synthetic peptide and regions of multiple axonal dynein heavy chain proteins (DNAH2, DNAH7, and DNAH12). Similar proteins may be present in some colorectal carcinomas and pituitary adenomas that cross-react with the VE1 antibody, significantly impacting the specificity of VE1 IHC in these tumors. It is also possible that tissue-specific factors unique to colonic mucosa (i.e., not present in melanoma or papillary thyroid carcinoma) influence expression or modification of BRAF proteins independent of mutation status, resulting in altered antigenicity (and nonmutation-related VE1 IHC reactivity). Moreover, the possible roles of pathway regulation, especially potential influences on the relationship between gene expression and mutation, in cancers remain to be determined. The Cancer Genome Atlas studies report that mutation at the DNA level without expression at the mRNA level is relatively common (48-50). The possibility remains that protein product expression is also influenced by these factors, such as regulatory noncoding RNAs and posttranslational modifications, thereby providing a biologic, rather than a purely methodologic, explanation of some of the discordances between IHC and sequencing results for BRAF in colorectal carcinoma. Multiple studies have suggested that one of the most important advantages of IHC is that tissue-limited samples that could not be analyzed by molecular techniques would be amenable to IHC (20, 21, 23, 24). In our study, we found significant heterogeneity in VE1 IHC staining in colorectal carcinoma. Among 480 colorectal carcinomas evaluated by IHC, 126 cases (26%) had focal (<20%) cytoplasmic staining in tumor cells. Similarly, Affolter and colleagues evaluated 14 colorectal carcinomas with BRAF V600E mutation and found heterogeneous staining in four cases (29%; ref. 20). Thus, significant sampling error may be encountered when VE1 IHC is performed on a biopsy specimen. In contrast, reliable molecular results may be achieved from limited tissue. Our laboratory has shown that 10 ng of formalin-fixed, paraffin-embedded DNA is sufficient to amplify mutation hotspots in 46 genes (including BRAF) using Ion Torrent Personal Genome Machine (NGS; ref. 33). In fact, we routinely perform DNA sequencing on biopsy specimens, including fine-needle aspiration specimens, in our laboratory.

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. Tetzlaff, 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. Tetzlaff, R.L. Basset Jr, M.D. Williams, S.R. Hamilton, R.R. Broaddus
References


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