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

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Abbreviations used: CAP, College of American Pathologists; CLIA, Clinical Laboratory Improvements Amendments; CRC, colorectal carcinoma; CRYSTAL, Cetuximab Combined with Irinotecan in First-Line Therapy for Metastatic Colorectal Cancer; IHC, immunohistochemistry; MAPK, mitogen-activated protein kinase; NGS, next-generation sequencing; OPUS, Oxaliplatin and Cetuximab in First-Line Treatment of Metastatic Colorectal Cancer; PTC, papillary thyroid carcinoma; TCGA, The Cancer Genome Atlas

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**ABSTRACT**

Although sequencing provides the gold standard for identifying colorectal carcinoma (CRC) 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 CRCs (323 *BRAF* wild-type, 142 *BRAF* V600E mutation, and 15 *BRAF* non-V600E mutation). We also compared the results to melanomas and papillary thyroid carcinomas (PTC).

With the Bond method, among 142 *BRAF* V600E-mutated CRCs, 77 (54%) had diffuse VE1 staining and 48 (33%) had heterogeneous staining, but 17 (12%) were negative. Among 323 *BRAF* wild-type CRCs, 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 CRCs, 36 (63%) had diffuse VE1 staining, while 6 (11%) had no or weak (<20% of tumor cells) staining. Among 33 *BRAF* wild-type CRCs, 16 (48%) had no or weak staining, while 15 (45%) had heterogeneous staining. In contrast to CRC, Bond and Ventana VE1 IHC in melanoma and PTC were highly concordant with sequencing results. We conclude that VE1 IHC produces suboptimal results in CRC and should not be used to guide patient management.
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 mitogen-activated kinase (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 RAS-independent activity of the kinase domain, in turn causing constitutive activation of the MAPK pathway (1). Using *in vivo* mouse models, Rad et al. 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 (CRCs) that harbor the *BRAF* V600E mutation are a distinct subset of tumors. They are frequently associated with poor differentiation, mucinous histology and advanced TNM stage (3). In patients with microsatellite-stable CRC (4, 5) and those with advanced CRC (6), *BRAF* V600E mutation confers worse survival compared to their wild-type counterpart. Furthermore, Lynch syndrome (hereditary non-polyposis colorectal cancer syndrome) is virtually excluded when a CRC 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 CRCs. Some clinical studies, supported by in vitro results (8), have reported a detrimental effect of *BRAF* V600E mutation in patients with CRC treated with the anti-epidermal growth factor 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 CRC patients with BRAF mutations and BRAF wildtype (13). *In vitro* and *in vivo* xenograft models have shown that CRC that harbor *BRAF* V600E may be sensitive to proteasome inhibitors (14). While CRCs 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 CRC 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, Pleasanton, CA) 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 CRC 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 CRCs 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 CRC 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 CRC 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 CRC 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 CRC (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 CRC and BRAF V600E CRC 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 CRC cases to include equal numbers of cases each year from over 1500 CRC 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 CRC 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 CRCs 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 \textit{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.

\textbf{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, Uppsala, Sweden; \(n=202\)) (34); (2) Sequenom matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Sequenom MassARRAY; Sequenom, San Diego, CA; \(n=148\)) (32); and (3) Next-generation sequencing (NGS) with the Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, CA; \(n=130\)) (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 Immunohistochemistry**

IHC was performed for this study on formalin-fixed, paraffin-embedded whole tissue sections of the 480 CRCs 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, Pleasanton, CA). For the Leica Bond method (n=480 CRC), 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, Newcastle Upon Tyne, UK). For the Ventana BenchMark system subset (n=92 CRC), parameters were as follows: antigen retrieval, Ultra cell conditioning 1 (64 minutes), and Ventana BenchMark Ultra automated system and standard reagents provided by Ventana (OptiView DAB IHC Detection Kit; Ventana Medical Systems, Tucson, AZ).

VE1 cytoplasmic staining was scored microscopically at the time of the study by semiquantitative analysis by one pathologist (JSE) 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 (MTT and RRB) to evaluate the initial interpretation. Difference in classification was resolved by a consensus agreement. None of the pathologists (JSE, MTT, and RRB) 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’s 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, since *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 immunohistochemistry 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 CRC**

Results of VE1 IHC using the Bond method are tabulated in Supplementary Table S1. Among the 142 CRC 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 CRC 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 CRC cases with BRAF V600E mutation by sequence analysis had unambiguously positive VE1 IHC.

Among the 323 CRC 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 CRCs exhibited VE1 IHC staining.

Among the 15 CRC cases with BRAF mutation other than V600E by sequence analysis, 12 cases (80%) did not stain with the VE1 antibody, while 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 Figure 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 to 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 CRC cases with *BRAF* V600E identified by sequencing was positive by VE1 IHC in ≥20% of tumor cells; specificities 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 CRC 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 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 (ρ=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 \textit{BRAF} V600E mutation by pyrosequencing, results of additional molecular analyses performed on the same tumor block were as follows: 21 of 22 were \textit{KRAS} wild-type in codons 12, 13 and 61, as expected since \textit{KRAS} and \textit{BRAF} mutation are usually mutually exclusive; 5 cases exhibited high levels of microsatellite instability by PCR and methylation of the \textit{MLH-1} promoter region, again expected because MSI-H secondary to somatic \textit{MLH-1} hypermethylation or mutation is commonly associated with \textit{BRAF} mutation; 2 cases exhibited methylation of the \textit{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 \textit{MLH-1} promoter methylation analyzed. Only two cases did not have additional molecular analysis.

We reviewed the sequencing result of 18 cases with \textit{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 \textit{BRAF} V600E mutation with allele burden ranging from 10\%-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 \textit{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), \textit{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
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 13 cases
are tabulated in Supplementary Table S3.

**VE1 IHC using the Ventana method in CRC**

Since 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
CRC 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 two cases
harboring *BRAF* mutations other than V600E. Results are summarized in
Supplementary Table S2. With the Ventana method, among the 57 CRC cases
that harbored *BRAF* V600E mutation by sequencing, 36 cases (63%) exhibited
diffuse cytoplasmic staining, while 6 cases (11%) had no staining or weak
staining in <20% of tumor cells. Among the *BRAF* wild-type CRCs, 16 (48%) had
no staining or weak staining in <20% of tumor cells, while 15 (45%) had positive
cytoplasmic staining, including five 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 (Figure 2). As depicted in the figure, there is a random distribution of individual cases, both \textit{BRAF} V600E and \textit{BRAF} wild-type/non-V600E, across VE1 IHC scores using the two different immunohistochemical staining platforms. Neither \textit{BRAF} V600E nor wild-type/non-V600E cases demonstrated concordance with respect to their BRAF status by VE1 immunohistochemistry.

**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 \textit{BRAF} mutation status by NGS (49 with \textit{BRAF} V600E, 74 \textit{BRAF} wild-type, and 21 with \textit{BRAF} non-V600E mutations). Among the 49 melanomas harboring \textit{BRAF} V600E mutation, 48 stained with the VE1 antibody, representing 98% sensitivity. Among the 95 \textit{BRAF} wild-type and non-V600E melanomas, 91 were negative with the VE1 antibody, representing 96% specificity (data presented in part in Tetzlaff, et al. (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 \textit{BRAF} mutation status by NGS (25 with \textit{BRAF} V600E, 48 \textit{BRAF} wild-type, and 17 with \textit{BRAF} non-V600E mutations). Among the 25 melanomas with \textit{BRAF} V600E mutation, 23 were positive for the VE1 antibody, representing sensitivity of 92%. Among the 65 \textit{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 contaminating 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 CRC and compared the results to melanoma and papillary thyroid carcinoma. We believe this is the largest study of its type to date.
in CRC. In CRC, 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 CRCs with BRAF V600E were negative with the VE1 antibody, and 18% of CRCs with BRAF wild-type exhibited diffuse cytoplasmic staining. Our findings show that in CRC, 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 CRC are summarized in Table 4. Our results are similar to those of three prior studies (19, 25, 30). Adackapara et al. evaluated 52 CRC whole tissue sections and reported a sensitivity of 71% and a specificity of 74% using a manual staining technique (19). Lasota et al. used the Leica Bond platform to evaluate 113 CRC 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 et al. evaluated 99 CRC 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 CRC 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 et al. (25) and Kuan et al. (29) suggested that weak staining (even diffuse) was an unreliable result, while the study by Bledsoe et al. (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 hours 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 to use of a basic solution (29, 31). In our laboratory, we used a basic solution for antigen retrieval (Tris-EDTA, pH 9.0) with the Bond method and used Ultra cell conditioning 1

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(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 et al. 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 CRC 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 CRC. In contrast, we found that VE1 IHC reliably detected BRAF V600E mutation in both melanoma and papillary thyroid carcinoma. Similarly, Loes et al. demonstrated high concordance between molecular and VE1 IHC results in melanoma (sensitivity, 89%; specificity, 100%) but suboptimal VE1 IHC results in CRC (sensitivity, 59%; specificity, 84%) (30). Together, these findings suggest that the IHC-sequencing discordance observed in CRC 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 non-tumoral tissue can produce a false-negative molecular result. While this may be the case in studies using Sanger sequencing in which the limit of detection is ~20% (i.e. at least 20% of tumor cells must be present in order to reliably detect the mutation of interest) (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 ~10%. Moreover, in our study, all 13 cases in which sequence analysis indicated wild-type $BRAF$ and VE1 IHC had either moderate to strong staining in 20% to 70% of tumor cells had adequate tumor for multiple additional molecular analyses (Supplementary Table S3), with informative molecular results reported for 9 of 13 cases, including four with $KRAS$ mutation, two with high levels of microsatellite instability by PCR, and two with $CTNNB1$ mutation.

While other studies reported excellent concordance between molecular analysis and VE1 IHC in some central nervous system tumors, specifically epithelioid glioblastomas (43), pleomorphic xanthoastrocytoma (44) and dysembryoplastic neuroepithelial tumor (45), other authors found that the utility of VE1 IHC is limited in pituitary adenomas (46), similar to what we found in CRC. Sperveslage et al. evaluated 78 pituitary adenomas using two different platforms (Leica Bond-Max and Ventana OptiView). They found 10 cases that exhibited VE1 cytoplasmic staining, including four with moderate to strong staining, but did not harbor $BRAF$ V600E mutation by Sanger sequencing. Similarly, we and others have reported non-specific VE1 staining on normal cells adjacent to CRC
(20, 24, 25, 28) and in normal anterior pituitary and adrenal cortex (47). Recently, Jones et al. showed sequence homology between the VE1 synthetic peptide and regions of multiple axonemal dynein heavy chain proteins (\textit{DNAH2}, \textit{DNAH7}, and \textit{DNAH12}). Similar proteins may be present in some CRCs 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 non-mutation-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 (TCGA) 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 non-coding RNAs and post-translational modifications, thereby providing a biological, rather than a purely methodological, explanation of some of the discordances between immunohistochemical and sequencing results for \textit{BRAF} in CRC.

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 immunohistochemical staining in CRC.
Among 480 CRCs evaluated by IHC, 126 cases (26%) had focal (<20%) cytoplasmic staining in tumor cells. Similarly, Affolter et al. evaluated 14 CRCs with \(BRAF\) V600E mutation and found heterogeneous staining in four cases (29%) (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 hot-spot regions in 46 genes (including \(BRAF\)) using Ion Torrent Personal Genome Machine (NGS) (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 CRC. We found that, in contrast to what has been reported in melanoma and papillary thyroid carcinoma, the sensitivity and specificity of VE1 IHC in CRC are suboptimal, indicating that this technique should not be used to guide therapy and clinical management of patients with CRC. In the era of personalized medicine, more rigorous validation of tests with prognostic and predictive importance is necessary to optimize care for cancer patients.

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29. Kuan SF, Navina S, Cressman KL, Pai RK. Immunohistochemical detection of BRAF V600E mutant protein using the VE1 antibody in colorectal carcinoma is highly concordant with molecular testing but requires rigorous antibody optimization. Hum Pathol. 2014;45:464-72.


Table 1. Colorectal carcinoma patient characteristics

<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>14 (10)</td>
<td>3 (20)</td>
</tr>
</tbody>
</table>
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&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Scores 1-6</td>
<td>Scores 3-6</td>
</tr>
<tr>
<td>WT or non-V600E mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>208</td>
<td>315</td>
</tr>
<tr>
<td>Positive</td>
<td>130</td>
<td>23</td>
</tr>
<tr>
<td>V600E mutation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>17</td>
<td>36</td>
</tr>
<tr>
<td>Positive</td>
<td>125</td>
<td>106</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>88%</td>
<td>75%</td>
</tr>
<tr>
<td>Specificity</td>
<td>62%</td>
<td>93%</td>
</tr>
</tbody>
</table>

Abbreviation: IHC, immunohistochemistry; WT, wild-type.

<sup>a</sup>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 immunohistochemistry in colorectal carcinoma using the Bond method according to sequencing method used as the criterion standard

<table>
<thead>
<tr>
<th>VE1 IHC result&lt;sup&gt;a&lt;/sup&gt;</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>79%</td>
</tr>
<tr>
<td>Specificity</td>
<td>89%</td>
<td>95%</td>
<td>95%</td>
</tr>
<tr>
<td>Spearman’s rho</td>
<td>0.487</td>
<td>0.707</td>
<td>0.576</td>
</tr>
</tbody>
</table>

Abbreviation: WT, wild-type.

<sup>a</sup>A positive VE1 IHC result was defined as staining in ≥20% of tumor cells.
Table 4. Studies of concordance between BRAF V600E immunohistochemistry (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>
</tr>
</thead>
<tbody>
<tr>
<td>Affolter, et al. [12]</td>
<td>Pyrosequencing</td>
<td>WS</td>
<td>Ventana Ultraview</td>
<td>14/14</td>
<td>0/17</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Day, et al. [15]</td>
<td>Sanger and SNapShot</td>
<td>TMA</td>
<td>Ventana OptiView</td>
<td>59/59</td>
<td>0/416</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dvorak, et al. [16]</td>
<td>Sanger, SNapShot, and NGS</td>
<td>TMA</td>
<td>Ventana OptiView</td>
<td>86/86</td>
<td>2/193</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>Kuan, et al. [21]</td>
<td>PCR</td>
<td>WS</td>
<td>Ventana BenchMark Ultra</td>
<td>74/74</td>
<td>3/54</td>
<td>100</td>
<td>94</td>
</tr>
<tr>
<td>Lasota, et al. [17]</td>
<td>Multiple analyses</td>
<td>WS</td>
<td>Leica Bond-Max Ultra</td>
<td>24/27</td>
<td>22/86</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>12/77</td>
<td>59</td>
<td>84</td>
</tr>
<tr>
<td>Rossle, et al. [18]</td>
<td>Sanger and ultra-deep sequencing</td>
<td>WS</td>
<td>Ventana OptiView</td>
<td>37/37</td>
<td>1/21</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>0/23</td>
<td>88</td>
<td>100</td>
</tr>
<tr>
<td>Sinicrope, et al. [19]</td>
<td>Multiplex allele-specific PCR</td>
<td>WS</td>
<td>Ventana OptiView</td>
<td>49/49</td>
<td>0/25</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Toon, et al. [20]</td>
<td>Multiplex PCR and RT-PCR</td>
<td>WS</td>
<td>Leica Bond-III</td>
<td>43/44</td>
<td>0/157</td>
<td>98</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: PCR, polymerase chain reaction; NGS, next-generation sequencing; 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.
FIGURE LEGENDS

Figure 1. Examples of VE1 immunohistochemistry 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 >70% of tumor cells (score = 5). D, Moderate to strong cytoplasmic staining in >70% of tumor cells (score = 6). A-D, Magnification, ×100.

Figure 2. Scatter plot of VE1 immunohistochemistry scoring results with the Bond method (x-axis) and Ventana method (y-axis) for 92 CRC cases. Blue represents $BRAF\ V600E$, and red represents $BRAF$ wild-type or $BRAF$ mutations other than V600E mutation. 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 immunohistochemical staining platforms.
Figure 2

![VE1 immunohistochemistry scoring by Ventana vs. VE1 immunohistochemistry scoring by Bond](image-url)

Legend:
- **BRAF V600E**
- **BRAF WT/non-V600E**
Molecular Cancer Therapeutics

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

Jeannelyn S. Estrella, Michael Tetzlaff, Roland L. Bassett, Jr., et al.

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