Published in final edited form as:

Am J Surg Pathol. 2014 October ; 38(10): 1418–1428. doi:10.1097/PAS.0000000000000263.

BRAF V600E immunohistochemistry is reliable in primary and metastatic colorectal carcinoma regardless of treatment status and shows high intratumoral homogeneity

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Abstract

In colorectal carcinoma the evaluation of *BRAF* mutation status is increasingly being performed given its utility as a prognostic and predictive biomarker. However, there are conflicting reports of the sensitivity and specificity of BRAF V600E immunohistochemistry, and little is known about its reliability in tissues collected from metastatic sites or following chemo/radiation or targeted therapy. The degree of intratumoral staining heterogeneity is also not well established. We performed immunohistochemistry for BRAF V600E (VE1) on 204 cases of colorectal carcinoma including 59 with the *BRAF V600E* mutation. These included primary (n=147) and metastatic/recurrent (n=57) tumors, collected before (n=133) or after (n=71) chemo/radiation or targeted therapy. Evaluation of a test cohort (39 cases) with knowledge of mutation status established a specific staining pattern for the mutation: diffuse cytoplasmic staining of near-uniform intensity, regardless of strength of staining. Using this pattern, pathologists at three levels of training independently performed blinded evaluation of the remaining cases. BRAF V600E staining was 96.3% sensitive and 98.5% specific for the mutation, including both pre- and post-treatment specimens. Fleiss' kappa for interobserver agreement was 0.96. Staining of whole sections of the *BRAF* mutants showed diffuse staining in all cases and uniform or near-uniform intensity in 91%. In 20 cases with both pre- and post-treatment specimens there was 100% accuracy and agreement in staining between samples. We conclude that BRAF V600E immunohistochemistry is reliable for the evaluation of mutational status in colorectal carcinoma regardless of site or prior treatment history, and staining shows a high degree of intratumoral homogeneity.

Keywords

BRAF; colorectal carcinoma; immunohistochemistry; VE1

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Conflicts of Interest and Source of Funding: The authors declare no conflicts of interest.

Introduction

Activating mutations in the *BRAF* gene - most frequently those resulting in the substitution of glutamate for valine at position 600 (V600E mutation) - occur in a range of human neoplasms including melanoma, hairy cell leukemia, papillary thyroid carcinoma, serous ovarian tumors, and colorectal carcinoma, among others.¹⁻⁴ *BRAF* mutation results in constitutive activation of the MAP-kinase signaling cascade leading to dysregulation of cell proliferation and apoptosis, thereby contributing to the neoplastic process. In colorectal adenocarcinoma, studies have shown that microsatellite stable tumors carrying the *BRAF* mutation are clinically aggressive and are associated with poor survival.⁵⁻⁷ Furthermore, as in *KRAS* mutant colorectal carcinomas, those with *BRAF* mutations may be unresponsive to targeted therapy with inhibitors to epidermal growth factor receptor (EGFR).⁸⁻⁹ Testing for *BRAF* V600E mutation has also been shown to be useful in distinguishing sporadic microsatellite-unstable colorectal carcinomas from those associated with Lynch syndrome.¹⁰⁻¹¹

Given that *BRAF* mutation status is clinically useful as a prognostic and predictive biomarker in colorectal adenocarcinoma, determination of mutation status is increasingly being performed as an adjunct to histopathologic examination. Mutation testing is commonly implemented through polymerase chain reaction (PCR)-based assays or sequencing modalities. Such molecular methodologies are relatively expensive and time-consuming, and may be insensitive in samples with a small quantity and low cellularity of tumor.¹²⁻¹³ This is particularly relevant in post-treatment settings, where residual tumor cells may be scant, resulting in suboptimal tissue samples for molecular testing. The recent development of mutation-specific BRAFV600E monoclonal antibodies has opened up new avenues for rapid immunohistochemical screening for *BRAF* mutations.¹⁴ Such antibodies are now commercially available and have been implemented in many institutions. Application of these antibodies has been shown to be a useful and reliable tool in almost all pathological entities carrying the *BRAF* V600E mutation, including melanoma, hairy cell leukemia, serous ovarian tumors, lung adenocarcinoma, and papillary thyroid cancer,^{3,15-19} and interobserver variability has generally been good.²⁰ Comparisons between specific antibodies have shown somewhat differing reliabilities, with a recent study suggesting a slight superiority of the VE1 antibody for clinical use.⁴

In colorectal neoplasia in particular, the availability of such antibodies is potentially useful not only for prompt determination of mutation status in carcinomas, but also for improved classification of pre-malignant serrated lesions.²¹ Furthermore, when used in a panel with immunostains for mismatch-repair (MMR) proteins, these antibodies facilitate rapid screening for Lynch syndrome and help define aggressive tumors with poor prognosis.^{6,10-11} However, studies on the utility of V600E antibodies in colorectal adenocarcinoma have shown mixed results. Though most have reported a high sensitivity and specificity,²²⁻²⁴ others have found a lower sensitivity and specificity using the same VE1 antibody.²⁵ Furthermore, while reported in small numbers,^{3-4,23,25} the relative reliability of *BRAF* V600E antibodies in samples of metastatic colorectal adenocarcinoma is not well established compared to primary tumors. Lastly, to our knowledge, the effect of traditional chemotherapy (neoadjuvant or adjuvant) or targeted *BRAF* inhibition on *BRAF*

staining in colorectal carcinoma has not been studied, and the degree of staining heterogeneity within an individual tumor is not well defined.

The objectives of this study were: 1. To examine the staining pattern, sensitivity, specificity, and interobserver variability of BRAF V600E immunohistochemistry performed on primary and metastatic colorectal adenocarcinomas, including treatment-naïve tumors and those procured following neoadjuvant, adjuvant, or targeted therapy; and 2. To assess intratumoral heterogeneity of the staining in samples from *BRAF V600E* mutation positive tumors.

Materials and methods

Tissue samples

This study was approved by the institutional review board of the Massachusetts General Hospital, Boston, Massachusetts.

Colorectal adenocarcinoma cases with known *BRAF* mutation status were identified from the pathology files of Massachusetts General Hospital (Boston, Massachusetts). These consisted of 204 cases diagnosed between 1997 and 2012, including 147 primary resections and 57 resections of metastatic or locally recurrent tumors. Of those, 26 (18%) primary tumors were resected following neoadjuvant chemo/radiation therapy and 45 (79%) metastatic/recurrent tumors were sampled following adjuvant chemo/radiation. In one case with a *BRAF V600E* mutation, the patient had been treated with combined targeted therapy with BRAF and MEK inhibitors prior to tumor sampling. In 20 cases, six of which harbored a *BRAF V600E* mutation, both pre- and post-treatment specimens from the same patient were examined. These included samples of ten primary resections and ten metastatic tumors before and after chemotherapy. The median age at initial diagnosis was 61 (range: 24–92) and the overall male:female ratio was 0.79. Patient demographics and tumor characteristics are listed in Table 1.

Original hematoxylin and eosin stained sections of all formalin-fixed paraffin-embedded (FFPE) colorectal carcinoma cases were reviewed by two pathologists (JRB and MM-K) for the presence of adequate material for tissue microarray (TMA) construction. TMAs were constructed using two to three (median three) 2 mm tissue cores of representative tumor from each case, along with multiple cores of non-neoplastic colonic mucosa, which served as controls.

Mutational analysis

In all cases tumor mutation status had been determined prior to the study as part of the clinical work-up by a multiplex PCR-based assay (SNaPshot platform; Applied Biosystems) to detect a panel of commonly mutated genes implicated in oncogenesis, including *BRAF*, *KRAS*, *APC*, and *TP53*, among others, as previously described.²⁶

Immunohistochemistry

Immunohistochemistry was performed on 5-μm sections of the TMAs using an automated immunostainer (BOND-III, Leica Microsystems, Bannockburn, IL). Briefly, FFPE sections were deparaffinized, antigen retrieval was performed with an EDTA-based solution (Leica)

at pH 9 for 40 minutes, and sections were stained with antibodies to BRAF V600E (clone: VE1, 1:100, Spring Bioscience, Pleasanton, CA). Positive and negative controls were included. Evaluation of immunohistochemical staining was performed by three pathologists at different stages of training [pathology resident (JRB), gastrointestinal pathology fellow (MK), and staff pathologist with a special interest in gastrointestinal pathology (MM-K)]. Consensus examination of a 'test cohort' consisting of two TMAs, including 7 *BRAF* mutant cases (20 tissue cores) and 32 *BRAF* wild-type cases (96 tissue cores), was performed with full knowledge of mutation status to establish the most sensitive and specific staining pattern. Subsequent blinded evaluation of a 'validation cohort' - consisting of 8 additional TMAs, including 40 *BRAF* mutant cases (99 tissue cores) and 99 *BRAF* wild-type cases (262 tissue cores), and 26 whole slide sections, including 12 *BRAF* mutant and 14 *BRAF* wild-type cases - was performed independently by each of the above observers.

To evaluate intratumoral uniformity/heterogeneity of BRAF immunostaining, whole-slide sections of 55 of the 59 *BRAF* mutant cases (those with sufficient tumor left in the blocks) were stained with the VE1 antibody and evaluated by two of the authors (JRB and MM-K). Whole slide sections on all cases with both pre- and post-treatment specimens were also examined to determine the degree of staining heterogeneity induced by treatment. Staining intensity was scored from 0–3: negative (0), weak (1), moderate (2), and strong (3). Diffuseness of staining was scored as: diffuse (all malignant cells fulfill staining criteria) or non-diffuse (lack of staining in any malignant cells). Uniformity/homogeneity of staining was scored as: uniform (all malignant cells having the same staining intensity), near-uniform (a minority of cells with ± 1 intensity of staining compared to the majority), and heterogeneous (variability of staining intensity).

Statistics

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the established immunohistochemical staining pattern for *BRAF V600E* mutation were calculated for each observer and averaged for overall values. Interobserver variability was calculated using Fleiss' kappa. Analyses were performed using Prism 6 statistical software (GraphPad Software, Inc., La Jolla, CA).

Results

BRAF V600E mutations were present in 59 of 204 cases (29%) as determined by molecular analysis. The other mutations identified in this cohort included *KRAS* (n=48), *TP53* (n=36), and/or *PIK3CA* (n=10) mutations with or without additional non-BRAF or *NRAS* mutations only (n=5). No mutations were detected in 54 cases, and there were no detectable *BRAF* mutations in 9 cases in which analysis for non-BRAF mutations was not performed.

Consensus examination of the test cohort revealed non-specific BRAF V600E immunostaining of the normal colonic mucosa, which was confined to the luminal/superficial region of the crypt and consisted of nuclear and occasional cytoplasmic staining. Importantly, nuclear staining was always more intense than cytoplasmic staining in the normal mucosa (Figs. 1A, B). A highly sensitive and specific staining pattern was observed for cases with the corresponding *BRAF V600E* mutation: diffuse cytoplasmic staining of

tumor cells with or without membranous accentuation, and staining of nearly uniform intensity. Absolute cytoplasmic staining intensity varied in the *BRAF* mutant cases from weak to strong, but importantly was uniform in all cores from an individual case (Figs. 1C–H). Weak but diffuse cytoplasmic staining was seen in 14 cores (7 cases) that were scored positive for *BRAF* staining (15% of all positive cores). Nuclear staining occurred only in a minority of *BRAF* mutants (Fig. 1F), was regarded as non-specific, and in the absence of the above cytoplasmic criteria, was taken as non-diagnostic. Non-specific nuclear and heterogeneous non-diffuse cytoplasmic staining of variable intensity was observed in occasional non-*BRAF* mutant cases.

Application of this staining pattern to establish positive *BRAF* V600E staining in the ‘validation cohort’ yielded an overall sensitivity of 96.3%, specificity of 98.5%, PPV of 96.3%, and NPV of 98.5%, averaged across the three observers, following independent blinded evaluation. This included both primary and metastatic tumors as well as both pre- and post-treatment specimens. In primary resection specimens, sensitivity and specificity were 95.9% and 99.3%, in metastatic or locally recurrent tumors 100% and 96.7%, in untreated specimens 96.1% and 99.4%, and in treated specimens 95.8% and 97.0%. Fleiss’ kappa for inter-observer agreement was 0.96. There was 100% consistency and accuracy of staining between pre- and post-treatment samples in the 20 cases with both.

BRAF immunostaining of whole-slide sections of the *BRAF* V600E mutants revealed diffuse staining of tumor cells in all cases. The results of scoring for staining intensity and uniformity are shown in Table 2. The majority of cases showed diffuse and uniform (69%) or diffuse and near-uniform (22%) staining of all malignant cells. In five cases (9%) the staining pattern was diffuse and convincingly positive but intensity was not uniform. In each of the latter five cases approximately half of the tumor cells showed weak (1+) cytoplasmic staining and the remaining half showed moderate (2+) staining intensity. Comparison of pre- and post-treatment whole section specimens from the six *BRAF* mutants who underwent neoadjuvant or adjuvant chemo/radiation therapy showed no difference in staining intensity and uniformity, indicating that treatment did not induce heterogeneous staining (Figs. 2A, B). In the *BRAF* mutant case in which the patient had undergone treatment with combined *BRAF* and MEK inhibitors, positive (2+) staining was seen in the tumor sample (Figs. 2C, D).

In one case with a *BRAF* mutation, *BRAF* staining of all three cores was interpreted as negative by the three observers. This case was from a primary resection specimen without prior neoadjuvant chemotherapy and was composed entirely of signet-ring cells with large intracytoplasmic vacuoles and abundant extracellular mucin (Figs. 3A–C). In another case of a *BRAF* mutant primary resection with no prior treatment, one tissue core was interpreted as negative for *BRAF* staining by two of three observers. This case showed medullary architecture with weak but distinct and uniform staining of tumor cells (Figs. 3D–F). A separate core from the same case was appropriately interpreted as positive for *BRAF* mutation by all the 3 observers (Figs. 1G–H). Finally, in another *BRAF* mutant case, one observer interpreted staining in one out of three cores as negative. In retrospect this case showed weak but uniform staining (Figs. 3G–I). In each of these three false negative cases the appearance of the tumor on the corresponding whole slide sections was nearly identical

to the tissue cores but the whole slide sections were correctly interpreted as positive. This was likely due to the presence of more tissue, allowing for a better appreciation of the diffuse nature of tumor cell staining despite the weak staining in the latter two cases, and a better appreciation of cytoplasmic staining in the case with signet-ring cells.

In one core each of two separate *BRAF* wild-type cases, staining was interpreted as positive by one observer (the same observer in both instances). Each of these tumors was resected following chemotherapy; one was a primary resection specimen and the other a hepatic metastasis. Both cases had limited evaluable tumor, dim staining of tumor cells and high background staining; crush artifact also hampered evaluation in one case (Figs. 4A, D). In hindsight both cases showed non-uniform staining of tumor cells though the scanty tissue made this determination difficult (Figs. 4B–C and 4E–F). Of note, smooth muscle in particular showed a high degree of background staining not infrequently in the cohort (as seen in Figs. 4B–C).

Finally the three observers scored positive all three tissue cores of one case that had no detectable *BRAF* mutation by the molecular analysis performed as part of the clinical work up. This case was from a 65-year-old man with a hepatic metastasis status post two cycles of chemotherapy, and showed limited clusters of carcinomatous cells floating in abundant extracellular mucin (Figs. 5A, C). Immunohistochemistry showed staining fulfilling all of the above staining criteria, including diffuse and uniform cytoplasmic staining, which was strong in intensity (Figs. 5B, D). Notably, this case had been shown to lack expression of mismatch repair proteins MLH1 and PMS2; a MLH1 methylation assay was not performed. Prompted by our immunohistochemical findings, we repeated *BRAF* mutational analysis on the area with the highest tumor cellularity and a *BRAF V600E* mutation was detected.

Discussion

Mutation-specific immunohistochemistry for *BRAF V600E* has been established as a rapid and relatively inexpensive assay for mutation screening of colorectal and non-colorectal neoplasms.^{4,15,22–24} Whereas *BRAF V600E* staining has previously been shown to be useful in primary colorectal carcinomas as well as in metastases of non-colorectal tumors such as melanoma,¹⁵ relatively few studies have examined *BRAF V600E* staining in metastatic colorectal carcinoma, and in these studies the number of metastatic cases examined has generally been low.^{3–4,23,25} Furthermore, to our knowledge little has been reported about the relative reliability of *BRAF V600E* immunostaining in post-treatment specimens. Here we show that the *BRAF V600E* VE1 antibody is sensitive and specific in a large cohort of colorectal carcinomas and that the high sensitivity and specificity of the stain is maintained in metastatic tumors as well as post-treatment specimens. Furthermore, we demonstrate 100% concordance and accuracy of staining in 20 cases in which both pre- and post-chemotherapy samples from the same patient were examined. Table 3 lists our findings along with those of prior studies that have investigated the reliability of *BRAF V600E* immunohistochemistry in colorectal carcinoma.

The discrepancy between the numerous studies demonstrating high sensitivity and specificity of the *BRAF V600E* antibody and those reporting poor reliability is difficult to

reconcile, but may be due to technical considerations such as variation in staining protocols, or potentially due to under-recognition of staining pitfalls or inaccurate discounting of weak but positive cytoplasmic immunostaining. In this study, adherence to a pre-defined staining criteria resulted not only in high concordance with mutational analysis but also in near-perfect interobserver agreement between pathologists at all stages of training, suggesting that the interpretation is straightforward and reproducible. Similarly high levels of agreement have been demonstrated with this antibody previously.^{10–11,20,22–23}

In this study, evaluation of the staining pattern of whole slide sections of the *BRAF* mutants revealed a high degree of intratumoral homogeneity, with diffuse staining of tumor cells in all cases and uniform or near-uniform staining in over 90% of cases. In the five cases with non-uniform staining, the overall staining pattern was clearly positive regardless. Understanding the importance of diffuseness and near-uniformity of cytoplasmic staining may help increase sensitivity, specificity and interobserver agreement, particularly in weakly staining cases. The latter point is critical since there appears to be case-to-case variability in the absolute intensity of cytoplasmic staining of *BRAF* mutants with the VE1 antibody, but uniformity or near-uniformity within an individual case.^{11,24–25} For example, in this study 14 cores (15% of all *BRAF* mutant cores) showed weak but diffuse staining, which if discounted would have decreased the sensitivity of the stain from 96% to 82%. Therefore, weak but convincingly diffuse and near-uniform cytoplasmic staining should not be disregarded based on low staining intensity.

Staining method is another potential source for discrepancy between prior studies. Adackapara et al. report a relatively low sensitivity and specificity using a manual staining method performed when initial attempts to stain with a Dako autostainer (Dako, Carpinteria, CA, USA) were unable to produce a sufficiently strong signal.²⁵ Many other studies on *BRAF* immunohistochemistry in colorectal carcinoma have used automated Ventana stainers (Ventana Medical Systems, Inc., Tucson, AZ, USA),^{10,22–24} and have shown decent sensitivity and specificity. Here we used a Leica BOND-III immunostainer, also with good sensitivity and specificity when weak but diffuse staining was considered positive. Weak staining was also seen in a minority of cases when using the Ventana platform in prior studies on colorectal carcinoma.^{22–24} Given these findings, we believe that weak staining in most cases reflects true staining heterogeneity between cases. However, there seems to be differences in staining quality and reliability between platforms, possibly due to variability in antigen retrieval methods or antibody specificity, and staining of colorectal carcinomas in particular seems to often be problematic. Therefore, before implementation of *BRAF* V600E IHC for clinical use, it is important to understand the issues potentially associated with each platform and to validate the antibody using a large cohort of tumor types including *BRAF* V600E mutant colorectal carcinomas.

Awareness of common pitfalls in staining interpretation is essential for the surgical pathologist. In our experience, misinterpretations in the evaluation of immunostaining were most likely to occur in cases with signet-ring cell morphology, cases with very limited amounts of evaluable tumor, and in cases with dim but uniformly positive cytoplasmic staining. Here, false negative interpretation of staining in cases with signet-ring morphology likely arose secondary to limited visualization of the cytoplasm, which was almost entirely

replaced by mucin.¹¹ Interestingly, others have reported false positive staining of intracellular mucin of signet-ring cells with the VE1 antibody.²² Additionally, caution should be taken when only a few neoplastic glands are seen, particularly in the setting of high background staining. Finally, as described previously in both normal colonic mucosa,²¹ and in carcinoma,³ non-specific nuclear staining is fairly common and should not be interpreted as connoting an underlying BRAF V600E mutation.

Interestingly, in one microsatellite-unstable case the BRAF immunohistochemical staining pattern seen in all 3 cores strongly suggested a *BRAF* mutation but mutational analysis was negative (Fig. 5). The carcinomatous tissue was scant and surrounded by abundant mucin. Repeat mutational analysis on this case revealed that the tumor did in fact carry the *BRAF* V600E mutation. The scant cellularity may have initially precluded optimal molecular evaluation thereby resulting in a false negative molecular result. It is well documented that many commonly used PCR- and sequencing-based mutational assays require a minimal absolute amount of tumor or tumor cellularity for analysis.^{12–13} For example, one study evaluating *BRAF* mutational testing platforms found that complete agreement between 5 molecular testing modalities required a sample with at least 10%, and in certain cases up to 50%, tumor cellularity.¹² As in the present study, prior studies have identified cases of colorectal carcinoma and melanoma that were originally *BRAF* wild-type by molecular analysis but were subsequently found to harbor *BRAF* mutations after repeat testing prompted by discordantly positive immunohistochemistry.^{11,15} Taken together, these results suggest that BRAF V600E immunohistochemistry may have greater sensitivity than molecular analysis in cases with scant tissue, such as post-treatment samples, since expression can be evaluated through direct visualization of the cells of interest.

One specific case included in this study is particularly informative. In this case, we show that treatment with a BRAF mutation-specific inhibitor does not preclude accurate determination of *BRAF* mutation status by immunohistochemistry. It should be noted that in this case the patient had progression of disease while on targeted therapy, and no pre-treatment sample was evaluated so we therefore cannot speculate on the relative change in BRAF expression induced by *BRAF* inhibition.

Finally, prior studies have shown that the detection of *BRAF* mutations in colorectal adenocarcinoma is highly concordant between biopsy samples and corresponding resection specimens when using traditional molecular methods.²⁷ Comparatively little is known about the sensitivity and specificity of BRAF immunohistochemistry on colorectal carcinoma biopsy specimens with only a small amount of tissue. Our results are based, in part, on the evaluation of TMAs with tissue cores of 2mm diameter, approximating the typical amount of tissue received in a standard biopsy of a colorectal mass.^{28–29} The uniformity of staining seen within a particular core, and between cores and whole slide sections from the same case, indicates that immunohistochemical examination of small amounts of diagnostic tissue should reflect the staining characteristics of the tumor as a whole. Therefore, we believe that the high sensitivity and specificity demonstrated in this study recapitulates that which might be expected in biopsy specimens. Furthermore, although limited, our experience with biopsy specimens (n=5, data not shown) supports the clinical use of BRAF immunohistochemistry on even small amounts of tissue.

In summary, we report that BRAF V600E immunohistochemistry is a highly sensitive and specific method for detecting the corresponding *BRAF* mutation in colorectal carcinoma, regardless of tumor site or treatment status. We also show that interpretation using pre-defined staining criteria, including diffuse and near-uniform cytoplasmic staining, results in excellent interobserver agreement and our findings suggest that this method may be sensitive enough to be used on small amounts of tissue such as endoscopic biopsies. Taken together, our data support the notion that mutation-specific IHC for the *BRAF V600E* mutation is a useful and relatively rapid assay that will serve well as a rapid screening test for the *BRAF V600E* mutation. For cases with scant tumor cellularity and/or purity, immunohistochemistry for the detection of the *BRAF V600E* mutation may in fact have an advantage over more laborious molecular methods.

Acknowledgments

The authors thank Dr. A. John Iafrate and Ms. Julie Batten for their technical support.

M.M.-K. was supported by National Cancer Institute grants P50 CA127003 and R01 CA169086.

References

1. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. *Nature*. 2002; 417:949–954. [PubMed: 12068308]
2. Tiacci E, Trifonov V, Schiavoni G, et al. BRAF mutations in hairy-cell leukemia. *N Engl J Med*. 2011; 364:2305–2315. [PubMed: 21663470]
3. Capper D, Berghoff AS, Magerle M, et al. Immunohistochemical testing of BRAF V600E status in 1,120 tumor tissue samples of patients with brain metastases. *Acta Neuropathol*. 2011; 123:223–233. [PubMed: 22012135]
4. Routhier CA, Mochel MC, Lynch K, et al. Comparison of 2 monoclonal antibodies for immunohistochemical detection of BRAF V600E mutation in malignant melanoma, pulmonary carcinoma, gastrointestinal carcinoma, thyroid carcinoma, and gliomas. *Hum Pathol*. 2013; 44:2563–2570. [PubMed: 24071017]
5. Samowitz WS, Sweeney C, Herrick J, et al. Poor survival associated with the BRAF V600E mutation in microsatellite-stable colon cancers. *Cancer Res*. 2005; 65:6063–6069. [PubMed: 16024606]
6. Pai RK, Jayachandran P, Koong AC, et al. BRAF-mutated, microsatellite-stable adenocarcinoma of the proximal colon: an aggressive adenocarcinoma with poor survival, mucinous differentiation, and adverse morphologic features. *Am J Surg Pathol*. 2012; 36:744–752. [PubMed: 22314188]
7. Toon CW, Chou A, DeSilva K, et al. BRAFV600E immunohistochemistry in conjunction with mismatch repair status predicts survival in patients with colorectal cancer. *Mod Pathol*. 2013
8. Di Nicolantonio F, Martini M, Molinari F, et al. Wild-type BRAF is required for response to panitumumab or cetuximab in metastatic colorectal cancer. *J Clin Oncol*. 2008; 26:5705–5712. [PubMed: 19001320]
9. Saridaki Z, Tzardi M, Sfakianaki M, et al. BRAFV600E mutation analysis in patients with metastatic colorectal cancer (mCRC) in daily clinical practice: correlations with clinical characteristics, and its impact on patients' outcome. *PLoS One*. 2013; 8:e84604. [PubMed: 24367680]
10. Capper D, Voigt A, Bozukova G, et al. BRAF V600E-specific immunohistochemistry for the exclusion of Lynch syndrome in MSI-H colorectal cancer. *Int J Cancer*. 2013; 133:1624–1630. [PubMed: 23553055]
11. Toon CW, Walsh MD, Chou A, et al. BRAFV600E immunohistochemistry facilitates universal screening of colorectal cancers for Lynch syndrome. *Am J Surg Pathol*. 2013; 37:1592–1602. [PubMed: 23797718]

12. Lade-Keller J, Rømer KM, Guldberg P, et al. Evaluation of BRAF mutation testing methodologies in formalin-fixed, paraffin-embedded cutaneous melanomas. *J Mol Diagn*. 2013; 15:70–80. [PubMed: 23159593]
13. Lewandowska MA, Jówicki W, urawski B. KRAS and BRAF mutation analysis in colorectal adenocarcinoma specimens with a low percentage of tumor cells. *Mol Diagn Ther*. 2013; 17:193–203. [PubMed: 23606169]
14. Capper D, Preusser M, Habel A, et al. Assessment of BRAF V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody. *Acta Neuropathol*. 2011; 122:11–19. [PubMed: 21638088]
15. Long GV, Wilmott JS, Capper D, et al. Immunohistochemistry is highly sensitive and specific for the detection of V600E BRAF mutation in melanoma. *Am J Surg Pathol*. 2013; 37:61–65. [PubMed: 23026937]
16. Andrulis M, Penzel R, Weichert W, et al. Application of a BRAF V600E mutation-specific antibody for the diagnosis of hairy cell leukemia. *Am J Surg Pathol*. 2012; 36:1796–1800. [PubMed: 22531170]
17. Bösmüller H, Fischer A, Pham DL, et al. Detection of the BRAF V600E mutation in serous ovarian tumors: a comparative analysis of immunohistochemistry with a mutation-specific monoclonal antibody and allele-specific PCR. *Hum Pathol*. 2013; 44:329–335. [PubMed: 23089489]
18. Ilie M, Long E, Hofman V, et al. Diagnostic value of immunohistochemistry for the detection of the BRAF V600E mutation in primary lung adenocarcinoma Caucasian patients. *Ann Oncol*. 2013; 24:742–748. [PubMed: 23131393]
19. Koperek O, Kornauth C, Capper D, et al. Immunohistochemical detection of the BRAF V600E-mutated protein in papillary thyroid carcinoma. *Am J Surg Pathol*. 2012; 36:844–850. [PubMed: 22592144]
20. Marin C, Beauchet A, Capper D, et al. Detection of BRAFp.V600E mutations in melanoma by immunohistochemistry has a good interobserver reproducibility. *Arch Pathol Lab Med*. 2014; 138:71–75. [PubMed: 23651150]
21. Mesteri I, Bayer G, Meyer J, et al. Improved molecular classification of serrated lesions of the colon by immunohistochemical detection of BRAF V600E. *Mod Pathol*. 2014; 27:135–144. [PubMed: 23887306]
22. Rössle M, Sigg M, Rüschhoff JH, et al. Ultra-deep sequencing confirms immunohistochemistry as a highly sensitive and specific method for detecting BRAF (V600E) mutations in colorectal carcinoma. *Virchows Arch*. 2013; 463:623–631. [PubMed: 24085553]
23. Affolter K, Samowitz W, Tripp S, et al. BRAF V600E mutation detection by immunohistochemistry in colorectal carcinoma. *Genes Chromosomes Cancer*. 2013; 52:748–752. [PubMed: 23650027]
24. Sinicrope FA, Smyrk TC, Tougeron D, et al. Mutation-specific antibody detects mutant BRAF V600E protein expression in human colon carcinomas. *Cancer*. 2013; 119:2765–2770. [PubMed: 23657789]
25. Adackapara CA, Sholl LM, Barletta JA, et al. Immunohistochemistry using the BRAF V600E mutation-specific monoclonal antibody VE1 is not a useful surrogate for genotyping in colorectal adenocarcinoma. *Histopathology*. 2013; 63:187–193. [PubMed: 23763264]
26. Dias-Santagata D, Akhavanfard S, David SS, et al. Rapid targeted mutational analysis of human tumours: a clinical platform to guide personalized cancer medicine. *EMBO Mol Med*. 2010; 2:146–158. [PubMed: 20432502]
27. Krol LC, 't Hart NA, Methorst N, et al. Concordance in KRAS and BRAF mutations in endoscopic biopsy samples and resection specimens of colorectal adenocarcinoma. *Eur J Cancer*. 2012; 48:1108–1115. [PubMed: 22446020]
28. Woods KL, Anand BS, Cole RA, et al. Influence of endoscopic biopsy forceps characteristics on tissue specimens: results of a prospective randomized study. *Gastrointest Endosc*. 1999; 49:177–183. [PubMed: 9925695]

29. Elmunzer BJ, Higgins PD, Kwon YM, et al. Jumbo forceps are superior to standard large-capacity forceps in obtaining diagnostically adequate inflammatory bowel disease surveillance biopsy specimens. *Gastrointest Endosc.* 2008; 68:273–278. [PubMed: 18155204]

Figure 1. BRAF V600E (VE1) immunohistochemistry in normal colonic mucosa and *BRAF* mutant colorectal carcinoma. Non-specific staining of normal mucosa was heterogeneous with negative staining in the crypt base and conspicuous nuclear and weak cytoplasmic staining of the luminal aspect of the crypts (A). Absolute intensity of staining in *BRAF* mutants varied from strong (B) to moderate (C) to weak (D) but was notably uniform in all cases. Nuclear staining occurred occasionally (C) in *BRAF* mutants, especially in the luminal aspect, but should be regarded as non-specific.

Figure 2. Staining for BRAF V600E (VE1) in pre-treatment (A) and post-treatment (B) tumors from the same patient showed nearly identical intensity and uniformity in the six *BRAF* mutants examined. One case of interest (C) carried a *BRAF V600E* mutation and had undergone treatment with a mutation specific *BRAF*-inhibitor prior to sampling. The VE1 antibody demonstrated convincingly positive staining (D) despite prior targeted therapy.

Figure 3.

False negative interpretations of BRAF V600E (VE1) immunohistochemistry in *BRAF* mutant colorectal carcinoma (A–B, C–D, and E–F). Cytoplasmic staining in signet-ring cells is confounded by the presence of intracytoplasmic mucin (B). Very weak but uniform staining was present in a case with medullary morphology that was called negative by two of three observers (D). Weak cytoplasmic staining (as in D and F) should not be disregarded. Rather, uniform cytoplasmic staining, even if dim, should be taken as positive.

Figure 4.

False positive interpretations of BRAF V600E (VE1) immunohistochemistry in *BRAF* wild-type colorectal carcinoma. In two non-*BRAF* mutant cases (A-B and C-D) interpreted as positive by one of three observers, tumorous tissue was scant and staining of malignant cells was weak and, in retrospect, not uniform across the tumor cells. High level background staining of stromal cells (B and D) was seen occasionally, particularly in smooth muscle (B), and in one case in an adjacent ganglion (D, top left).

Figure 5.

This case had no detectable *BRAF* mutation by molecular analysis, but multiple tissue cores (A, C) showed scant groups of tumor cells that fulfilled all positive BRAF V600E staining criteria (B, D): strong and uniform cytoplasmic staining with or without membranous accentuation. Repeat molecular analysis was performed and was positive for the *V600E* mutation, consistent with an initial false negative molecular result, likely due to the limited tumor purity and low tumor cellularity.

Table 1

Patient demographics

	No. of Patients (%)		
	Test cohort, n=39	Validation cohort, n=165	Total, n=204
Age (years): Mean $\pm$ SD ¹	55 $\pm$ 13.8	63 $\pm$ 13.8	61 $\pm$ 14.2
Sex			
Men	20 (51)	70 (42)	90 (44)
Women	19 (49)	95 (58)	114 (56)
Mean primary tumor size, cm	4.3	4.7	4.7
Primary T stage			
T1 or T2	6 (15)	31 (19)	37 (18)
T3 or T4	33 (85)	134 (81)	167 (82)
Tumor site sampled			
Primary tumor	27 (69)	120 (73)	147 (72)
Metastasis/recurrence	12 (31)	45 (27)	57 (28)
Treatment status			
Treated	17 (44)	54 (33)	71 (35)
Untreated	22 (56)	111 (67)	133 (65)
<i>BRAF</i> mutation status			
<i>BRAF</i> V600E mutant	7 (18)	52 (32)	59 (29)
Non- <i>BRAF</i> V600E	32 (82)	113 (68)	145 (71)
No. of tissue cores ²			
<i>BRAF</i> mutant	20 (17)	99 (27)	119 (25)
<i>BRAF</i> wild-type	96 (83)	262 (73)	358 (75)
MMR ³			
MMR deficient	4 (10)	50 (30)	54 (26.5)
MMR proficient	35 (90)	110 (67)	145 (71)
Unknown	0	5 (3)	5 (2.5)

¹ Standard deviation;² Not inclusive of the whole slide sections;³ Mismatch repair

Table 2

Uniformity and intensity of BRAF VE1 immunostaining in *BRAF* mutant colorectal carcinoma

	Total (%)	Intensity		
		Weak	Mod	Strong
Uniform	38 (69%)	5	30	3
Near-uniform	12 (22%)	2	9	1
Heterogeneous	5 (9%)	0	5*	0
Total	55	7	44	4

* The five cases with non-uniform staining showed regions of both weak and moderate cytoplasmic staining

Table 3

Studies correlating BRAF V600E immunohistochemistry with mutational status in colorectal carcinoma

Authors	Tissue type/volume	No. of BRAF mutants	Antibody/IHC platform	Positive BRAF V600E staining criteria	Results	Notes on staining
Adackapara et al. 2013 ²⁵	46 primary resections, 6 metastases	17	VEI; Manual staining ¹	Cytoplasmic staining	Sensitivity: 71%; Specificity: 74%;	Moderate to strong cytoplasmic staining was more specific; relatively uniform staining throughout all positive cases
Affolter et al. 2013 ²³	22 primary resections, 5 biopsies of colonic primary, and 4 metastases	14	VEI; Ventana Benchmark Ultra ²	Cytoplasmic staining	Sensitivity and Specificity: 100%; 100% agreement between three pathologists	Staining in the majority of BRAF mutant cases was strong and diffuse (n=8). Heterogenous (n=4) or weak (n=2) staining occurred infrequently
Capper et al. 2013 ¹⁰	91 microsatellite-unstable colorectal cancers	11	VEI; Ventana Benchmark XT ²	Staining of >80% tumor cells above background	Sensitivity: 100%; Specificity: 98.8%; 100% agreement between three observers	Homogenous finely granular cytoplasmic staining was seen in most cases
Rösle et al. 2013 ²²	71 tumors in the antibody establishment cohort; 264 in the test cohort	Establishment cohort: 3; Test cohort: 37	VEI; Ventana Benchmark XT ²	Unequivocal cytoplasmic staining of a majority of tumor cells	Sensitivity: 100%; Specificity: 95%; Kappa: 0.921 between two pathologists	Diffuse staining of variable intensity (from weak to strong) in most cases. False positive staining noted in signet ring tumor cells
Routhier et al. 2013 ⁴	18 primary colorectal resections and 7 metastases	11	VEI and anti-B-Raf(V600E) mouse monoclonal antibody ³ ; Bond III immunostainer ⁴	Diffuse and moderate to strong cytoplasmic staining of tumor cells	Sensitivity (VEI): 100%; Specificity (VEI): 100%; Sensitivity (anti-B-Raf): 88%; Specificity (anti-B-Raf): 93%	Non-specific staining included: isolated nuclear staining of tumor cells and weak staining of occasional tumorous cells, and low level background staining
Sinicrope et al. 2013 ²⁴	75 primary resections	50	VEI; Ventana Benchmark XT ²	Cytoplasmic staining of in all BRAF mutant cases	100% concordance of VEI staining and BRAF V600E mutational analysis	Homogenous staining seen in the majority of cases. Medium to strong intensity was noted in at least 70% of tumor cells in all BRAF mutant cases.
Toon et al. 2013 ¹¹	Two cohorts with 1403 colorectal resections (201 with mutational analysis and IHC results), and 51 colorectal carcinomas	Cohort 1: 44/201; Cohort 2: 23	VEI	Diffuse strong positive staining of >75% of malignant cells	Overall percentage agreement between IHC and mutational analysis: 96.5%; Kappa: 0.964 - 1 between any two pathologists	Patchy non-specific staining in smooth muscle cells, mucin, and colonic mucosa (with nuclear staining). Weak but diffuse staining seen in occasional positive cases

Authors	Tissue type/volume	No. of BRAF mutants	Antibody/IHC platform	Positive BRAF V600E staining criteria	Results	Notes on staining
Current study	204 cases of primary and metastatic colorectal carcinoma including pre- and post-treatment cases (n=39 in the test cohort; n= 165 in the validation cohort)	Test cohort: 7; Validation cohort: 52	VE1; Bond III immunostainer ⁴	Diffuse cytoplasmic staining of tumor cells of uniform to near uniform intensity, ranging from weak to strong in strength	Sensitivity: 96.3%; Specificity: 98.5%; Kappa: 0.96 between three pathologists with various levels of experience	Pitfalls include signet-ring cell morphology and over calling scant tumor cells with high background. Dim but uniform staining should not be disregarded

IHC: immunohistochemistry;

- ¹
- : Initial staining with a Dako immunostainer (Dako, Carpinteria, CA, USA) was noted to be insufficiently strong;
- ²
- : Ventana Medical Systems, Inc., Tucson, AZ, USA;
- ³
- : New East Biosciences, Malvern, PA, USA;
- ⁴
- : Leica Microsystems, Bannockburn, IL, USA