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ORIGINAL RESEARCH

A mutant BRAF V600E-specific immunohistochemical assay: correlation with molecular mutation status and clinical outcome in colorectal cancer

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Abstract The B-type Raf kinase (BRAF) V600E mutation is a well-established biomarker for poor prognosis in metastatic colorectal cancer (mCRC) and is a highly attractive drug target. A barrier to the development of new therapies targeting BRAF V600E in mCRC is the low prevalence of mutations (approximately 10 %) and the current need for access to sequencing-based technologies which are not routinely available outside of large cancer centres. Availability of a standardised immunohistochemistry (IHC) test, more suited to routine pathology practice, would provide much broader access to patient identification. We sought to evaluate the accuracy and clinical utility of a recently developed BRAF V600E IHC method as a prognostic biomarker in a large cohort of community-based CRC patients. Archival tumour samples from 505 patients with stage I–IV CRC were immunohistochemically tested with two antibodies, pBR1 for total BRAF and VE1 for BRAF V600E. Cases were assessed by two blinded pathologists, and results were compared to *BRAF* V600E mutation status determined using DNA sequencing. Discordant cases were retested with a *BRAF* V600E SNaPshot assay. *BRAF* mutation status was correlated with overall survival (OS) in stage IV CRC. By DNA sequencing and IHC, 505 and 477 patients were respectively evaluable. Out of 477 patients, 56 (11. 7 %) had *BRAF* V600E mutations detected by sequencing and 63 (13.2 %) by IHC. Using DNA sequencing results as the reference, sensitivity and specificity for IHC were 98.2 % (55/56) and 98.1 % (413/421), respectively. IHC had a positive predictive value (PPV) of 87.3 % (55/63) and a negative predictive value (NPV) of 99.8 % (413/414). Compared to DNA sequencing plus retesting of available discordant cases by SNaPshot assay, IHC using the VE1 antibody had a 100 % sensitivity (59/59), specificity (416/416), NPV (416/416) and PPV (59/59). Stage IV CRC patients with BRAF V600E protein detected by IHC exhibited a significantly shorter overall survival (hazard ratio = 2.20, 95 % CI 1.26–3.83, p=0.005), consistent with other published series. Immunohistochemistry using the BRAF V600E VE1 antibody is an accurate diagnostic assay in CRC. The test provides a simple, clinically applicable method of testing for the *BRAF* V600E mutation in routine practice.

Paul Waring and Jayesh Desai contributed equally to this study.

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Introduction

A serine/threonine kinase of the MAPK-ERK signalling pathway, B-type Raf kinase (BRAF), and other RAF family members are usually activated by GTP-bound RAS signalling downstream of the epidermal growth factor receptor (EGFR) or in response to other mitogens [1]. Mutant BRAF, however, displays constitutive activation when affected by missense mutation [2], most commonly V600E. Approximately 10 % of colorectal cancers (CRCs) harbour *BRAF* V600E [2–4], and this subset is associated with a significantly poorer survival [5–7] in patients with metastatic disease. *BRAF* mutation may also predict lack of benefit from anti-EGFR therapy [8, 9] in metastatic CRC, although reports are conflicting [10]. The current clinical value of *BRAF* V600E detection is the delineation of hereditary non-polyposis colorectal cancer (HNPCC)-associated tumours (BRAF wild type) from sporadic CRCs (BRAF V600E mutant) in mismatch repair-deficient colorectal disease [11–13] and rational patient enrolment to clinical trials testing BRAF inhibitors [6].

Various methods of genotyping tumour samples for *BRAF* status are currently used in diagnostic and research laboratories, ranging from traditional Sanger sequencing [14] to quantitative pyrosequencing [15], mutation-specific real-time polymerase chain reaction (RT-PCR) assays [16] and mass spectrometry-based methods [17]. Common to all these methods, however, is the requirement for DNA extraction from tissue and the need for rigorous protocols to minimise the impact of contamination of non-tumour cells on the overall tumour to non-tumour cell ratio.

Importantly, at this point in time, the expertise and infrastructure required for DNA-based genotyping methods are frequently available only at academic centres and reference laboratories. Testing for *BRAF* V600E therefore requires multiple steps and coordination between the primary site and reference laboratory, resulting in sample transit costs and diagnostic delays. Until genomic-based testing becomes available in routine community-based pathology laboratories, the complexity involved in such testing will continue to serve as an impediment to providing a patient's *BRAF* status to their treating clinician.

A monoclonal antibody specific to the BRAF V600E kinase, VE1, has recently been described [18] and offers the advantages of immunohistochemical determination of tumour *BRAF* mutation status, no requirement for DNA purification, low cost and the ability to perform testing on formalin-fixed paraffin-embedded (FFPE) tissue in routine histopathology laboratories. To date, immunohistochemistry with VE1 has been applied to the detection of BRAF V600E in brain metastases of varied primary sites [19], papillary thyroid carcinoma [20, 21], Langerhans cell histiocytosis [22, 23], ovarian carcinomas [24, 25], melanoma [26–28], lung adenocarcinoma [29] and hairy cell leukemia [30]. A recently published study by Sinicrope et al. explored the VE1 antibody in a carefully preselected group of 75 patients with stage III colorectal cancer, for whom *BRAF* mutation status had already been determined [31]. In another recent study examining the utility of BRAF immunohistochemistry (IHC) in microsatellite unstable CRC, Toon et al. [32] compared BRAF IHC with conventional PCR-based molecular methods for *BRAF* V600E detection in 216 patients with CRC. In a further cohort, they also performed IHC to mismatch repair (MMR) proteins and BRAF V600E in a larger cohort of 1,403 patients with CRC but failed to also validate this using conventional sequencing-based molecular techniques.

In this study, we aimed to determine the sensitivity, specificity and predictive values of VE1 immunohistochemistry for BRAF V600E in a large community-based and unselected cohort (n=505) of patients with CRC, with the intent of determining how this could inform the use of this IHC-based antibody in routine practice. FFPE tumour samples were annotated for clinical outcomes and had been previously assessed for *BRAF* status by direct (Sanger) sequencing.

Methods

Colorectal tissue samples

Primary tumour and matched normal tissue samples were obtained from an unselected community-based cohort of 505 patients with CRC undergoing surgery at three hospitals in Melbourne, Australia: the Royal Melbourne, Melbourne Private and Western Hospitals. Resected tumours included those from the proximal colon, distal colon and rectum, and all disease stages (I–IV) were represented. This study was approved by the ethics committees of these hospitals and the University of Melbourne.

Tissue microarrays (TMAs) comprised of 1-mm-diameter tissue cores were constructed from the FFPE surgical specimens. Up to four tumour and two normal colon tissue cores were embedded per patient. Tumour cores were harvested from the areas of densest tumour cell percentage. Based on examination of hematoxylin and eosin (H&E)-stained TMA sections by two anatomical pathologists, cores were deemed to contain sufficient tumour sample for immunohistochemical analysis in 491 patients (97 %) (Fig. 1).

Sanger sequencing

All patients of the cohort were characterised for *BRAF* codon 600 mutation status based on Sanger sequencing at the

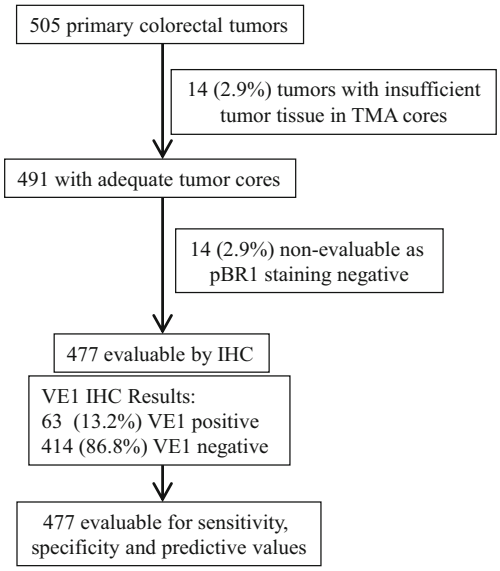

Fig. 1 Workflow and results for immunohistochemistry on tissue microarray (TMA) sections

Ludwig Institute Parkville, Melbourne, prior to immunohistochemical analysis of TMAs. Tumours were microdissected from the originating FFPE blocks, and the purified DNA was subjected to PCR and sequencing using primers and methods previously described [6]. Samples with indeterminate sequencing traces on first analysis were subjected to repeat PCR and sequencing.

Immunohistochemistry

Total BRAF and BRAF V600E-mutant proteins were detected by immunohistochemistry using rat anti-panBRAF monoclonal antibody (clone pBR1) and mouse anti-BRAF V600E monoclonal antibody (clone VE1), respectively, both kindly provided by Dr A. von Deimling from Ruprecht-Karls-University, Heidelberg [18]. All assays were performed on a BenchMark XT automated slide stainer at Ventana Medical Systems, Inc., Tucson, AZ.

Sections from TMAs were freshly cut to 4 μm and dried at 80 °C for 15 min. The presence of total BRAF protein was detected using ultraView Universal DAB Detection Kit (Ventana) where ultraView Universal HRP Multimer was substituted for an HRP-conjugated goat anti-rat secondary antibody. The staining procedure included deparaffinisation, pretreatment using standard Cell Conditioning 1, incubation with pan-BRAF antibody (diluted 1:8) at 37 °C for 16 min and treatment with ultrawash.

The BRAF V600E mutation-specific IHC assay was completed with OptiView DAB IHC Detection Kit (Ventana). Briefly, the tissue sections were deparaffinised, heat pretreated in Cell Conditioning 1 for 64 min and followed by inactivation of the endogenous peroxidases. Specimens were incubated with VE1 hybridoma supernatant (diluted 1:3) at 37 °C for 16 min.

Following the chromogenic detection, all slides were counterstained with Hematoxylin II and Bluing Reagent (Ventana) for 4 min each and coverslips were applied.

The immmunostained slides were evaluated independently by two pathologists (S.S. and D.W.) blinded to the *BRAF* V600E mutation status as determined by sequencing. First, pan-BRAF IHC was assessed for the presence of total BRAF cytoplasmic staining within invasive tumour cells. Cases were scored as unevaluable when total BRAF expression could not be detected, and they were excluded from further immunohistochemical analysis. Next, BRAF V600E (VE1) immunostained slides were evaluated for the presence or absence of BRAF V600E protein expression. Immunoreactivity was scored positive when there was unequivocal cytoplasmic staining above background in the majority of invasive viable tumour cells. Any nuclear staining, weak cytoplasmic staining of isolated tumour cells or focal confluent staining of tumour cells in a tumour that otherwise showed no staining was scored as immunonegative.

SNaPShot assays

DNA was extracted with QIAamp DNA FFPE Tissue Kit (Qiagen) from tumour cells microdissected from unstained FFPE tissue slides and then quantitated using a Qubit® fluorometer (Invitrogen, Grand Island, NY, USA). Purified tumour DNA was PCR-amplified for 30 cycles at 94 and 72 °C, for 45 s each, using BRAF exon 15 forward (TTCATAATGCTTGCTCTGATAGG) and reverse (AGTA ACTCAGCAGCATCTCAGG) primers (GeneWorks, Thebarton, SA, Australia) and AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Grand Island, NY, USA). The 246-bp products were treated with ExoSAP-IT (USB) at 37 °C for 30 min and 80 °C for 15 min to remove excess nucleotides and primers. HPLC-purified detection primer ($(C)_5$TGATTTTGGTCTAGCTACAG) (GeneWorks) was added to the cleaned product together with PRISM SNaPshot Multiplex Ready Reaction Mix (Applied Biosystems) containing fluorescent ddNTPs. The detection primer was extended by thermocyling for 35 cycles at 96 °C for 10 s, 48 °C for 1 min and then 60 °C for 30 s. Excess nucleotides and primers were removed by shrimp alkaline phosphatase (Sigma, St. Louis, MO, USA) treatment at 37 °C for 1 h followed by 75 °C for 15 min, and the cleaned fragments were run in Hi-Di Formamide (Applied Biosystems) through an ABI 3130xl genetic analyser (Applied Biosystems) and analysed by GeneMapper® fragment analysis software. A mutation was called when the area under the fluorescent peak for the mutant allele was greater than five times the background relative fluorescent units. This method has been reported to detect mutations in tissue samples containing ≤5 % tumour cells [33–35].

Statistical analyses

Sensitivity, specificity and predictive values for IHC in comparison to direct DNA sequencing were determined as per convention [36, 37]. Overall survival (OS) in stage IV disease was defined as the time from diagnosis of metastatic CRC to death from any cause. Analyses included patients with de novo stage IV disease and those diagnosed with distance recurrence after prior treatment of earlier stage disease. Survival times were estimated using the Kaplan-Meier method, and results were compared using the log-rank test. Statistical significance was defined as $p<0.05$.

Results

BRAF V600E status

Using Sanger sequencing, 11.7 % (59/505) of stage I–IV CRC patients were *BRAF* V600E mutation positive and the remainder were wild-type (WT). No tumour carried non-V600E mutations at codon 600. Of the 491 patients with adequate TMA tumour tissue for interpretation of total BRAF (pBR1 antibody) and BRAF V600E (VE1 antibody) immunohistochemistry, 14 patients (2.9 %) were negative for pBR1 staining and were accordingly deemed unevaluable for *BRAF* status by IHC (Fig. 1). Of the 477 'evaluable' (pBR1 positive) patients, 63 (13.2 %) were positive for VE1 ('BRAF V600E') and 414 (86.8 %) were negative for VE1 staining ('BRAF WT') (Fig. 2). Of the 63 pBR1- and VE1-positive cases, the majority showed moderate to strong homogeneous cytoplasmic staining with the VE1 antibody. Occasional cases showed uniformly weak staining or focal patches of no staining in otherwise clearly positive tumours. Of the 414 pBR1-positive and VE1-negative cases, 8 (1.9 %) showed weak patchy positivity for VE1 (Fig. 3). All eight such heterogeneous cases were *BRAF* WT by sequencing. In normal colonic mucosa, nuclear VE1 staining was frequently observed in surface epithelial cells (Fig. 4).

The clinicopathological features and IHC results for the evaluable patient population are shown in Table 1. A higher frequency of BRAF V600E positivity was seen in female patients and CRCs resected from the right colon, exhibiting poor differentiation or with microsatellite instability (MSI). These results are consistent with the findings of multiple prior cohort studies typing V600 mutations using DNA-based methods [38–40].

The sensitivity, specificity and predictive values for IHC were determined in the evaluable population as compared to the results from Sanger sequencing, which was considered to be the current gold standard for the determination of *BRAF* genotype. The sensitivity of VE1 staining for *BRAF* V600E was 98.2 % (55/56), specificity 98.1 % (413/421), negative predictive value (NPV) 99.8 % (413/414) and positive predictive value (PPV) 87.3 % (55/63) (Table 2).

Further investigation of discordant cases

Based on the Sanger sequencing results and IHC conducted on TMA cores, there were eight apparent IHC false-positive cases and one apparent IHC false-negative case (Table 2, Fig. 5). We sought to resolve these nine discordant cases by retesting and applying additional methods to detect or exclude *BRAF* V600E. To exclude the possibilities of sample mismatches or sampling error arising from the use of TMA cores for IHC, whole tissue sections were cut from the original FFPE tissue blocks and reprocessed for *BRAF* sequencing and IHC. In addition, to increase the sensitivity of DNA-based determination of *BRAF* mutation status, *BRAF* SNaPshot assays [34, 35] were performed on DNA extracted from tumour cells microdissected from sections adjacent to the original tissue sections utilised.

The original archival FFPE tissue blocks could be obtained for these further analyses from seven of the nine patients with discordant results, comprising six IHC false-positive and the single IHC-false negative case (Fig. 5). For three of the initial IHC false-positive cases, reanalyses confirmed the presence of the *BRAF* V600E mutation upon IHC and SNaPshot (allele frequencies of 33, 27 and 3 %), but Sanger sequencing again missed these mutant cases. In contrast, the other three initial IHC false-positive cases were found to be WT upon reanalysis with Sanger sequencing, SNaPshot and IHC on the whole mount slides (two with 0 % and one with 10 % of cells staining). Of the three cases erroneously VE1 positive on TMA IHC, review of the tissue core harvest sites on the VE1 whole sections showed sampling from heterogeneously stained areas in two of the three cases.

The single initial IHC false-negative case was confirmed to be *BRAF* V600E by resequencing and SNaPshot analysis (mutant allele frequency of 17 %) and showed corresponding VE1-positive staining in 47 % of tumour cells in the whole tissue section. Most discrepancies were, therefore, due to either low sensitivity of Sanger sequencing and/or heterogeneous VE1 antibody staining compounded by sampling error from the use of TMAs. When the two discordant cases with unavailable blocks for retesting were excluded, the VE1 antibody had a 100 % sensitivity, specificity, NPV and PPV in the population of reevaluable cases ($n=475$) (Table 3).

Prognostic significance of VE1 positivity

We determined the prognostic significance of VE1 positivity in patients of the evaluable cohort with de novo ($n=108$) or recurrent ($n=87$) metastatic disease using final results after investigation of the discordant cases with whole mount IHC and SNaPShot assays. Survival data was available for 188

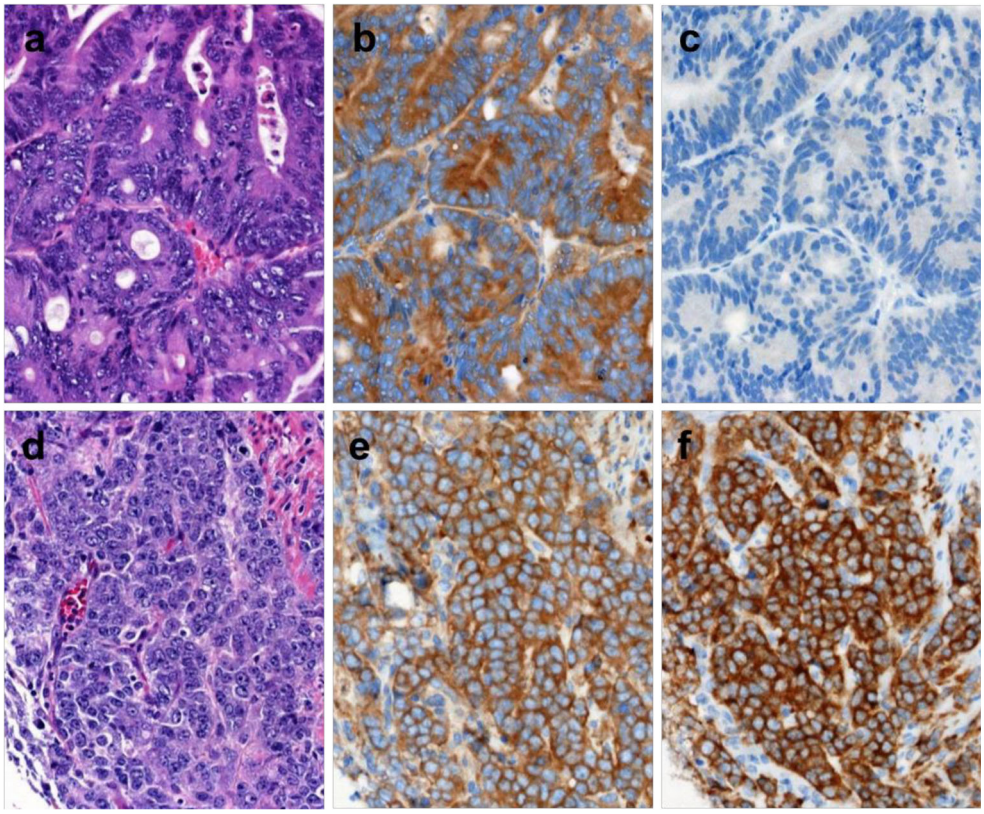

Fig. 2 Representative images of pBR1-positive tumours either positive or negative for VE1. The *upper panels* represent a BRAF wild-type tumour, showing **a** hematoxylin and eosin (H&E), **b** total BRAF (pBR1) expression and **c** lack of BRAF V600E (VE1) protein. The *lower panels* demonstrate a confirmed BRAF V600E mutant tumour displaying **d** H&E and **e** total BRAF (pBR1) and **f** BRAF V600E (VE1) protein expression

(96 %) patients of this subgroup. The median OS with metastatic disease for patients with *BRAF* V600E tumours on IHC was 275 days compared to 518 days for those with *BRAF* WT. The hazard ratio for OS with *BRAF* V600E was 2.20 (95 % CI 1.26–3.83, p=0.005) (Fig. 6).

Discussion

Mutation of *BRAF* V600E is a well-validated poor prognostic marker in metastatic CRC [5–7], as well as a discriminator between HNPCC-related disease and sporadic MSI CRCs

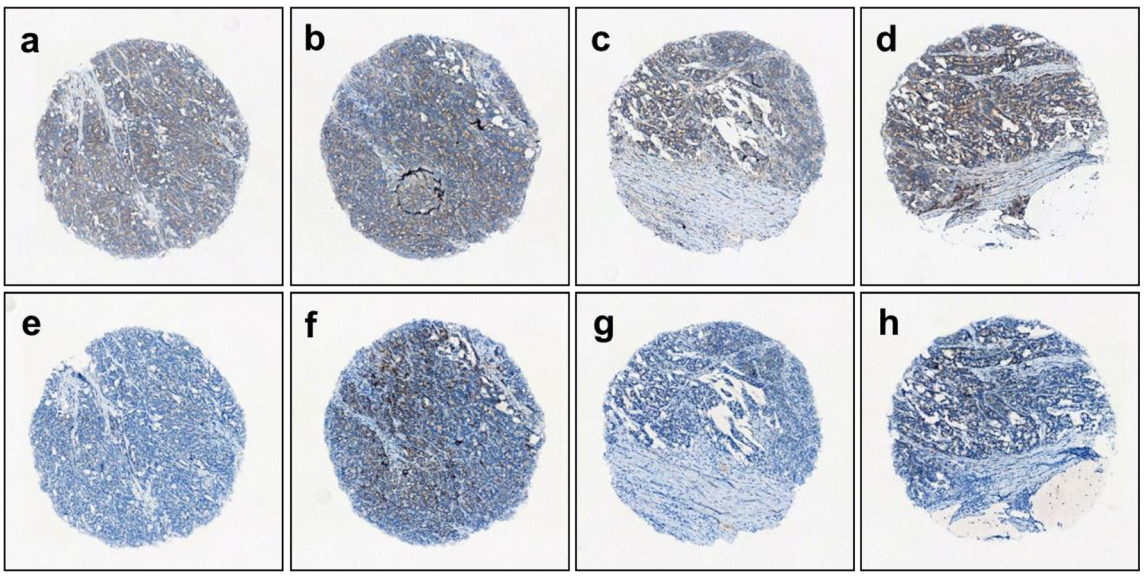

Fig. 3 Representative images demonstrating heterogeneous staining of mutant BRAF V600E protein. **a**–**d** pan-BRAF IHC (pBR1) detects total BRAF within all cores of a single case. **e**–**h** Using the mutation-specific antibody VE1, mutant BRAF V600E is only present within cores **f** and **h** and absent within cores **e** and **g** of the same case

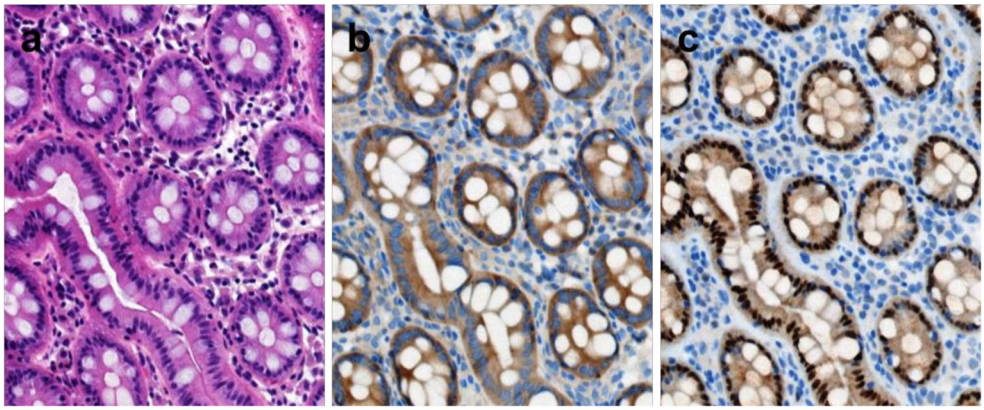

Fig. 4 Representative images of normal colon indicating **a** hematoxylin and eosin (H&E), **b** cytoplasmic total BRAF expression (pBR1) and **c** both cytoplasmic and nuclear staining in normal colonic epithelium (VE1)

[11–13]. Perhaps the greatest promise of *BRAF* V600E detection, however, is in the application of targeted therapies in the emerging era of personalised medicine. While *BRAF*-mutant colorectal cancer has not demonstrated the same responsiveness to single-agent BRAF inhibitors seen in metastatic melanoma [41, 42], recent research findings show encouraging headway in the characterisation of CRC resistance mechanisms [42–44]. Clinical studies combining BRAF inhibitors with EGFR inhibitors, PI3K inhibitors or MEK inhibitors are now underway based on these recent data and will require accurate, efficient identification of patients with *BRAF*-mutant CRC.

We report a large series of community-based colorectal cancers screened for *BRAF* V600E mutation using the novel, mutation-specific monoclonal antibody VE1. In this study, blinded interpretation of VE1 IHC on CRC cores embedded in TMAs showed 98.2 % sensitivity, 98.1 % specificity, 87.3 % PPV and 99.8 % NPV against *BRAF* genotype determined by direct sequencing. Similar sensitivities and specificities for VE1 have been reported in papillary thyroid cancer, lung cancer and melanoma [20, 27–29]. Given that positive IHC results are likely to be verified by gene-based mutation detection methods and accurate identification of *BRAF* mutation is expected to facilitate access to novel targeted therapies, the NPV of VE1 IHC is of the highest importance. Only one sample in this study was falsely negative on VE1 TMA IHC and subsequently shown to be clearly VE1 positive on whole-section staining. The resolution of discordant findings for this case, and the further six available TMA IHC false-positive cases, by additional methods is reasonable in the context of this particular study. It is important to note that whole-section IHC would be conducted in routine clinical practice, rather than via the creation of TMAs. In addition, numerous genotyping methods have been recently demonstrated to be more sensitive than Sanger sequencing [15–17, 27]. The sensitivity, specificity, PPV and NPV were each recalculated as 100 % in the fully evaluable patient population. As

Table 1 Clinical characteristics of patients with colorectal cancer evaluable by immunohistochemistry (n=477)

Feature	n (%)	*BRAF* V600E IHC positive, n (%)
Mean age (years)	70.5	
Gender		
Male	193 (40)	10 (5)
Female	282 (59)	53 (19)
Unknown	2 (<1)	0 (0)
Tumour site		
Right colon	200 (42)	52 (26)
Left colon	193 (40)	8 (4)
Rectum	82 (17)	3 (4)
Unknown	2 (<1)	0 (0)
Tumour stage		
I	27 (6)	2 (7)
II	148 (31)	23 (16)
III	192 (40)	23 (12)
IV	108 (23)	15 (14)
Unknown	2 (<1)	0 (0)
Differentiation		
Well moderate	304 (64)	25 (8)
Poor	161 (34)	35 (22)
Unknown	12 (3)	3 (25)
Microsatellite status		
Microsatellite stable	371 (78)	24 (6)
Microsatellite unstable	96 (20)	39 (41)
Unknown	10 (2)	0 (0)

Table 2 Initial determination of *BRAF* V600E using immunohistochemistry on TMA sections versus determination by Sanger sequencing

BRAF V600E results (n=477)	IHC positive	IHC negative
Sequencing positive	55*a*	1*b*
Sequencing negative	8*c*	413*d*

Sensitivity ($a/a+b$)=55/56 (98.2 %); specificity ($d/d+c$)=413/421 (98.1 %); negative predictive value ($d/d+b$)=413/414 (99.8 %); positive predictive value ($a/a+c$)=55/63 (87.3 %)

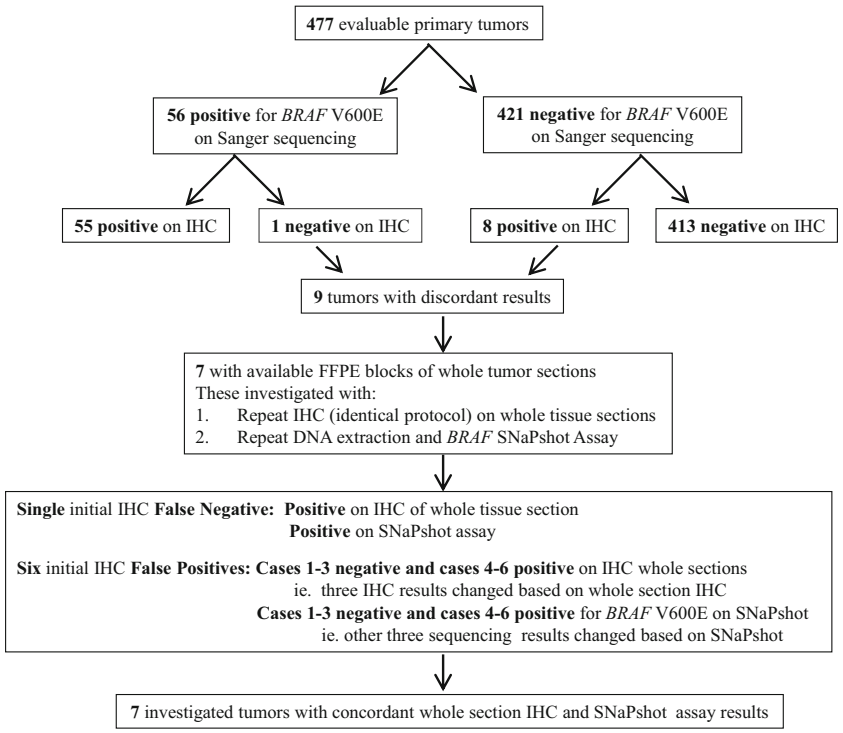

Fig. 5 Initial results and subsequent investigation of discordance between TMA IHC and Sanger sequencing results. *IHC* immunohistochemistry, *FFPE* formalin-fixed, paraffin-embedded

expected, clinical correlations with BRAF V600E positivity confirmed the same patient and tumour associations in this patient cohort seen with *BRAF* genotype as determined by DNA-based mutation detection methods [38–40], further supporting the validity of this diagnostic approach in community-based patient cohorts. Although we restricted survival data to the subgroup of patients with metastatic disease (either de novo stage IV or recurrent stage IV disease, $n=188$), given this is the group of patients for which the presence of a BRAF V600E mutation is a clear prognostic factor, our survival data was again consistent with that seen in other series.

In studies forerunning to those using anti-BRAF antibodies, the ability to apply immunohistochemistry for the detection of mutated gene products has been recognised in the management of gliomas. A monoclonal antibody specific for the mutant protein *IDH1* R132H, important diagnostically and prognostically [45], has been developed and applied to clinical samples [46, 47]. Similarly, determination of *BRAF* mutation status by IHC is anticipated to confer significant benefits in the clinical setting. Unlike genotyping methods, IHC is routinely performed in all hospital histopathology departments. The cost of reagents, equipment and labour is projected to be lower than that of currently available sequencing methods. Additionally, the time to results may be shorter due to the on-site sample processing and absence of requirement for DNA extraction. Routine profiling of all newly diagnosed CRCs for *BRAF* V600E mutation may therefore now be feasible with an inexpensive and widely available methodology. This takes on added significance when considering *BRAF* V600E's relatively low population prevalence. Detection of *BRAF* V600E mutation at the time of primary CRC resection and histopathological analysis has the advantages of excluding underlying

Table 3 Revised determination of *BRAF* V600E using immunohistochemistry on TMAs and/or whole mount sections versus determination by Sanger sequencing and/or SNaPshot assay

BRAF V600E results ($n=475$)	IHC positive	IHC negative
Sequencing/SNaPshot positive	59*a*	0*b*
Sequencing/SNaPshot negative	0*c*	416*d*

Sensitivity ($a/a+b$)=59/59 (100 %); specificity ($d/d+c$)=416/416 (100 %); negative predictive value ($d/d+b$)=416/416 (100 %); positive predictive value ($a/a+c$)=59/59 (100 %)

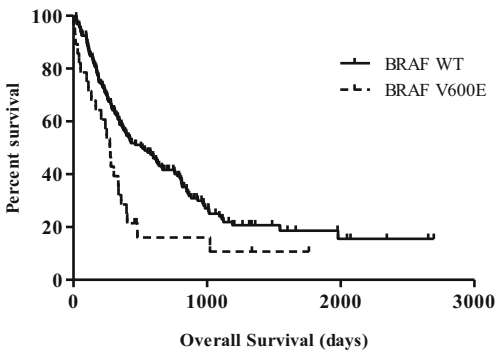

Fig. 6 Overall survival in metastatic colorectal cancer according to *BRAF* status as determined by IHC

HNPCC in MSI CRCs [11–13], with resultant screening implications [48], and prevention of lag time awaiting *BRAF* mutation results in patients later diagnosed with metastatic disease. Given that patients with metastatic *BRAF*-mutant CRC have a significantly poorer prognosis, early recognition of their *BRAF* status may alter clinical decisions regarding disease observation and, in the future, administration of molecularly directed therapies.

As per the original description of VE1 IHC on clinical samples [18], this study applied both VE1 and pBR1 primary antibodies to all tumours. While VE1 had been selected from 2,234 hybridoma clones as the only immunoglobulin specific for *BRAF* V600E on IHC, pBR1 had been chosen from pan-BRAF clones for its demonstrated ability to bind both BRAF wild type and BRAF V600E on IHC and Western blots [18]. The utility of positive pBR1 staining is in its confirmation of BRAF protein expression, reducing the likelihood of false-negative VE1 IHC findings, i.e. the presence of *BRAF* V600E genotype but negative VE1 staining due to the absence of BRAF protein expression in the tumour. In this cohort, only 14 of 491 primary CRCs (2.9 %) were negative on pBR1 IHC, suggesting that failure to express BRAF is rare in CRC and the routine use of pBR1 concurrently with VE1 may be unnecessary for this tumour type. More important than concurrent pBR1 staining may be preservation of tissue quality, as tissue areas subject to diathermy, showing necrosis, or frozen and then formalin-fixed have all been reported to show lower antigenicity for VE1 [18]. The use of freshly cut tissue sections is also recommended [19]. We noted strong staining of normal colonic surface epithelial cell nuclei for VE1, which could be used as a positive internal control for VE1 IHC protocols. The reason for nuclear staining with the VE1 antibody is unknown.

Immunohistochemistry may be an optimal method for determining the *BRAF* V600E mutation status of tissue samples with low cellularity due to its single-cell-level resolution. In contrast, for DNA-based detection methods, even microdissected tissue samples contain significant proportions of stromal cell-derived DNA, posing challenges in the detection of diluted tumour cell-derived mutant alleles. BRAF V600E-specific IHC has some limitations, however. Samples collected by fine-needle aspiration (FNA) may pose a challenge because of the fixatives used. Caution is urged until the validity of this approach in FNA samples has been adequately investigated. Due to the heterogeneity of VE1 staining seen in a proportion of CRCs, 1.7 % of the evaluable population in this study, small biopsy (e.g. core biopsies) and tissue specimens may be erroneously interpreted due to sampling error. This phenomenon was documented in our patient cohort with the initial use of TMAs for VE1 IHC; for one sample, IHC findings were changed from positive to negative, and for three samples, the reverse occurred when TMA IHC findings were compared to staining on whole mount sections. Although in our patient cohort <3 % of CRCs were negative on pan-BRAF IHC, other malignancies with low BRAF protein expression may not be amenable to IHC testing. VE1 is highly specific for *BRAF* V600E and does not detect the protein products of other *BRAF* aa.600 mutations [19, 27, 28]; hence for diseases with a higher burden of *BRAF* non-V600E mutations, DNA-based genotyping or alternative monoclonal antibodies may be indicated, particularly should these mutations demonstrate drug sensitivity. In CRC, <4 % of *BRAF* mutations are non-V600E [4, 9], and no other aa.600 mutations were detected by sequencing in the 505 patients of this cohort. However, we cannot exclude the possibility that a small number of patients may have carried other non-aa.600 *BRAF* mutations, as DNA sequencing was limited to the region of aa.600.

Two recently published studies exploring the VE1 antibody in colorectal cancer have also shown a high degree of specificity and sensitivity, but included either small series [32] and/or a highly preselected patient population [31]. However, the consistent findings in both of these studies add considerable weight to our findings in supporting the incorporation of the antibody as the initial method of detection of *BRAF* V600E status in patients with colorectal cancer. As opposed to the findings of strong concordance between the VE1 IHC antibody and sequencing-based techniques in these two studies and ours, another study examining VE1 IHC in colorectal cancer was also published recently and reports low sensitivity (71 %) and specificity (74 %) of VE1 tested on 52 FFPE CRC samples [49]. Methodological differences in the antigen retrieval, staining and signal amplification protocols resulting in poor signal intensity and background staining likely account for this discrepancy [49]. We found that the use of OptiView DAB IHC Detection Kit resulted in clear discrimination between background tissue and *BRAF* V600E-positive tumour cell cytoplasmic staining using the VE1 antibody. In our experience, patchy focal staining, rather than the level of staining intensity, can lead to difficulties with interpretation, particularly in tissue core samples. Patchy focal staining was occasionally seen (1.7 %) in otherwise clearly negative tumours and could lead to false-positive results (Fig. 3). Patchy focal non-staining was occasionally seen in otherwise clearly positive tumours and could lead to false-negative results. These staining anomalies were often present in the same areas in restained sections and did not correspond to any anatomical features such as tumour nodules. Interpretive difficulties were often resolved by examining additional tumour areas by staining whole mount sections. In such cases, confirmation of *BRAF* status should be verified by IHC on a different specimen from that resection or by a sensitive molecular method.

The observation that the vast majority of CRCs stained in this study showed either homogeneously negative or positive BRAF V600E expression argues against the clonal acquisition of *BRAF* activating mutations in primary CRCs. Interestingly,

lack of clonality for BRAF V600E expression has also been noted in other malignancies including metastatic lesions [19–21, 28], despite suggestions of polyclonality for *BRAF* genotype in melanoma and thyroid cancer using alternative experimental methods [50–52]. The use of mutation-specific antibodies to address this controversial cancer biology question is but one of their potential research applications. Busam et al. [53] and Sahm et al. [23] utilised VE1 IHC in co-staining experiments to further characterise *BRAF* mutation-positive melanocytic lesions and Langerhans cell histiocytosis respectively. Exploration of staining intensity has also been suggested as a potentially fruitful translational research application [54].

In conclusion, the BRAF V600E VE1 antibody is an accurate immunohistochemical diagnostic assay in patients with CRC and should serve as a simple method for the detection of the *BRAF* V600E mutation in routine practice.

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