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ORIGINAL PAPER

Assessment of *BRAF* V600E mutation status by immunohistochemistry with a mutation-specific monoclonal antibody

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Abstract Activating mutations of the serine threonine kinase v-RAF murine sarcoma viral oncogene homolog B1 (BRAF) are frequent in benign and malignant human tumors and are emerging as an important biomarker. Over 95% of BRAF mutations are of the V600E type and specific small molecular inhibitors are currently under preclinical or clinical investigation. *BRAF* mutation status is determined by DNA-based methods, most commonly by sequencing. Here we describe the development of a monoclonal BRAF V600E mutation-specific antibody that can differentiate BRAF V600E and wild type protein in routinely processed formalin-fixed and paraffin-embedded tissue. A total of 47 intracerebral melanoma metastases and 21 primary papillary thyroid carcinomas were evaluated by direct sequencing of *BRAF* and by immunohistochemistry using the BRAF V600E mutation-specific antibody clone VE1. Correlation of VE1 immunohistochemistry and BRAF sequencing revealed a perfect match for both papillary thyroid carcinomas and melanoma metastases. The staining intensity in *BRAF* V600E mutated tumor samples ranged from weak to strong. The generally homogenous VE1 staining patterns argue against a clonal heterogeneity of the tumors investigated. Caution is essential when only poorly preserved tissue is available for VE1 immunohistochemical analysis or when tissues with only little total BRAF protein are analyzed. Immunohistochemistry using antibody VE1 may substantially facilitate molecular analysis of BRAF V600E status for diagnostic, prognostic, and predictive purposes.

Keywords BRAF · V600E · Mutation · Monoclonal antibody · Immunohistochemistry · Melanoma · Papillary thyroid carcinoma · Biomarker

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Introduction

Activating mutations of BRAF are frequent in benign and malignant human tumors. It has been estimated that 5–7% of all human neoplasm carry BRAF alterations. According to the Catalogue of Somatic Mutations in Cancer (http://www.sanger.ac.uk/cosmic) the vast majority of BRAF alterations (15174 of 15682 *BRAF* mutations in database with recorded mutation type; ~97%) are characterized by a missense substitution of valine by glutamic acid in a mutational hotspot at amino acid position 600 (referred to as

BRAF V600E). This exchange mimics the phosphorylation of amino acid residues T599 and S602 and induces a conformational change of the activation segment leading to a constitutive kinase activity of BRAF and consecutive phosphorylation of downstream targets [34]. Particularly high *BRAF* V600E mutation rates have been detected in melanoma (~30–70%), papillary thyroid carcinoma (40–70%), pleomorphic xanthoastrocytomas (~60–70%), and Langerhans cell histiocytosis (~50–60%), and in some benign lesions, for example melanocytic nevi and serrated polyps of the large intestine [5, 11, 12, 24, 30–32]. *BRAF* V600E mutations are also frequently observed in various other cancers, in particular borderline ovarian cancer (~30%), ganglioglioma (~20%), colorectal carcinoma (5–10%), and pilocytic astrocytoma (~5–10%) [32, 33, 35].

Detection of *BRAF* mutations is emerging as an important biomarker with diagnostic, prognostic, and predictive potential in several clinical settings. In papillary thyroid carcinoma the presence of *BRAF* V600E mutation is associated with a higher rate of tumor recurrence and tumor-related mortality and was shown to be a diagnostic aid in fine-needle aspirants of thyroid nodules [29]. For colorectal carcinoma, *BRAF* mutation is a strong negative prognostic factor and there is increasing evidence that *BRAF* V600E mutation corresponds with resistance to anti-epidermal growth factor receptor (EGFR) therapy [13, 15]. Most importantly, mutated BRAF V600E protein has recently emerged as a promising therapeutic target. Several specific small-molecule BRAF V600E inhibitors are currently under pre-clinical or clinical investigation (reviewed in Ref. [4]). Of these, the V600E-specific inhibitor PLX4032 (Vemurafenib) has remarkable clinical activity in patients with *BRAF* V600E mutated metastasizing melanoma [19]. The compound GSK 2118436 also resulted in clinical response in metastasized melanoma and may also be effective in less frequent BRAF mutations, for example V600K [23].

The gold standard for *BRAF* mutation analysis is direct sequencing of tumor DNA. A variety of additional DNA-based assays are available, most of which rely on polymerase chain reaction (PCR) techniques such as quantitative PCR, locked nucleic acid-PCR sequencing, pyrosequencing, or assays using SNaPshot® technology [3, 16, 26]. Thus, currently all testing required for pre-selection of patients eligible for targeted therapy is based on nucleotide analyses and not on assessment of the mutated target protein.

We have recently demonstrated that mutation specific antibodies can reliably detect the exchange of single amino acids in routinely processed, formalin-fixed, and paraffin-embedded (FFPE) tumor tissue [9, 10]. Here we describe the development of a BRAF V600E mutation-specific antibody that can be used to detect BRAF V600E mutated protein in FFPE tissue samples and that may be of help in a wide range of diagnostic applications.

Materials and methods

Antigen peptide, immunization, and hybridization

For immunogen preparation, an 11-amino-acid synthetic peptide representing the BRAF V600E mutated amino acid sequence from amino acid 596 to 606 (GLATEKSRWSG) was coupled to keyhole limpet hemocyanin (Peptide Specialty Laboratories, Heidelberg, Germany). The HUSAR software package (DKFZ, Heidelberg, Germany) was used to select the appropriate sequence region. Six-week-old female C57BL/6 mice (Charles River, Sulzfeld, Germany) were immunized with 20 µg coupled peptide on day 1 and boosted on days 5, 11, 18, and 46. Immunoreaction was enhanced with complete Freund's Adjuvant on day 1 and with incomplete Freund's Adjuvant on day 5. Sera of mice were tested for immunoreaction with full length BRAF V600E protein by Western blot on days 19 and 47. Popliteal lymph node cells of anti-BRAF V600E seropositive mice were fused with mouse myeloma SP2/O cells by polyethylene glycol fusion. Sprague–Dawley rats were immunized with recombinant protein spanning full length wt BRAF fused to a hexahistidine tag for the generation of a pan-BRAF antibody detecting both mutated and wt BRAF. The monoclonal antibodies were raised according to the method described by Kohler and Milstein [25]. Consecutive subcloning, isotyping, and purification were performed in accordance with published procedures [20].

Screening of clones and purification of monoclonal antibody

V600E mutation was introduced in full length *BRAF* (RefSeq DNA: NM_004333) using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, USA), following the supplier's instructions. For hybridoma screening we engineered HEK 293T cells to transiently over-express full length BRAF V600E in a pFLAG-CMV-D11 vector. Hybridomas were screened on transiently BRAF V600E expressing HEK 293T cells plated on 96-well dishes (Becton–Dickinson Labware, Franklin Lakes, USA) by immunofluorescence using hybridoma supernatant as primary antibody. Alexa Fluor 568 donkey anti-mouse IgG (Invitrogen, Carlsbad, Germany) was used for visualization. Clones with detectable immunofluorescence signals were further tested in Western blot of frozen tissue and immunohistochemistry of routinely processed paraffin-embedded tissue samples. The same screening procedures were performed for selection of rat pan-BRAF

clones, except that Cy3 labeled donkey anti-rat IgG (Dianova, Hamburg, Germany) was used as secondary antibody.

Human tumor specimens

For Western blot analysis, six frozen tissue samples of intracerebral melanoma metastases with known *BRAF* mutation status (3 BRAF wt and 3 BRAF V600E) were selected from the archives of the Department of Neuropathology at the University of Heidelberg.

Immunohistochemical and genetic analyses were performed from 47 routinely processed FFPE intracerebral melanoma metastases diagnosed at the Department of Neuropathology and 21 routinely processed paraffin-embedded primary papillary thyroid carcinomas diagnosed at the Institute of Pathology, both Heidelberg University Hospital, Heidelberg, Germany. All samples were routinely fixed in 4% neutral buffered formalin for approximately 12–24 h immediately after surgical resection. The cases of this series were not selected for tissue quantity and quality but represent continuously diagnosed cases from 1993 (for melanoma metastases) and 1995 (for papillary thyroid carcinomas) onward. Diagnoses were made by histological assessment and additional immunohistochemical staining, as appropriate, by an experienced pathologist (GM) or neuropathologist (AvD). All cases were analyzed for *BRAF* exon 15 mutations by direct sequencing as previously described [32]. Utilization of tissue samples was in accordance with the faculty of medicine, Heidelberg, ethics committee.

PCR amplification and direct sequencing

A fragment of 173-bp length including codon 600 of *BRAF* (RefSeq DNA: NM_004333) was amplified using 60 ng each of the sense primer BRAFf TGCTTGCTCTGA TAGGAAAATG and the antisense primer BRAFr CCA CAAAATGGATCCAGACA. PCR using standard buffer conditions, 100 ng DNA, and GoTaq DNA polymerase (Promega, Madison, USA) employed 35 cycles with denaturing at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 40 s in a total volume of 25 µl. Two microliters of the PCR amplification product were submitted to the sequencing reaction using the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, Foster City, USA). Twenty-five cycles were performed employing 12 ng of the sense primer BRAFf, with denaturing at 95°C for 30 s, annealing at 56°C for 15 s, and extension at 60°C for 240 s. For selected cases, a second round of sequencing analysis was performed using the antisense primer BRAFr and the sequencing reaction conditions as described above. Sequences were determined using a semiautomated sequencer (ABI 3100 Genetic Analyzer; Applied Biosystems) and Sequence Pilot version 3.1 software (JSI-Medisys, Kippenheim, Germany).

Western blot

Western blot lysates were prepared as previously described [9] and stored at −80°C for further processing. 30 µg protein diluted in NuPAGE-sample buffer and reducing reagent (Invitrogen, Carlsbad, Germany) were denatured at 95°C for 5 min and electrophoretically separated on 4–12% Bis–Tris mini gels (Invitrogen). Blotting to nitrocellulose membranes was followed by blocking in 5% nonfat dried milk for 1 h and incubation with undiluted hybridoma supernatant of BRAF V600E specific clone VE1 (internal number 263) overnight at 4°C. After repeated washing, membranes were incubated with peroxidase-labeled anti-mouse IgG antibody (Cell Signaling Technology) then incubated with LumiGLO peroxidase chemiluminescent substrate Kit (KPL, Gaithersburg, USA) for signal visualization. For loading control, total BRAF was visualized on the same membrane using undiluted hybridoma supernatant of pan-BRAF (clone pBR1, internal number 101) using anti-rat IgG antibody (KPL) as secondary antibody. Protein lysates from *BRAF* V600E mutated melanoma cell line A375 and HEK 293T cells served as mutated and wild-type control, respectively [17].

Immunohistochemistry

Sections cut to 4 µm with a Microm HM 355 S microtome (Thermo Fisher Scientific, Waltham, USA) were dried at 80°C for 15 min and stained with BRAF V600E specific clone VE1 on a Ventana BenchMark XT immuno stainer (Ventana Medical Systems, Tucson, USA). The Ventana staining procedure included pretreatment with cell conditioner 1 (pH 8) for 60 min, followed by incubation with undiluted VE1 hybridoma supernatant at 37°C for 32 min. Antibody incubation was followed by standard signal amplification including the Ventana amplifier kit, ultraWash, counterstaining with one drop of hematoxylin for 4 min and one drop of bluing reagent for 4 min. For chromogenic detection, ultraView Universal DAB detection kit (Ventana Medical Systems) was used. Subsequently, slides were removed from the immunostainer, washed in water with a drop of dishwashing detergent, and mounted. No chromogen was detected when primary antibody BRAF V600E clone VE1 was omitted.

Automated immunohistochemistry was also performed with pan-BRAF antibody clone pBR1. The staining conditions were as above; except that undiluted pBR1 was used as primary antibody and a monoclonal rabbit anti-rat IgG bridging antibody (Epitomics, Burlingame, USA) was

applied directly after the incubation with the primary antibody.

Three observers (DC, MP, AvD) evaluated the immunostained slides simultaneously on a multiheaded microscope. All three observers were unaware of the genetic results. Immunoreaction was scored positive when unambiguous cytoplasmic staining for VE1 or pBR1 was observed for a substantial fraction of viable tumor cells. Of positive cases, the intensity of staining was recorded as weak, moderate, or strong (for illustration see Fig. 3d). Faint diffuse staining, any type of isolated nuclear staining, weak staining of single interspersed cells, or staining of monocytes/macrophages was scored negative. For evaluation of immunostaining results in melanomas, heavily pigmented areas were avoided. Photomicrographs were taken with a Zeiss (Jena, Germany) Axioplan 2 microscope equipped with a Zeiss AxioCam and AxioVision software (Release 4.8; Zeiss). All photomicrographs were taken with the same software settings; light intensity was adjusted on the microscope for the individual micrographs.

Results

Selection of BRAF V600E mutation-specific clone VE1 and pan-BRAF clone pBR1

In total, 2,234 clones were investigated for the desired immunoprofile for detecting BRAF V600E protein but not BRAF wt protein. Only one of these clones (clone VE1, internal number 263) demonstrated specific reaction with BRAF V600E in immunofluorescence, Western blot, and immunohistochemistry in FFPE tissue.

The screening of pan-BRAF clones led to the identification of pBR1 (internal number 101) that detects both BRAF wt and BRAF V600E protein in Western blot and immunohistochemistry. The isotype of both monoclonal antibodies VE1 and pBR1 was classified as IgG2a.

Identification of BRAF mutated tumors by Western blot

Fresh frozen tissue samples of six intracerebral melanoma metastases and lysates of one *BRAF* V600E mutated (A375) and one BRAF wt cell line (HEK 293T) were analyzed by Western blot. VE1 detected a strong band at the predicted molecular weight of BRAF (~95 kD) only in tumors with *BRAF* V600E mutation (Fig. 1). Interestingly, an additional, weaker, band was detected at ~75 kD in all three mutated melanoma specimens and in the mutated cell line A375. A band at ~60 kD was detected in one BRAF V600E mutated melanoma; an additional lower band at ~50 kD was observed in cell line A375. To control for the presence of BRAF protein in all samples, the blots were re-incubated with the pan-BRAF antibody pBR1. A strong band of comparable intensity was detected at ~95 kD in all samples. An additional weaker band was detected in all tissue samples at a molecular weight of ~60 kD. A further faint band was detected in most samples at ~70 kD. Additional weak bands appeared only in single lysates. The overlay of both antibodies showed that the band at ~95 kD was at exactly the same level. The weaker band at ~75 kD observed in V600E mutated lysates with VE1 was slightly higher than the faint band detected with pBR1 at ~70 kD. The band at ~60 kD seen in one melanoma lysate with VE1 also appeared when incubated with pBR1.

Correlation of *BRAF* sequencing and VE1 immunohistochemistry

A total of 47 intracerebral melanoma metastases and 21 primary papillary thyroid carcinomas were evaluated by direct sequencing of *BRAF* and by immunohistochemistry

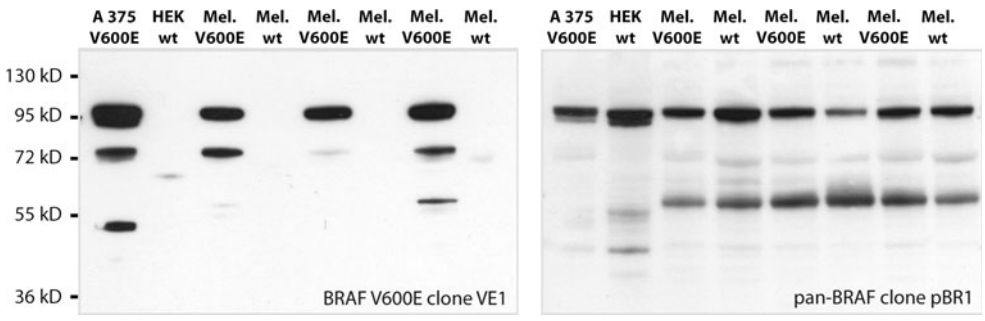

Fig. 1 Representative Western blot of *BRAF* V600E mutated and wt melanoma metastases with a band at the predicted molecular weight of ~95 kD only in mutated tumors (*left panel*). A second strong band at ~75 kD is detected in the mutated samples; this is most likely to be a splice variant of BRAF that includes the mutated kinase domain. A band at ~60 kD was detected in one *BRAF* V600E mutated melanoma, an additional lower band at ~50 kD was observed in cell line A375. In the *right panel* the same blot has been incubated with an antibody (pBR1) detecting both mutated and wt BRAF. pBR1 does not detect the band at ~75 kD, indicating that the pBR1 binding region is not present in this supposed splice variant. *Mel*. melanoma metastasis

using the BRAF V600E mutation-specific antibody VE1. Genetic analysis was successful in 43/47 (91%) melanoma metastases and in 18/21 (86%) papillary thyroid carcinomas. In the unsuccessful cases, either no amplifiable DNA could be extracted or the sequencing reaction did not yield an evaluable sequence. In melanoma metastases and papillary thyroid carcinomas heterozygous *BRAF* V600E mutations were detected in 16/43 (37%) and 9/18 (50%) of cases, respectively. In each mutant case, exchange of T to A at c.1799 was observed (c.1799T > A). No other *BRAF* codon 600 hotspot mutations were identified in this series.

Immunohistochemistry with VE1 was evaluable in all 47 melanoma metastases (Fig. 2a, b, e) and all 21 papillary thyroid carcinomas (Fig. 2c, d). 18/47 (38%) melanoma metastases and 12/21 (57%) thyroid carcinomas were scored immunopositive using VE1. Correlation with genetic data revealed a perfect match for both papillary thyroid carcinomas and melanoma metastases (Table 1). All 25 *BRAF* V600E mutated cases were scored immunopositive, all 36 wt cases were scored negative. Thus, the sensitivity of VE1 for correct identification of *BRAF* V600E mutation in this series was 100% and specificity was 100%. Among the cases in which genetic analysis failed, VE1 was positive in 2 of 4 melanomas and in 3 of 3 papillary thyroid carcinomas.

Immunohistochemical staining patterns of VE1 and pBR1

To control for total BRAF expression, all tumor tissues of this series were analyzed with pBR1, an antibody detecting both mutated and wt BRAF (Fig. 2b, d, f). All 21 papillary thyroid carcinomas and all 47 melanomas were positive for pBR1. Among melanomas, expression in 9 cases was scored weak, in 21 moderate, and in 17 strong. In papillary thyroid carcinomas intensity was weak in 5 cases, moderate in 11, and strong in 5. When immunohistochemistry of VE1-positive cases was compared with pBR1 a relationship with staining intensity was evident not only for distribution of staining (i.e., in heterogeneously stained tumors, areas with stronger pBR1 labeling also had stronger VE1 labeling in same regions) but also for the general intensity. As could be expected for a heterozygous mutation, the staining intensity of pBR1 (detecting both wt and V600E mutated BRAF) was often slightly more intense than VE1 (detecting only BRAF V600E).

A VE1 staining feature that was evident in several tumors was a focal loss or strong reduction of staining intensity. This was clearly associated with areas of artificial tissue damage, especially in areas of surgical coagulation (Fig. 3a). This effect was most obvious in coagulated tissue with elongation and streaming of nuclei with homogenous chromatin but was also manifest in directly adjacent areas with minimum cellular damage. The only repeatedly observed change in these areas was a loss of the otherwise often prominent nucleoli. Necrotic areas were also negative for VE1. Adjacent pre-necrotic areas were also either negative or had a marked reduction of VE1 staining (Fig. 3b). In several cases stronger staining intensity of perivascular tumor cells was observed. Interestingly, the areas with a loss or reduction of VE1 staining generally also showed reduced staining in immunohistochemical preparations for total BRAF protein using pBR1 as primary antibody. To evaluate the utility of this antibody in tissues initially subjected to frozen section analysis, two BRAF V600E melanoma metastases were frozen, followed by routine formalin fixing, paraffin-embedding, and then VE1 immunohistochemistry (Fig. 3c). We observed a strong reduction of staining intensity in the post-cryofixed tissue compared with the directly formalin fixed tissue that might have led to a negative interpretation of VE1 immunohistochemistry.

For positive melanoma metastases ($n = 18$, of these 16 with successful genetic analysis), intensity of VE1 staining was weak in 6 cases, moderate in 10, and strong in 2 (Fig. 3d). When areas of coagulation and pre-necrosis were avoided, the staining was homogenous in most cases of melanoma metastases with equal intensity of VE1 in the vast majority of tumor cells (Fig. 2a). In two cases single VE1-negative cells surrounded by positive tumor cells were observed. These cells did not mark for other melanoma markers (MELAN A, HMB45) and are most likely to represent infiltrating inflammatory cells (not shown). In papillary thyroid carcinoma with BRAF V600E mutation variation of staining intensity was low; 11 cases were scored as moderate and one as weak. As in melanoma, intratumoral heterogeneity was minimal.

Discussion

We here report a mutation-specific monoclonal mouse antibody enabling detection of BRAF V600E mutated protein in Western blot and in immunohistochemistry of routinely processed paraffin-embedded tissue. For assessment of the general properties of this antibody we chose melanoma and papillary thyroid carcinoma known for frequent BRAF mutations. For both tumor types detection of *BRAF* mutation has strong clinical implications, either as a diagnostic and prognostic marker or potentially as a predictive marker for BRAF V600E-targeted therapy.

Analysis of melanoma metastasis lysates in Western blot revealed high specificity of BRAF mutation specific clone VE1 for mutated BRAF protein with a strong band at the predicted molecular weight of ~95 kD exclusively in mutated cases. In addition, a strong band at ~75 kD was detected in all mutated lysates, and additional lower weight

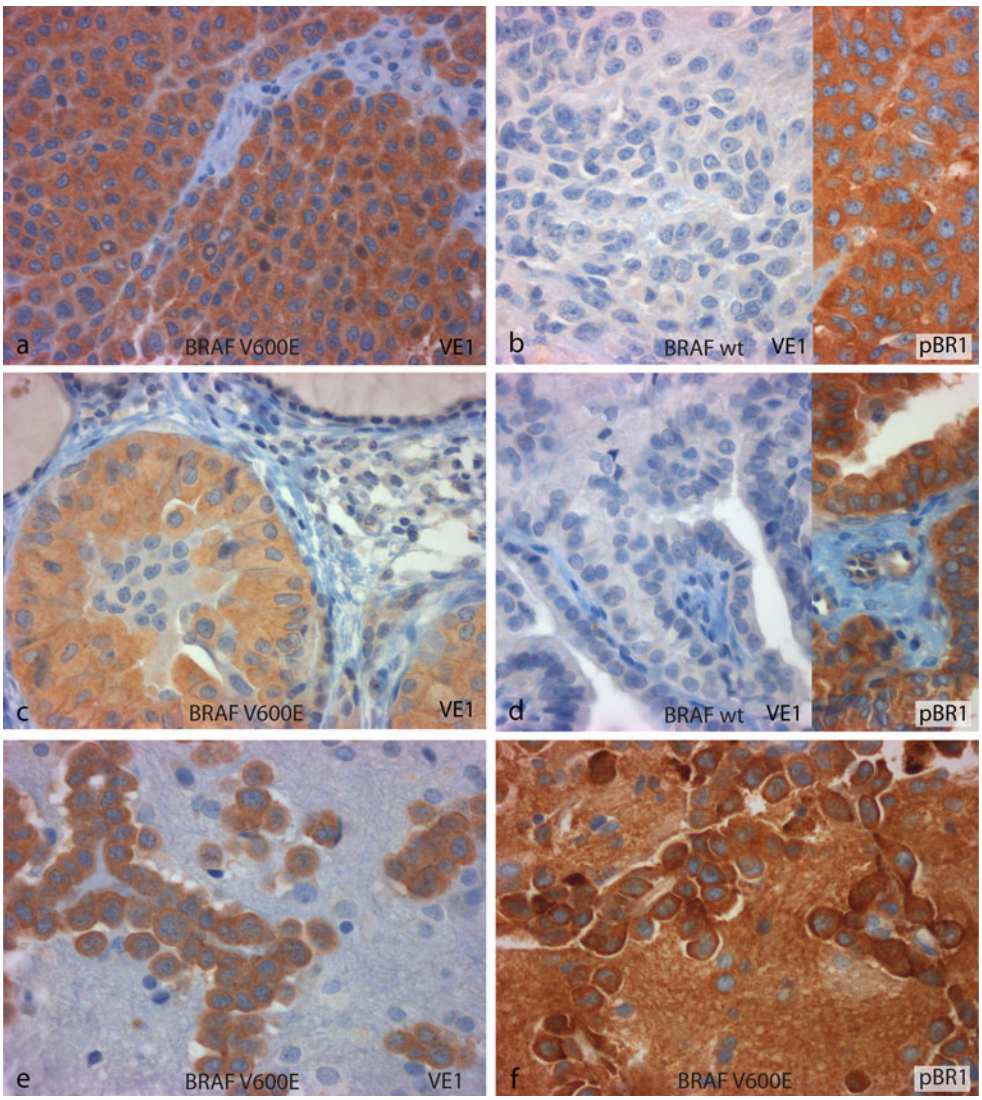

Fig. 2 VE1 and pBR1 immunohistochemistry in melanoma and papillary thyroid carcinoma. **a** *BRAF* V600E mutated melanoma metastasis shows strong cytoplasmic staining for BRAF V600E specific antibody VE1. **b** BRAF wt melanoma metastasis is VE1-negative but is strongly stained with antibody pBR1, an antibody detecting total BRAF. **c** *BRAF* V600E mutated papillary thyroid carcinoma with moderate cytoplasmic staining of VE1. Note VE1-negative residual thyroidal follicles in the upper part of the picture. **d** BRAF wt papillary thyroid carcinoma is VE1-negative but is strongly stained with antibody pBR1. **e** Border of BRAF V600E mutated melanoma metastasis with mainly perivascular infiltration into brain tissue. Tumor cells are strongly VE1-positive. **f** Same tumor as in **e** shows strong expression of total BRAF protein in tumor cells and surrounding brain tissue with an antibody for total BRAF (pBR1). Original magnification 400×

Table 1 VE1 immunohistochemistry and *BRAF* codon 600 sequencing data

	Melanoma metastasis	Papillary thyroid carcinoma
Cases analyzed (*n*)	47	21
Genetic analysis successful	43 (91%)	18 (86%)
BRAF V600E	16/43 (37%)	9/18 (50%)
VE1-positive (sensitivity)	16/16 (100%)	9/9 (100%)
VE1-negative (specificity)	27/27 (100%)	9/9 (100%)

bands were detected in single lysates. Complex alternative splicing has been reported for mammalian BRAF and in mouse at least ten protein isoforms have been characterized, some of which have also been observed in human cell lines [7, 18]. Thus, the multiple bands detected by VE1 most likely represent splice variants of BRAF that include the BRAF V600E mutated epitope. Interestingly, an investigation of BRAF isoforms in papillary thyroid carcinoma could not demonstrate splice variants including exon 15 spanning codon 600 of *BRAF* [6]. Utilization of

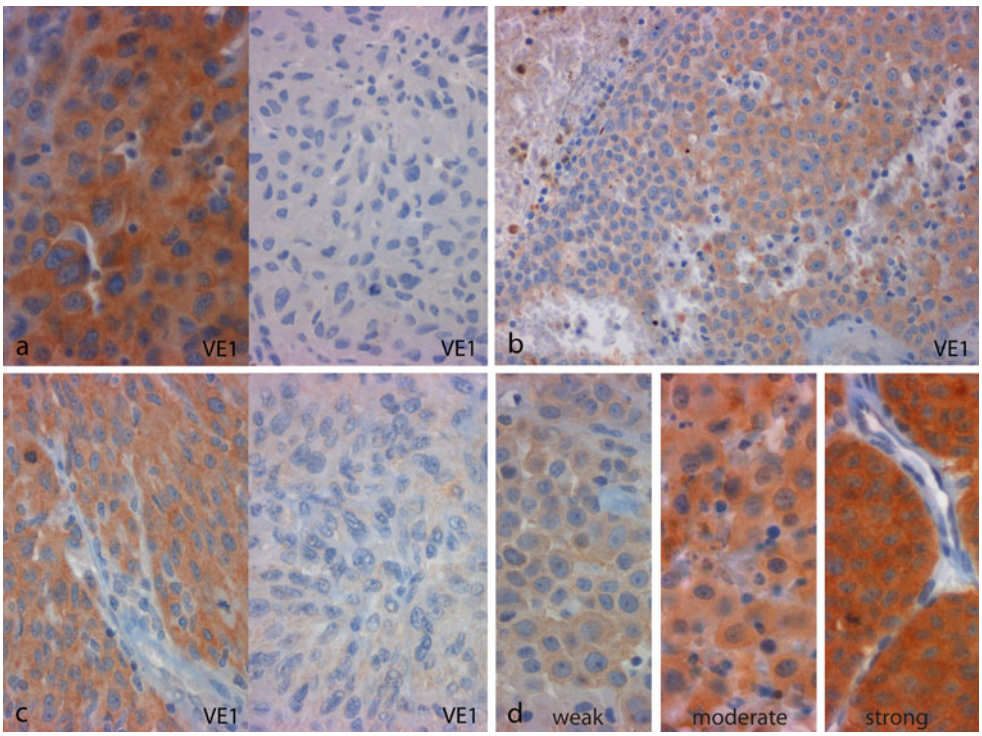

Fig. 3 Caveats of VE1 immunohistochemistry. **a** BRAF V600E mutated melanoma metastasis with strong expression of VE1-positive mutated BRAF (*left*) and loss of immunogenicity in areas with surgical coagulation (*right*). **b** VE1 staining is weaker in pre-necrotic tumor cells (vital tumor on the right, pre-necrosis and necrosis on the left). **c** VE1 immunogenicity is impaired by temporary cryofixing. Left tumor fraction was directly fixed in formalin; right tumor fraction was investigated as intra-operative frozen section and later transferred to formalin. **d** VE1 staining intensity may vary in BRAF V600E mutated tumors. Intensity was scored weak (*left*), moderate (*middle*), or strong (*right*) in these three melanoma cases. Weak staining may bear the risk of a false negative interpretation. Original magnification: 200× in **b**, 400× in **a**, **c**, and **d**

VE1 may help to clarify whether different patterns of splice variants including V600E mutated codon 600 exist between melanoma and thyroid carcinoma.

The main focus of this study was to identify antibody clones that reliably differentiate wt and BRAF V600E mutated protein by immunohistochemistry in routinely processed FFPE tissue. Although 30 of the screened clones were specific for mutated BRAF protein in Western blot, only one among 2,234 screened clones had specific immunoreactivity in FFPE tissue. This clone (VE1) detected all cases of *BRAF* V600E mutated melanomas and papillary thyroid carcinomas with specificity and sensitivity of 100%. Normal tissue surrounding the tumor was negative and investigation of the wt cases for total BRAF using antibody pBR1 revealed equally large amounts of total BRAF protein as in BRAF V600E mutated cases. We have previously developed an antibody specific for R132H mutated IDH1 protein that has been successfully implemented in a variety of tumor entities [1, 2, 10]. Besides the obvious advantages of immunohistochemistry (e.g. fast, inexpensive, no need for DNA extraction) the antibody enables assessment of mutation status in specimens of small size or low tumor cell content that may not be suitable for genetic analysis [8, 9, 14]. It has previously been estimated for *BRAF* that the mutant peak in conventional direct sequencing is only reliably discernable when heterozygous mutant cells comprised 20% of total sample cells [21]. With a mutation-specific antibody the threshold for detecting a mutation is likely far lower, in well preserved cases possibly as low as single infiltrating cells.

In this study 7 of 68 samples were not assessable by direct sequencing, either because no PCR product or no clear sequence could be obtained. We did not preselect our tissue samples; instead continuously diagnosed cases were analyzed. Only a small amount of tissue was available in several cases in which no amplifiable DNA could be extracted. In addition, formalin fixing and consecutive DNA fragmentation is a well recognized and likely cause of failure in our series [22, 26]. In contrast, analysis of these cases was unequivocal using VE1, and 5 BRAF V600E mutations were detected by immunohistochemistry among the seven cases.

In our series, no BRAF mutations other than V600E were observed, so we cannot make a definite statement about the binding of the antibody to other types of mutated BRAF protein, for example V600K. We have previously

demonstrated that IDH1 R132H mutation-specific antibody clone H09 is highly specific for the R132H mutation and does not detect other amino acid exchanges at the same position [9]. So it seems likely that VE1 is also specific for V600E and will not be able to detect other mutation types at hotspot codon 600. This may be of advantage for patient selection for therapy with V600E-specific inhibitors such as PLX4032. Other small molecules, for example GSK 2118436, also have activity against other forms of BRAF mutations [4]; it should, however, be kept in mind that 97% of BRAF mutations are of the V600E type.

Our investigation of BRAF staining patterns revealed three technical problems that must be kept in mind when BRAF mutation status is assessed using VE1. First, the antigenicity of the epitope seems to be impaired by tissue coagulation or early necrosis (Fig. 3a, b). This effect is likely to be more prominent than for other diagnostic antibodies used in our institution and may bear the risk of false negative interpretation if only poorly preserved tissue is available. A histological aspect generally correlated with good tissue preservation was the presence of distinct nucleoli. Staining was equally lost in pre-necrotic areas (areas with condensed tumor cell nuclei and loss of nucleoli) and areas directly adjacent to necrosis (Fig. 3b). Second, antigenicity seems to be greatly reduced in "post-cryofixed samples" as in tissue first investigated as a frozen section during intraoperative diagnostic evaluation and later subjected to formalin-fixing and paraffin-embedding for definite tumor typing (Fig. 3c). As known for other antibodies, false negative results may be possible in such a setting. Third, an immunohistochemical assay can only function reliably if the target protein is expressed in sufficient amounts. Although all mutated cases were scored positive for VE1 by three observers unaware of the genetic results, six melanoma metastases and one thyroid carcinoma had a weak staining reaction that may lead to wrong conclusions in some instances. Although we tested the total BRAF expression of this series using parallel immunostaining with antibody pBR1, it seems possible that other benign or malignant tumors may have so little total BRAF expression that detection of mutated protein may be below the detection limit of the antibody.

These problems may be circumvented by use of an additional antibody detecting total BRAF expression which is helpful in identification of tissues not adequate for BRAF V600E detection by an antibody assay. We used pBR1 to control for total BRAF and observed relatively similar staining pattern for VE1 and pBR1 in V600E mutated cases, e.g. weak VE1 positivity was associated with weak pBR1 positivity, thus confirming altogether low BRAF expression. In cases of faint diffuse staining of VE1, a clearly positive pBR1 was useful to confirm unspecific background staining and exclude weak positive staining of the mutation-specific antibody.

The intensity of BRAF immunohistochemistry may be important additional information in BRAF mutation screening. Approximately 40% of patients with *BRAF* V600E mutated melanomas have de-novo resistance toward BRAF V600E-specific inhibitor PLX4032 [19]. It should be tested whether cases with low level expression are less dependent on MAPK pathway activation by BRAF V600E and may thus be resistant to targeted therapy. This may, in fact, be an advantage of immunohistochemistry over genetic testing modalities that test a surrogate only, i.e. the genetic mutation and not the actual target, i.e. the mutated protein. It has recently been demonstrated that melanomas may acquire secondary resistance to PLX4032 treatment by receptor tyrosine kinase-mediated activation of alternative survival pathways and reactivation of the MAPK pathway via N-RAS up-regulation [28].

Mutation specific antibodies have the advantage that the presence of mutated protein can be assessed at the single-cell level. Interestingly, we found hardly any evidence of clonal heterogeneity in our series of melanoma metastases and papillary thyroid carcinomas. Only two melanoma cases had single interspersed viable cells that were VE1-negative. These cells did not form clonal expansions and are most unlikely to be tumor cells. Our observations can thus not confirm the recent results of Lin and colleagues [27] who observed strong heterogeneity of *BRAF* mutation status in primary melanoma and melanoma metastasis at the single-cell level. In cases sequenced as wt we did not observe single clearly VE1-positive cells. This, also, speaks against clonal heterogeneity of these tumors. Our observations are indirectly supported by recent results of Nazarian et al. [28], who did not detect selection of a *BRAF* wt sub-population in cell cultures from *BRAF* V600E mutated melanomas by chronic treatment with the BRAF V600E-specific inhibitor PLX4032.

In conclusion we report the development of a mutation-specific antibody enabling reliable detection of an important diagnostic, prognostic, and predictive biomarker as a further step toward personalized medicine. This antibody may supplement the genetic detection of *BRAF* status, possibly for cases with uncertain results or insufficient material, or may be used as a substitute for genetic testing in selected clinical settings. Future studies should investigate the role of BRAF expression and response to BRAF V600E targeted therapy.

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Conflict of interest Dr Zentgraf, Dr Capper, and Dr von Deimling have applied for a patent on the diagnostic use of BRAF V600E mutant-specific antibody VE1.

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