ORIGINAL ARTICLE

Diagnostic value of immunohistochemistry for the detection of the BRAF V600E mutation in colorectal carcinoma

Maria Ioannou¹, Roidoula Papamichali¹, Maria Samara¹, Efrosini Paraskeva², Constantina Papacharalambous¹, Korina Baxevanidou¹, George Koukoulis¹

¹Department of Pathology and ²Department of Physiology, University of Thessaly, Faculty of Medicine, Biopolis 41110, Larissa, Greece

Summary

Purpose: V600E is the most common activating BRAF mutation in colorectal carcinomas (CRCs). It is a crucial biomarker for patient selection and response to targeted therapy with BRAF V600E inhibitors. Previous studies using immunohistochemistry (IHC) have shown different results. In this study, we evaluated the IHC expression of the mutated BRAF protein in archival material from CRC specimens and correlated it with DNA sequence analysis.

Methods: 51 cases of primary colon adenocarcinoma were stained with BRAF V600E-specific clone VE1 antibody against mutated BRAF protein. DNA sequence analysis was performed and the results were compared.

Results: BRAF V600E protein was detected in the cytoplasm of neoplastic cells in 15 of the 51 examined cases (29.4%). The correlation between IHC staining and DNA sequence analysis showed 93.75% sensitivity and 100% specificity.

Conclusions: Our data show that IHC could be used in routine clinical practice as a screening method for BRAF V600E mutant protein detection in CRC patients.

Key words: BRAF V600E mutation, colorectal carcinoma, DNA sequence analysis, immunohistochemistry

Introduction

The serine threonine kinase v-RAF murine sarcoma viral oncogene homolog B1 (BRAF) proto-oncogene is an activator of the mitogen-activated protein kinase (MAPK) pathway and is commonly mutated in a variety of cancers. More than 95% of mutated cases carry the V600E point mutation, which results in constitutive tyrosine kinase activity [1,2]. The BRAF V600E mutation causes a substitution of valine by glutamic acid in the activating segment of the kinase domain of BRAF, thus leading to constitutive kinase activity [2]. Activation of downstream targets leads to increased tumor growth and metastatic activity, and specific inhibitors of BRAF V600E-mutated protein have been developed.

Activating mutations in BRAF are found in

5-25% of CRCs, with the vast majority carrying the *BRAF V600E* mutation [3-7]. *BRAF V600E* mutation in microsatellite unstable (MSI) CRCs virtually excludes Lynch syndrome (LS). In microsatellite-stable (MSS) CRCs it predicts poor prognosis [5,8,9-15]. *BRAF V600E* is also a marker of resistance to anti-EGFR therapy in metastatic tumors [16].

Determination of the *BRAF V600E* mutational status in CRCs can be analysed by molecular methods, such as PCR, mass spectroscopy, and various sequencing technologies. Recently, a monoclonal mouse antibody (clone VE1), which recognizes the BRAF V600E protein by IHC has been developed [17]. Previous studies have shown that this antibody detects *BRAF V600E* mutations

Correspondence to: Maria Ioannou, MD, PhD. Department of Pathology, University of Thessaly-Faculty of Medicine, Biopolis 41110, Larissa, Greece. Tel: +30 2410 685645¬¬, Fax: +30 2410 685548, E-mail: mioan@med.uth.gr Received: 07/12/2015; Accepted: 24/12/2015 in different types of tumors [18-26].

In the last few years, the detection of the *BRAF V600E* mutation by IHC in CRC has also been studied. Most investigators suggest it as an alternative to a DNA-based assay screening test of CRCs in routine clinical practice [21,27-30]. However, other authors have reported limited sensitivity of this approach, indicating that IHC with VE1 is not a useful surrogate for genotyping in CRCs [31,32].

In this context, the aim of our study was to evaluate the IHC expression of the mutated BRAF protein in CRC and to correlate the results with the mutation status by DNA sequence analysis. Furthermore, we aimed to assess the utility of IHC in the prediction of BRAF mutations in CRC and consequently, in the selection of patients most likely to respond to targeted therapeutic intervention with BRAF V600E inhibitors.

Methods

Patients

In this retrospective study 51 cases of primary colon adenocarcinoma (30 male and 21 female patients, median age 74 years) were evaluated, for whom archival specimens from the resected primary tumor were available. None of the patients had received chemotherapy or radiation before surgery. All surgical specimens were retrieved from the files of the Pathology Department of the University of Thessaly on the basis of *BRAF* and *KRAS* genotype as established through clinical testing in our laboratory.

In total, 16 *BRAF* mutant (*KRAS* wild-type), 20 *KRAS* mutant (*BRAF* wild-type tumors) and 15 *BRAF/ KRAS* wild-type tumors were evaluated. The samples had been routinely fixed in 10% buffered formalin, processed, and embedded in paraffin.

Immunohistochemical analysis

Anti-BRAF V600E immunostaining was performed on the same tissue block that was used for molecular analysis using the monoclonal mouse antibody VE1 as previously described on 4µm-thick tissue sections of formalin-fixed, paraffin-embedded (FFPE) tumor tissue blocks [17]. All histologic slides were freshly cut before IHC analysis. Sections were dried at 60 °C for 15 min and stained with the undiluted hybridoma supernatant of BRAF V600E-specific clone VE1 (Spring Bioscience, Pleasanton, CA) on a Ventana BenchMark XT immunostainer (Ventana Medical Systems, Tucson, AZ). The Ventana staining procedure included pretreatment with cell conditioner 1 (pH 8) for 60 min, followed by incubation with the VE1 antibody at 37 °C for 16 min. After that, incubation with OptiView DAB IHC Detection Kit (Ventana Medical Systems) followed. Slides

were counterstained with hematoxylin and Bluing reagent for 4 min each. The negative controls were created by omitting the primary antibody or using nonspecific immunoglobulins from the same species instead of the first antibody. For positive control, melanoma with a known BRAF V600E mutation was used.

All immunostained slides were independently evaluated by two pathologists (R.P. and M.I.) without previous knowledge of the clinical, histopathologic and genetic data. The VE1 antibody staining was scored as positive when the majority of viable tumor cells showed clear, uniform granular cytoplasmic staining. The VE1 antibody staining was scored as negative when there was no staining or there was weak staining of single interspersed cells or heterogeneous faint staining of tumor cells without granular quality.

Genomic DNA isolation

Hematoxylin and eosin-stained sections of each specimen were reviewed by a pathologist, followed by macrodissection to ensure the percentage of tumor cells was enriched to at least 70%. Five to 10 sections of 10µm thickness were used for DNA extraction. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions. Paraffin was dissolved in xylene and removed. The samples were lysed under denaturing conditions with proteinase K digestion. Incubation at 90 °C for one hr was performed to reverse formalin crosslinking. The DNA was eluted in distilled water and quantified by both agarose gel electrophoresis and absorption spectrophotometry at 260/280 nm.

Polymerase chain reaction

Genomic DNA was amplified for exon 15 (codon 600) of BRAF gene using specific primers and Ampli-Taq Gold DNA polymerase (Applied Biosystems, USA). The amplification mixture consisted of 5 µL of 10x reaction mix, 2.5 mM MgCl₂, 1.6 mM dNTPs, a 0.4 μ M concentration of each oligonucleotide primer, 2.0u AmpliTaq Gold DNA polymerase and 5 µL of template DNA in a final volume of 50 µL. Samples were amplified as follows: an initial denaturation step at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 2 min and a final extension step at 72 °C for 10 min. In all reactions a non-template control was included. PCR amplified products of 224 bp were analysed in 3% agarose gel, using 100bp DNA ladder (Invitrogen, Life Technologies, USA) and ethidium bromide staining. 5' -> 3' sequences of the primers used were the following:

BRAF exon 15: F- TCATAATGCTTGCTCTGATAGGA BRAF exon 15: R- GGCCAAAATTTAATCAGTGGA

Sequencing

Prior to sequence analysis of BRAF gene (exon 15,

codon 600), all PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Subsequently, the purified products were subjected to bidirectional sequence analysis on an ABI 3500 Genetic Analyzer (Applied Biosystems, USA). All positive samples that were analyzed for exon 15 of BRAF gene, had the same nucleotide base substitution for c.1799 T>A (ref Seq GenBank NM_004333.4), resulting in an amino acid change p.Val600Glu (p.V600E).

Western blot

40 μ g of protein were resolved by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blot with an anti-human BRAF V600 mouse monoclonal antibody (Clone VE1, 1:50 and 1:500, Spring Biosciences) or an anti- β -actin mouse monoclonal antibody (1:5000, Sigma). Membranes were then incubated with horseradish peroxidase conjugated anti-mouse IgG followed by enhanced chemiluminescence (ECL) (Thermo Scientific).

Statistics

Calculation of VE1 antibody sensitivity and specificity was performed using the SPSS software (v13.0, SPSS Inc., Chicago, Ill, USA). PHI correlation coefficient was also used in order to test the association between immunostaining and mutational status.

A p value <0.05 was considered as statistically significant.

Results

Expression of BRAF V600E oncoprotein in colorectal carcinomas

The two independent observers were concordant in VE1 antibody staining assessment in all cases (Figures 1,2,3).

BRAF V600E protein was detected in the cytoplasm of neoplastic cells in 15 of the 51 examined specimens (24.4%). Due to the absence of background staining in our samples, any intensity of staining was specific to the tumor cells and assessed as positive when most or all of the tumor cells were stained. In some cases a nuclear positive immunoexpression was also seen together with the specific granular cytoplasmic staining. In the majority of *BRAF V600E* positive cases [10/13] the staining was diffuse and strong throughout the tumor. Two of the remaining cases displayed diffuse moderate granular cytoplasmic staining and one case showed weak diffuse granular cytoplasmic immunostaining (Figure 1a-d).

The BRAF V600E staining with the VE1 antibody was absent in 36 cases (70.5%). Thirty three of these cases showed complete uniform absence (Figure 2). In the remaining 3 cases a nonspecific focal faint staining was observed at low power magnification giving the false impression of heterogeneous positive immunoexpression (Figure 3a). However, the staining was not granular and there was complete independent final agreement by the two pathologists to consider these cases as negative (Figure 3b).

An interesting observation was the cytoplasmic staining detected in smooth muscle cells from the intestinal wall. This staining was diffuse, granular weak to moderate and it was seen in all the examined cases (Figure 4).

Normal colorectal mucosa was present in 40



Figure 1. 1a: BRAF V600E–mutated carcinoma with *strong cytoplasmic* expression of the mutated BRAF protein (original magnification x10). **1b:** BRAF V600E–mutated carcinoma with *moderate* cytoplasmic expression of the mutated BRAF protein (original magnification x10). **1c:** BRAF V600E–mutated carcinoma with *weak* cytoplasmic expression of the mutated BRAF protein (original magnification x10). **1d:** High power magnification of a positive case. Note the granular quality of the staining (original magnification x40).



Figure 2. Negative BRAF V600E immunostaining in a case of BRAF wild-type CRC (original magnification x10).



Figure3. 3a: A discordant case showing focal faint BRAF immunostaining in low magnification. This tumor did not reveal a T1799A point mutation of the BRAF gene (original magnification x4). **3b:** High power magnification of the discordant case. Note the lack of granular quality of staining in the high power magnification (original magnification x40).



Figure 4. Unspecific staining of smooth muscle by VE1 immunohistochemistry (original magnification x10).

of the 51 (78.4%) examined slides and displayed non specific BRAF V600E immunoreactivity in the nuclei of normal colonic cells. In addition, cytoplasmic staining was observed in tumor-associated macrophages (data not shown).

Associations between BRAF V600E oncoprotein expression levels with BRAF and/or KRAS mutational status

Of the 51 samples, 16 (38.4%) had a mutation in BRAF exon 15, whereas 20 (39.2%) had a KRAS mutation (BRAF wild-type) and 15 were BRAF/ KRAS wild-type (Figures 5a-d, Table 1).

Fifteen of 16 tumors with previously identified BRAF V600E mutation, showed positive IHC results (93.75% of *BRAF* mutant tumors overall).

Negative BRAF V600E immunostaining was detected in 36 (70.6%) cases. One of the immunohistochemically considered as negative cases was BRAF mutant (false negative). This case showed complete absence of immunostaining. In addition, 35 of 35 cases which carried wild type copies of BRAF V600E (15 BRAF/KRAS wild-type and 20 BRAF wild-type/ KRAS mutant) showed negative results (100% of BRAF wild-type tumors overall). Thirty two of the 35 BRAF wild-type cases showed complete absence of immunostaining and 3 cases showed equivocal faint heterogeneous staining which did not show a granular quality and they finally were evaluated as negative (Figures 2,3).

The results of the correlation between the IHC and molecular data of our study are present-



Figure 5. 5a: BRAF codon 600 wild type sequence; **5b:** BRAF codon 600 mutant (c.1799 T>A, p.V600E) sequence; **5c:** KRAS codon 12, 13 wild type sequence; **5d:** KRAS codon 12 mutant (c.35 G>T, p.G12V) sequence.

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	KRAS mutation	BRAF V600E mutation	KRAS/ BRAF wild type
BRAF V600E cytoplasmic expression	0	15	0
Absence of BRAF V600E cytoplasmic expression	20	1	15
Total	20	16	15

Table 1. Correlation of the presence of KRAS and BRAFgene mutations with immunohistochemical results

ed in Table 1. The sensitivity was 93.75% (15/16) and the specificity 100% (35/35). The results were statistically significant (p<0.001), with positive correlation (PHI value 0.955).

Western blot

In a band corresponding to 95kDa, the predicted molecular weight of the BRAF protein was not detected in extracts derived from intestine smooth muscle tissue. Instead, immunostaining with the BRAF V600 antibody led to the detection of multiple bands of apparent lower molecular weight indicating the presence of other proteins cross-reacting with the BRAF V600 antibody in smooth muscle (data not shown).

Discussion

The V600E mutation of BRAF is a biomarker and a therapeutic target for selected CRC. It is typically determined by DNA-based techniques including allele-specific PCR and direct DNA sequencing. However, recent studies have proposed the use of newly developed antibodies against the V600E protein for identification of this mutation [21,27-32]. In our study we demonstrated high agreement between BRAF VE1 IHC and a sequencing PCR-based assay in the determination of BRAF status on formalin fixed and paraffin embedded tissue. Fifty one genotyped CRC cases were examined and cytoplasmic staining with VE1 was detected in 93.75% of tumors with BRAF V600E mutation, whereas absence of immunostaining was seen in 100% of tumors without BRAF V600E mutation. There was 100% independent agreement among the two pathologists for the IHC interpretations. Our results indicate that the IHC detection of the mutant BRAF V600E protein is a reliable, highly specific method for the detection of the BRAF V600 mutation in CRC.

Previous investigations regarding the VE1 IHC on CRC specimens have shown similar re-

sults [21,27-30]. In addition, there are some reports of limited diagnostic value [31,32]. In the study by Affolter et al., all 14 tumors with BRAF V600E mutation were positive by IHC whereas 17 tumors without the mutation were negative by IHC. Sinicrope et al. [29] reported all 49 tumors with BRAF V600E mutation being positive by IHC, whereas staining was absent in 25 tumors without the mutation. Rössle et al. [28] reported a very high sensitivity of 100% and a specificity of 95.24% of IHC detecting BRAF V600E mutations in CRC. Toon et al, compared BRAF V600E IHC with multiplex polymerase chain reaction (PCR) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry and they concluded that IHC for BRAF V600E mutation is highly concordant with PCR-based methodology [33]. IHC was also highly reliable in the diagnosis of Lynch syndrome in the same study. IHC for V600E-mutant BRAF protein in metastatic tumors has been proposed as highly reliable for patient stratification [21]. In a recent study, Kuan et al. demonstrated 100% sensitivity and 94% specificity of VE1 in a large series of CRCs, indicating that VE1 IHC might be used as a useful tool alternative to PCR [30]. In contrast, Adackapara et al. demonstrated sensitivity and specificity of 71% and 74%, respectively. The authors reported that BRAF IHC is insufficiently sensitive to serve as a screening tool for BRAF mutation in CRC [31].

These conflicting data could be, at least in part, explained by the use of different primary antibodies and fixatives. Differences in the interpretation of staining could also be responsible for different results. Most of the previous studies as well as our study have used automated systems for IHC. A manual technique with overnight incubation for VE1 has been performed in the study by Adeckapara et al. [31]. In addition, preanalytic variables and differences in the antigen expression levels between tumor specimens could also result to false-negative results.

When an IHC assay is critical for the clinical management, it is important to be validated in order to be optimized. We found that the VE1 antibody performed very well in Ventana automated system. We used the criterion of diffuse and granular cytoplasmic staining for positivity [28]. The vast majority of BRAF mutant cases showed diffuse homogeneous granular staining and the majority of the BRAF wild-type cases showed complete absence of immunoreaction. Thus, semiquantitive analysis was not required in our study to diagnose true positive and true negative cases. It is of note that one mutant case did not show immunoreaction. Although preanalytical parameters regarding the fixation and tissue preparation could explain this result, we suggest that any negative result should be confirmed by molecular testing before treatment. In addition, few BRAF wild-type cases in our study showed a heterogeneous faint, weak and patchy immunostaining. These cases were considered as negative based on the absence of granular quality of the cytoplasmic staining which was constantly observed in the BRAF mutant cases.

Other studies have also demonstrated weak immunoreaction observed in BRAF mutant cases and in BRAF wild-type cases, suggesting that weak staining with VE1 is not diagnostic of BRAF V600E protein expression and requires additional testing by PCR [30]. It is obvious that the necessity of both methods - IHC and molecular is indicated for the objective evaluation of BRAF gene and protein. We suggest that any laboratory that plans to use BRAF mutation specific antibody clinically should validate its use against a valid molecular assay. Antibody optimization as well as strict criteria for positivity should be provided in order to establish a reliable and reproducible inhouse methodology of detecting and evaluating the BRAF mutant status in tumor specimens of CRC. After BRAF V600 antibody establishment, the lab should communicate its clinical performance characteristics (eg. sensitivity, specificity) to the clinicians, and make available the referral to molecular laboratory for molecular testing to exclude mutations in IHC-negative tumors.

Using an automated system for immunostaining in our study, we provided standardization of staining conditions and we achieved reproducible granular staining in the genotyped positive cases, without nonspecific background staining. We also detected nonspecific staining of mucus, non-neoplastic epithelium and macrophages. An interesting finding of our study was the BRAF immunoexpression in smooth muscle cells of the intestinal wall.

In order to investigate the meaning of this immunoreaction we performed Western blot analysis in normal smooth muscle tissue, isolated from the intestinal wall of a surgical specimen received in our laboratory. In Western blot analysis, the antibody did not detect a band corresponding to 95kDa, the predicted molecular weight of the BRAF protein. Our results are in keeping with other investigators [33] and suggest that BRAF staining of muscle is non-specific.

IHC offers the advantage of a fast, easy to perform, and cost-effective assay that can be performed in most hospital pathology laboratories, particularly for cases with tissue heterogeneity and a low percentage of neoplastic tumor content. Using of the VE1 antibody allows the correlation of the tumor histology with subcellular localization of BRAF oncoprotein and might be performed on all CRC patients as diagnostic testing on surgical excision specimens. Further genetic confirmation might be used only as the gold standard in equivocal cases or to confirm the negative results before treatment in selected patients. Moreover, this approach is useful for the identification of the poor prognostic group of BRAF V600E-mutated, microsatellite stable tumors.

In conclusion, we confirmed the high reliability of the VE1 monoclonal antibody for the detection of BRAF V600E mutant protein in CRC tissue samples. We suggest that the use of BRAF V600E protein is an alternative to a DNA-based methodology that can facilitate the screening of CRCs in routine clinical practice. In addition, our data further validate the potential role of IHC as a reliable tool for patient stratification and selection for targeted therapy, particularly in resource-poor settings.

Conflict of interests

The authors declare no confict of interests.

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