

Immunohistochemistry as an accurate tool for the assessment of *BRAF V600E* and *TP53* mutations in primary and metastatic melanoma

STEFAN RUSU¹, CAMILLE VEROCQ¹, ANNE LAURE TREPANT^{1,2}, CALLIOPE MARIS^{1,2}, NANCY DE NÈVE¹, ORIANE BLANCHARD¹, CLAUDE VAN CAMPENHOUT¹, SARAH DE CLERCQ¹, SANDRINE RORIVE^{1,2}, OVIDIU SIMION COTOI³, CHRISTINE DECAESTECKER^{4,5}, ISABELLE SALMON^{1,2,4} and NICKY D'HAENE^{1,2}

¹Department of Pathology, Erasme University Hospital, Université Libre de Bruxelles, B-1070 Brussels;

²Centre Universitaire Inter Regional d'Expertise en Anatomie Pathologique Hospitalière (CurePath), B-6040 Charleroi (Jumet), Belgium; ³Department of Pathology, Clinical County Hospital of Targu Mures, University of Medicine, Pharmacy, Science and Technology 'George Emil Palade' of Targu Mures, Targu Mures, RO-540139 Mures County, Romania; ⁴DIAPath-Center for Microscopy and Molecular Imaging, Université Libre de Bruxelles, B-6041 Gosselies; ⁵Laboratory of Image Synthesis and Analysis, Ecole Polytechnique de Bruxelles, Université Libre de Bruxelles, B-1050 Brussels, Belgium

Received September 25, 2020; Accepted July 15, 2021

DOI: 10.3892/mco.2021.2432

Abstract. Metastatic melanoma is a fatal disease with poor prognosis. Ever since targeted therapy against oncogenic *BRAF* was approved, molecular profiling has become an integral part of the management of such patients. While molecular testing is not available in all pathology laboratories, immunohistochemistry (IHC) is a reliable screening option. The major objective of the present study was to evaluate whether IHC detection of *BRAF* and the tumor (suppressor) protein 53 gene (*TP53*) are reliable surrogates for mutation detection. Formalin-fixed paraffin-embedded samples of melanomas for which molecular data were previously obtained by targeted next-generation sequencing (NGS) between January 2014 and February 2019 were immunostained with *BRAF* V600E and p53 antibodies. A blinded evaluation of the IHC slides was performed by two pathologists in order to evaluate inter-observer concordance (discordant cases were reviewed by a third observer). The associations between the results of IHC and molecular profiling were evaluated. The study included a series of 37 cases of which 15 harbored a *BRAF* mutation and five a *TP53* mutation. IHC had an overall diagnostic accuracy of 93.9% for *BRAF* V600E and 68.8% for *TP53* compared to NGS. A statistically significant association between the two diagnostic methods

was obtained for *BRAF* V600E ($P=0.0004$) but not for p53 ($P=0.3098$) IHC. The κ coefficient for IHC assessment of p53 was 0.55 and that for *BRAF* V600E was 0.72. In conclusion, the present results evidenced that IHC staining is a reliable surrogate for NGS in identifying the *BRAF* V600E mutation, which may become an efficient screening tool. Aberrant expression of p53 on IHC is at times associated with *TP53* mutations but it was not possible to establish a direct link.

Introduction

Melanoma represents a minority of skin cancers but it is associated with an increased risk of death compared with other skin malignancies (1-4), being responsible for ~90% of mortalities reported in cutaneous tumors (5). The worldwide incidence has risen rapidly over the last five decades (1-4). Among all newly diagnosed cancers, melanoma was the 17th most common type according to World Cancer Research Fund International in 2019 (6). Melanoma is a heterogeneous tumor with alterations in specific genes involved in pathways controlling cell proliferation, differentiation and survival (7). The major pathways involved are the cyclin-dependent kinase inhibitor 2A (CDKN2A)-dependent pathway and MAPK- and PI3K-dependent pathways (8,9).

As ~50% of advanced melanomas [unresectable Stage III or metastatic Stage IV, according to the American Joint Committee on Cancer Staging Manual, 8th edition (3)] harbor a mutation in the *BRAF* gene and since the Federal Drug Administration and the European Institutions approved targeted therapy with *BRAF* and MAPK kinase inhibitors, molecular testing for codon V600 of the *BRAF* oncogene became an integral part of the management of such cases. Several approaches are available, from single gene testing for the *BRAF* V600 mutation, such as PCR or immunohistochemistry (IHC)

Correspondence to: Dr Nicky D'Haene, Department of Pathology, Erasme University Hospital, Université Libre de Bruxelles, 808 Route de Lennik, B-1070 Brussels, Belgium
E-mail: nicky.d.haene@erasme.ulb.ac.be

Key words: melanoma, *BRAF*, *TP53*, immunohistochemistry, next-generation sequencing

using anti-BRAF (mutated V600E) antibody (AB) [VE1 clone (BRAF VE1)], to whole-genome sequencing. The targeted next-generation sequencing (NGS) technique allows the detection of abnormalities across multiples genes. The identification of multiple molecular aberrations provides several treatment targets. Despite the valuable provided data, NGS remains expensive and not widely distributed and requires sophisticated bioinformatics systems, fast data processing and large data storage capabilities. On the other hand, IHC has a shorter turnaround time (TAT), is cheaper and widely available, but it detects only specific antigens and false-positive and -negative results may be obtained. IHC using BRAF VE1 has emerged as a powerful tool in assessing the *BRAF V600E* mutation status; validation studies performed to date reported high specificity (95.4-100%) and sensitivity (94.4-100%) (10-14).

The tumor (suppressor) protein 53 gene (*TP53*), 'the guardian of the genome', is inactivated in ~90% of melanomas and only 10-20% of cases are carrying disabling point mutations (15), but its role remains controversial in melanoma and melanoma progression. IHC is used routinely as a surrogate for *TP53* mutation analysis, particularly in ovarian cancer (16), Barrett's esophagus (17) and other cancer types (18-22). Its application in melanoma requires further study (23-29).

In light of the importance of identifying the molecular profile for therapeutic purposes, the main objective of the present study was to evaluate whether BRAF VE1 and p53 immunoexpression are reliable surrogates for mutation detection. For this purpose, *BRAF* and *TP53* status were analyzed by IHC in a series of melanoma cases for which molecular data were previously obtained by NGS. Furthermore, the inter-observer concordance for the IHC assessment was assessed and a cost-effectiveness analysis for the BRAF VE1 AB was performed.

Materials and methods

Samples. Formalin-fixed paraffin-embedded (FFPE) samples of 37 melanomas from 37 patients were retrospectively analyzed, including 8 (21.6%) primary tumors and 29 (78.4%) metastatic tumors. The samples were provided by the Pathology Department of Erasme University Hospital (Brussels, Belgium). All tumor sample sites are summarized in Table SI.

The selection criteria were as follows: Patients with melanoma for whom molecular testing was required and performed between January 2014 and February 2019. The sample types were either biopsies (n=6), cell blocks (n=3) or surgical resections (n=28). The samples were retrieved for the NGS results and subsequently, the FFPE blocks were checked to assess if there is sufficient residual tissue to perform IHC. Thus, the exclusion criterion was an insufficient amount of residual tumor tissue as determined by the pathologist and the cases were further excluded from the statistical analyses.

NGS

DNA extraction. DNA extraction from FFPE samples was performed as previously described (30,31), using the QIAamp FFPE tissue kit (Qiagen GmbH), according to the manufacturer's protocol. The obtained DNA was quantified using the

Qubit® fluorometer in combination with the Qubit® dsDNA HS assay kit (Thermo Fisher Scientific, Inc.).

Library preparation, cluster amplification and sequencing. NGS was performed as previously described (30,31). DNA (10 ng) was amplified using the Cancer Panel (Ampliseq™; Thermo Fisher Scientific, Inc.). An amplicon library was generated for sequencing 2,850 mutations in 50 genes, including the following: *ABL1*, *AKT1*, *ALK*, *APC*, *ATM*, *BRAF*, *CDH1*, *CDKN2A*, *CSF1R*, *CTNNB1*, *EGFR*, *ERBB2*, *ERBB4*, *EZH2*, *FBXW7*, *FGFR1*, *FGFR2*, *FGFR3*, *FLT3*, *GNAI1*, *GNAQ*, *GNAS*, *HNFA1A*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KDR*, *KIT*, *KRAS*, *MET*, *MLH1*, *MPL*, *NOTCH1*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PTEN*, *PTPN11*, *RBI*, *RET*, *SMAD4*, *SMARCB1*, *SMO*, *SRC*, *STK11*, *TP53* and *VHL*. Library construction was performed using the Ion AmpliSeq™ Library kit 2.0 and Ion Xpress™ barcode adapters kit (Thermo Fisher Scientific, Inc.), while the quantification was performed with the Qubit® fluorometer and the Qubit® dsDNA HS assay kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The library was then quantified using the Qubit® fluorometer and the Qubit® dsDNA HS assay kit (Thermo Fisher Scientific, Inc.). Libraries were multiplexed and clonally amplified by emulsion PCR using the Ion One Touch 2 instrument with the Ion PGM™ template OT2 200 kit (Thermo Fisher Scientific, Inc.) and sequenced using a PGM™ sequencer with the Ion PGM™ sequencing 200 kit v2. Quality control was performed using the Ionsphere™ quality control kit (Thermo Fisher Scientific, Inc.). All steps were performed according to the manufacturer's protocols.

Data analysis. The raw data analysis was performed as previously described (30,31), using Torrent Suite software v4.0.2-v5.10.0 (Thermo Fisher Scientific, Inc.). The coverage analysis was performed using the Coverage Analysis plugin v4.0- v5.10. Cases for which the average base coverage was <500x were considered non-informative. Detection of mutations was performed using the Variant Caller plugins v4.0-v5.10 (Thermo Fisher Scientific, Inc.). Each mutation was verified in the Integrative Genome Viewer from the Broad Institute (<http://www.broadinstitute.org/>). Only mutations reported in the COSMIC database (<http://cancer.sanger.ac.uk/>) were taken into account, while silent or intronic mutations were not reported. Mutations were then classified into three categories based on data obtained from the literature and from the COSMIC database: Mutations with known clinical impact (with a targeted therapy on the market, e.g., *BRAF V600* mutation); mutations with potential clinical impact (clinical trials are ongoing, e.g., *NRAS*, *EZH2*, *BRAF nonV600E*); and mutations with unknown clinical impact (e.g., *TP53*).

The cases with poor-quality sequencing and/or insufficient material for IHC staining were considered non-contributory.

IHC

IHC procedure. Sections (4 µm thick) of the original FFPE block used for molecular analysis were subjected to anti-BRAF (mutated V600E) IHC staining (cat. no. ab228461; clone VE1; dilution, 1/100; Abcam) and anti-p53 IHC staining (cat. no. M7001; clone DO-7; dilution, 1/200; Agilent Technologies, Inc.) on a Dako Omnis (Agilent Technologies,

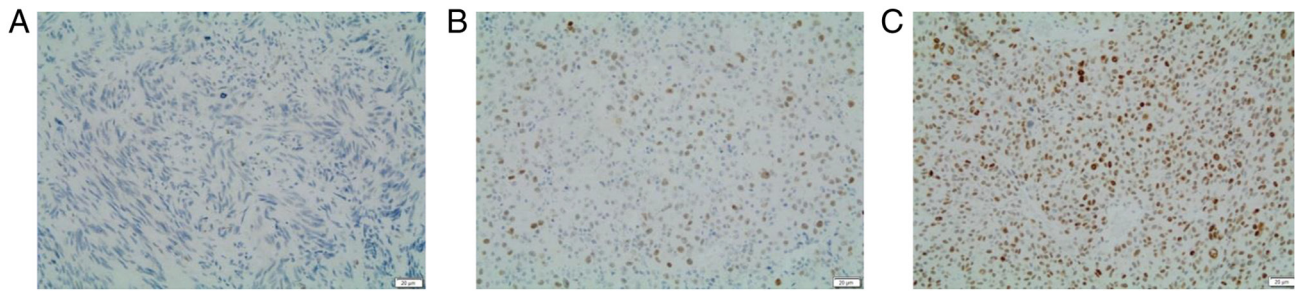


Figure 1. Representative images for three-tier score for immunohistochemical assessment of p53. (A) Score 0-no staining (loss of expression); (B) score 1-≤25% of cells with variable, heterogeneous cytoplasmic and nuclear staining (wild-type); (C) score 2->25% of cells with high homogeneous nuclear staining (overexpression). Hematoxylin counterstaining was applied and positive staining is brown (original magnification, x200; scale bar, 20 μ m).

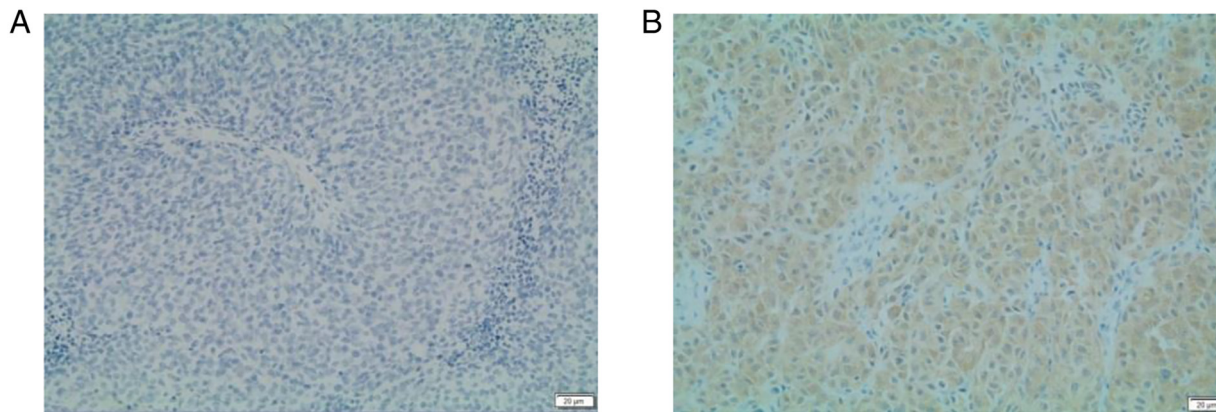


Figure 2. Qualitative assessment score for BRAF V600E immunohistochemistry. (A) Negative-no cytoplasmic staining; (B) positive-presence of cytoplasmic staining. Hematoxylin counterstaining was applied and positive staining is brown (original magnification, x200; scale bar, 20 μ m).

Inc.). Heat-induced epitope retrieval was performed using Dako Target Retrieval Solution pH 9 (cat. no. GV804; Agilent Technologies, Inc.) 30 min at 97°C, followed by primary antibody incubation 20 min at 32°C and detection with Dako Envision Flex detection system (cat. no. GV800; Agilent Technologies, Inc.) according to the manufacturer's protocol. The sections were counterstained with hematoxylin (cat. no. GC808; Agilent Technologies, Inc.). Control tissues (tonsil for p53 IHC and BRAF V600E mutated tumor for BRAF IHC) and universal negative control antibody (negative control Mouse IgG1; cat. no. X0931; dilution, 1/200; Agilent Technologies, Inc.) were processed in parallel with tissues exposed to the primary as described above.

Pathology scoring. Immunostained slides were evaluated by two trainee pathologists (one and two years of experience) blinded to the molecular data. For discordant cases, a third pathologist (>10 years of experience) blinded to previous results re-evaluated the p53 and/or BRAF V600E IHC staining.

A semi-quantitative assessment of p53 IHC (Fig. 1) expression was performed by using a three-tier score as previously described (32): 0-no staining (loss of expression); 1-≤25% of cells with variable, heterogeneous cytoplasmic and nuclear staining (wild-type); 2->25% of cells with high homogeneous nuclear staining (overexpression). This semi-quantitative evaluation is similar to the ones used by Kastelein *et al* (17) in Barrett's esophagus or Guedes *et al* (20) in prostate cancer.

A qualitative assessment of BRAF VE1 (Fig. 2) was used as previously described (10-13): Negative-no cytoplasmic staining; positive-presence of cytoplasmic staining.

Pathological data. The pathological data collected were sample type (biopsy/cell block/surgical resection), tumor sample site (skin/conjunctiva/brain/subcutaneous tissue/liver/lymph node/adrenal glands/digestive tract/lung/undetermined), IHC analysis (sufficient/insufficient material), IHC BRAF V600E (positive/negative), IHC p53 (no staining/wild-type/overexpression/non-contributory), NGS analysis (contributory/non-contributory), results of NGS (gene mutation, exon, coverage, % of mutated DNA, clinical impact). Non-contributory IHC means lack of positive internal control, while non-contributory NGS means that the sequencing quality was too poor to allow for analysis. Clinicopathological data, age and sex distributions were not available for the present study in line with the Ethics Committee consent.

Cost analysis. Information regarding the costs associated with IHC using BRAF VE1 AB Idylla™ (PCR-based method) and NGS were retrieved. A comparison of the costs was performed between these three methods.

Statistical analysis. The results from IHC and NGS testing were cross-tabulated and, using the online calculator provided by Medcalc (https://www.medcalc.org/calc/diagnostic_test.php), the sensitivity, specificity, accuracy, positive predictive

Table I. Results of immunohistochemical assessment of p53 and BRAF V600E by pathologists.

| Protein/result | 1st Pathologist | 2nd Pathologist | Final result |
|-------------------------------|-----------------------|-----------------------|--------------|
| p53 | | | |
| 0 (loss of expression) | 0 (0) | 1 (3) | 1 (3) |
| 1 (wild-type) | 23 (67.6) | 25 (73.5) | 22 (64.6) |
| 2 (overexpression) | 10 (29.4) | 7 (20.5) | 10 (29.4) |
| Non-contributory ^a | 1 (3) | 1 (3) | 1 (3) |
| BRAF V600E | | | |
| Absence of staining | 30 (88.2) | 30 (88.2) | 30 (88.2) |
| Presence of staining | 4 ^b (11.8) | 4 ^b (11.8) | 4 (11.8) |

^aNon-contributory p53 IHC means absence of internal positive cells; ^bTwo of the cases were discordant and reviewed by the third pathologist. Values are expressed as n (%).

value and negative predictive value were calculated. The association between NGS and IHC was performed using Fisher's exact test with Statistica® (StatSoft). $P < 0.05$ was considered to indicate statistical significance.

To assess the inter-observer coefficient, Cohen's unweighted κ coefficient was calculated by running an online test, provided by Vassarstats (<http://vassarstats.net/kappa.html>). The scale used for the interpretation of the κ coefficient was the following: 0-0.2, slight agreement; 0.21-0.40, fair agreement; 0.41-0.60, moderate agreement; 0.61-0.80, substantial agreement; and 0.81-1, almost perfect agreement.

Results

IHC results and inter-observer concordance. The IHC staining was performed on 34 of 37 cases (three cases did not have sufficient material for immunostaining). A total of 34 cases were further considered for statistical analyses. The IHC profile assessment for p53 and BRAF V600E is summarized in Table I.

Regarding p53, six of the 34 cases were discordant (17.6%) and were reviewed by a third pathologist, who agreed three times with the first pathologist and three times with the second. One case was not assessable due to the lack of internal positive control. The κ coefficient for evaluating the inter-observer concordance was 0.554, suggesting a moderate agreement.

Concerning BRAF V600E, two of 34 analyzed cases were discordant (5.90%) and were reviewed by a third pathologist, who agreed with the first pathologist in both cases. The Cohen unweighted κ coefficient was 0.717, which represents a substantial agreement.

NGS results and NGS-IHC correlation. Sequencing was optimal in 36 out of 37 cases (97.3%). It failed in one sample (2.7%) due to poor quality of sequencing (non-contributory). No mutation in the gene panel was detected for 5 of the 36 cases (13.9%), 15 (41.7%) harbored a BRAF mutation and 16 (44.4%) had other mutations than BRAF (potentially clinically actionable mutations or mutations with unknown impact). Of the 15 cases harboring a BRAF mutation, eight were V600E (two cases had insufficient residual tissue for IHC staining, leading to six cases included for NGS-IHC correlation analysis),

Table II. Cross-tabulated results for tumor suppressor protein 53 gene and BRAF V600E mutation status determined by IHC and NGS.

| IHC result | NGS result | |
|----------------------|-------------|---------|
| | Non-mutated | Mutated |
| p53 | | |
| Wild-type | 19 | 2 |
| Aberrant expression | 8 | 3 |
| BRAF V600E | | |
| No staining | 27 | 2 |
| Presence of staining | 0 | 4 |

Loss of expression and overexpression of the p53 protein were considered as positive results (aberrant IHC result); physiological protein expression according to IHC indicated wild-type p53 expression. IHC, immunohistochemistry; NGS, next-generation sequencing.

followed by three V600 non-E (two V600K and one V600R), two D594N, one K601E and one K601N. The most frequent mutations among the non-BRAF mutations were NRAS (19.4%, 7/36) and TP53 (13.9%, 5/36). BRAF V600E and TP53 mutations were not mutually exclusive and one case harbored BRAF V600E and TP53 mutations.

When performing the statistical analysis, loss of expression and overexpression of the p53 protein on IHC analysis was considered as a positive result (aberrant IHC result-expression of mutation in the tumor suppressor gene TP53) and the non-contributory cases were eliminated from the analysis. NGS was the reference technique for the analysis of both ABs. NGS and IHC results were available in 32 cases for the evaluation of TP53 protein expression and in 33 cases regarding BRAF V600E protein expression. The cross-tabulated results of the NGS and IHC assessment are summarized in Table II.

Considering the NGS technique to be the reference for the TP53 mutation status, IHC had an overall diagnostic accuracy of 68.8% for the TP53 mutation with 31.2% of cases being misclassified (10/32). The overall sensitivity of IHC was 60% and two cases were false-negative. The overall specificity of

Table III. NGS-IHC correlation results for both genes tested.

| NGS-IHC correlation | <i>TP53</i> | | <i>BRAF V600E</i> | |
|---------------------------|-------------|-----------|-------------------|------------|
| | Value (%) | 95% CI | Value (%) | 95% CI |
| Diagnostic accuracy | 68.8 | 50.0-83.9 | 93.9 | 79.8-99.3 |
| Overall sensitivity | 60.0 | 14.7-94.7 | 66.7 | 22.3-95.7 |
| Overall specificity | 70.4 | 49.8-86.3 | 100.0 | 87.2-100.0 |
| Positive predictive value | 27.3 | 13.0-48.5 | 100.0 | 51.0-100.0 |
| Negative predictive value | 90.5 | 76.0-96.6 | 93.1 | 81.3-97.7 |
| P-value | 0.3098 | | 0.0004 | |

The NGS technique served as the reference technique (gold standard). The table summarizes all of the values obtained for diagnostic accuracy, overall sensitivity, overall specificity and positive and negative predictive value for each antibody. *TP53*, tumor (suppressor) protein 53 gene; IHC, immunohistochemistry; NGS, next-generation sequencing.

IHC was 70.4% and eight cases were false-positive. The positive predictive value of IHC for p53 was 27.3% (3/11), while the negative predictive value was 90.5% (19/21). No statistically significant association between the two diagnostic methods was obtained ($P=0.3098$).

Considering the NGS technique to be the reference for the *BRAF V600E* mutation status, IHC had an overall diagnostic accuracy of 93.9% for the *BRAF V600E* mutation with 6.1% of cases being misclassified (2/33). These two misclassified cases were cases harboring a *BRAF V600E* mutation and negative BRAF VE1 IHC staining. The overall sensitivity of IHC was 66.7% and two cases were false-negative. The overall specificity for IHC was 100% with no false-positive results obtained. The positive predictive value of BRAF VE1 was 100%, while the negative predictive value was 93.1% (27/29). A statistically significant association between the two diagnostic methods was determined ($P=0.0004$). All related results are summarized in Table III.

Cost-effectiveness analysis for *BRAF* status testing. In our laboratory, the *BRAF V600E* status may be tested by IHC, Idylla™ (PCR-based method) or NGS. It was decided to perform the cost analysis only based on the reagents' cost to ensure harmonized results. Amortization costs, maintenance or human resources were not considered, as these elements vary among laboratories. It should be noted that the fact that reagents' costs may also vary depending on the test volume, supplier or country, so that the present cost-effectiveness analysis is only indicative. All of the costs per test for each technique are summarized in Tables SII and SIII. The cost per patient of an NGS analysis considering only the reagents is ~280 €, that of Idylla™ 120 € and BRAF VE1 IHC costs ~22 €. For the present case series, several simulations were performed and it was concluded that performing BRAF V600E IHC staining for all cases first followed by NGS for negative BRAF V600E IHC cases saves ~5% of costs compared to performing only NGS. Performing Idylla™ DNA-testing only was revealed to cost less than performing BRAF V600E IHC staining for all cases first followed by Idylla™. However, in larger case series, IHC staining followed by a DNA-based test for negative cases would cost less than only DNA-based testing.

Discussion

Melanoma is the most aggressive form of skin cancer with ~200,000 new cases diagnosed each year worldwide (33). The identification of *BRAF* mutations through DNA sequencing in the early 2000s led to a revolution in the treatment of melanoma, allowing the development of therapies targeting the BRAF oncogene and applications of other kinase inhibitors (34). The *BRAF* mutation status is critical for advanced-stage melanoma and molecular testing should be performed routinely for stage III and IV (unresectable and metastatic) (35) to promptly start the therapy, particularly in melanomas with aggressive behavior. Several *BRAF* mutation tests are available: DNA-based tests (including PCR and NGS) and one AB-based test for mutant BRAF V600E protein using the mouse monoclonal VE1 clone to detect the protein expression by IHC.

BRAF mutations are observed to occur in numerous different types of cancer, including thyroid, lung, colon cancers, glioblastomas and certain hematological malignancies (16-22,34). *BRAF* mutations are identified in 40-60% of melanomas (14,34,36). *BRAF V600E* is the most common mutation and it accounts for 60-90% of all *BRAF* mutations, followed by *V600K* mutation and *V600D/R* accounting for 10-30 and 3%, respectively (37,38). The present results overlap with those described in the literature, as *BRAF* mutations were identified in ~40% of the present cases and among the mutated cases, *V600E* mutation accounts for 53.3% of cases, followed by *V600K* mutation with 13.3%. The present study demonstrated a sensitivity of 66.7% and a specificity of 100% for the BRAF VE1 IHC as the method of detection for *BRAF V600E* compared to NGS analysis (considered the gold standard) in FFPE tumor tissue samples. Specimens with the *V600 non-E* mutation were not immunoreactive with the VE1 clone, as already described (10,11). The present results are comparable to previous validation studies in which a qualitative assessment scale for VE1 staining was employed regarding the specificity (95.4-100%), but the sensitivity was inferior to previously reported results (94.4-100%) (10-14).

Different scoring systems have been used for the evaluation of *BRAF* by IHC (two or more than two categories, based on the intensity of the IHC staining or based on the pattern of staining) in previous studies, leading to major

difficulties in comparing the results. For instance, Lo *et al* (10), Long *et al* (11), Colomba *et al* (12) and Boursault *et al* (13) used the same evaluation method as that employed in the present study, while Marin *et al* (39) and Pearlstein *et al* (40) scored BRAF IHC expression in more than two categories. The establishment of an objective and widely accepted consensus scoring system will lead to the uniformization of results and a decrease in interpretation discrepancies and bias.

The inferior sensitivity may be explained by the small number of cases harboring *BRAF V600E* included in the present study. Immunostaining with BRAF VE1 AB did not produce any false-positive final result. At the first assessment, one pathologist misclassified a negative case as positive on IHC. This false-positive result may be explained by the lack of experience of the trainee pathologist and may be remedied by providing training programs to correctly evaluate IHC profiles. In the present study, two false-negative results occurred, characterized by the absence of IHC staining but *BRAF V600E* mutation on NGS. In one of the cases, the interpretation of immunostaining was difficult due to the presence of a high quantity of melanin. In order to increase the specificity of the test, the brown chromogen may be replaced with red chromogen (40). Discordance between pathologists in assessing the BRAF VE1 IHC was determined in two of the 34 cases where a third pathologist's opinion was required. The Cohen's κ coefficient obtained (0.717) indicates a substantial agreement between the two pathologists. Although molecular testing is considered the gold standard for the detection of *BRAF* mutations, the monoclonal VE1 AB is emerging as a reliable option (10-14,40-42). In the present study, a statistically significant association was demonstrated between the IHC and NGS analysis ($P=0.0004$), which confirmed the hypothesis that IHC may be used as a surrogate in the evaluation of the *BRAF V600E* mutation status. The advantages of IHC are as follows: Shorter TAT, lower cost, small quantity of tumor tissue required for detection and the service may be offered by more histopathology departments than the genomics analysis. Relying on the results obtained, the algorithm incorporating both IHC and DNA-based analysis, as previously proposed by Pearlstein *et al* (40) or the Anglian Cancer Network Protocol (10), may be considered to be used in current clinical practice (Fig. 3). In this algorithm, BRAF VE1 IHC analysis is performed in all cases of unresectable or metastatic melanoma, providing a quick and inexpensive way to detect *BRAF V600E* mutations. Furthermore, it is the only modality of *BRAF* mutation detection in cases with limited tumor tissue that may otherwise not be tested by DNA-based analysis due to insufficient material. Afterwards, cases with negative BRAF VE1 staining should be tested by a DNA-based test in order to identify a possible false-negative result or a *BRAF* mutation other than V600E. The use of the proposed algorithm would offer a cost-saving of ~5% compared to the NGS test in all cases. However, testing only by Idylla™ in the present case series compared to prior BRAF VE1 testing followed by Idylla™ was more economical, but this is highly dependent on the number of cases. Concerning DNA-based testing, the NGS technique provides information beyond *BRAF*, revealing more 'actionable' mutations leading to other therapeutic options. However, NGS is not widely available, its interpretation requires highly trained staff and it has a longer

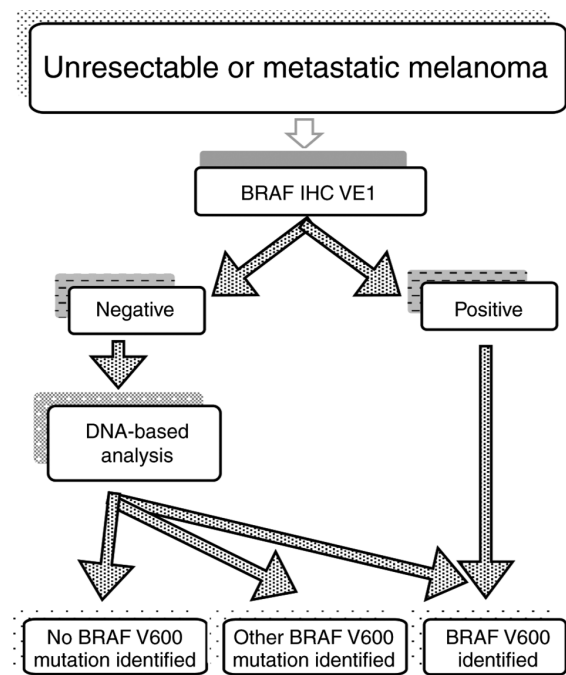


Figure 3. Algorithm proposed for the diagnosis of advanced-stage melanoma. BRAF V600E IHC is routinely applied for all unresectable and metastatic melanomas. In the case of a positive result, targeted therapy may be initiated. DNA-based analysis should be employed when a negative result is obtained in order to further guide the treatment. IHC, immunohistochemistry.

TAT. The Idylla™ test is a PCR-based test and an alternative to IHC or NGS for *BRAF V600* mutation, with the following advantages: It is less laborious, faster and allows the detection of *BRAF V600 non-E* mutations; however, it has a higher cost and it is not as widely available as IHC (43).

p53 is a transcription factor with a powerful tumor suppressor function. Wild-type p53 is a potent inducer of apoptosis, of cell cycle arrest and of cellular senescence (14). A review published by Lu *et al* (44) in 2013 highlights the potential role of targeting p53 in melanoma treatment, stating that reactivation of p53 by simultaneously blocking the p53 E3 ubiquitin ligase, MDM2 and inhibitor of apoptosis-stimulating protein of p53, together with BRAF V600E inhibition, induces apoptosis and suppresses melanoma growth in cell lines and animal models. Furthermore, a novel hypothesis regarding the importance of the p53 status emerges, stating that *TP53* mutation is a potential negative predictor of metastatic melanoma treated by CTLA-4 blockade (45,46). Thus, it may be worthwhile to routinely evaluate the TP53 status. The frequency of *TP53* mutation in melanoma reported in the literature is ~20% (9,47). A slightly inferior rate (14%) was obtained in the present cohort. The association between *TP53* mutations and p53 nuclear accumulation remains to be fully elucidated. IHC is used routinely as a surrogate for *TP53* mutation analysis in several types of cancer (17-22,48), but its interpretation is at times difficult (48). In the present study, the κ coefficient obtained was 0.554, suggesting a moderate inter-observer concordance, underlining the difficulty in interpreting p53 protein expression. The IHC-NGS association was not statistically significant, indicating that the use of p53 IHC to replace a DNA-based test should not be considered for routine use. In the present study, false-negative (two identified in the present

cohort) and false-positive (eight identified) results were obtained. The false-negative results harbored different *TP53* mutations. Furthermore, not all the mutations are leading to corresponding transcriptional or translational changes in p53 protein products (28). The false-positive results may be explained by the fact that p53 IHC expression may result from malfunction of other components of the p53 pathway other than the gene mutation or the fact that targeted NGS focuses on genomic regions of particular interest, with various regions of the *TP53* gene remaining unsequenced. Besides these, the tumors also exhibit genetic heterogeneity (29). Other studies (23-27) also reported no correlation between the protein expression and the *TP53* mutational status, suggesting that a direct link between aberrant protein expression of p53 and the presence of *TP53* mutation was not able to be established.

As at the present time, determination of the *TP53* status is not a standard of care for patients with melanoma in contrast to the *BRAF* status, expanded molecular testing is not required for all patients. Considering that IHC analysis of p53 is not highly sensitive or specific, when determination of the *TP53* mutation will be warranted, it should be assessed by a molecular method.

In conclusion, molecular profiling has become a key piece of information in the management of advanced-stage melanoma with the introduction of 'personalized medicine'. The results obtained demonstrated that IHC staining with BRAF VE1 AB is a reliable surrogate for NGS in identifying the *BRAF V600E* mutation, becoming an efficient screening tool prior to DNA-based analysis. Loss of p53 expression/overexpression determined by IHC is at times associated with *TP53* gene mutations, but it was not possible to establish a root-cause relationship. Aside from that, BRAF VE1 allows the detection of a specific hotspot mutation, while p53 expression on IHC is related to protein expression that may be wild-type but may also mirror a large spectrum of *TP53* mutations.

Acknowledgements

Not applicable.

Funding

This work was supported by the Fonds Yvonne Boël (Brussels, Belgium).

Availability of data and material

The original targeted NGS data were submitted to the European Nucleotide Archive. They are available as 'Rusu *et al* 2021: The use of immunohistochemistry as an accurate tool in the assessment of BRAF V600E and TP53 mutations in primary and metastatic melanoma' with the following study ID: PRJEB42810 (ERP126721) (<https://www.ebi.ac.uk/ena/browser/view/PRJEB42810?show=related-records>).

Authors' contributions

NDH, IS, CD and OSC contributed to the conception and design of the study. Material preparation, data collection and IHC analysis were performed by SRU, CV and NDH. SDC,

CVC, OB and NDN performed IHC and molecular analyses. ALT, CM and SRo performed the pathological diagnoses. SRU, CV, ND and CD analyzed the data. SRU, CV, NDH and CVC approved the authenticity of the raw data. The first draft of the manuscript was written by SRU and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Approval was granted by the Ethics Committee of the Erasme-Université Libre de Bruxelles (Brussels, Belgium) in May 2019 (no. P2019/310). The Ethics Committee waived the requirement of informed consent under the condition that the researchers check for the absence of any objection of the patient to use their material for research.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Glazer AM, Rigel DS, Winkelman RR and Farberg AS: Clinical diagnosis of skin cancer: Enhancing inspection and early recognition. *Dermatol Clin* 35: 409-416, 2017.
2. De Vries E, Elder DE, Bray F, Thompson JF, Coebergh JW, Barnhill RL, Cerroni L, van Muijen GNP, Ruiter DJ, Scolyer RA, *et al*: Chapter 2: Melanocytic tumours. Malignant melanoma: Introduction. In: World Health Organization Classification of Tumours. Pathology and Genetics of Skin Tumours. LeBoit PE, Burg G, Weedon D and Sarasin A (eds). IARC Press, Lyon, pp52-65, 2006.
3. Gershenwald GE, Scolyer RA, Hess KR, Thompson JF, Long GV, Ross MI, Lazar AJ, Atkins MB, Balch CM, Barnhill RL, *et al*: Part X. Skin-47. Melanoma of the Skin. In: AJCC Cancer Staging Manual, 8th edition. Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, *et al* (eds). Springer, Chicago, IL, pp563-585, 2017.
4. Lazar A and Bastian B: Melanoma. In: McKee's Pathology of the Skin. 4th edition. Calonje JE, Brenn T, Lazar A and McKee P (eds). Elsevier/Saunders, Edinburgh, pp1221-1267, 2012.
5. Matthews N, Li W, Qureshi A, Weinstock M and Cho E: Epidemiology of melanoma. In: Cutaneous Melanoma: Etiology and Therapy. Ward WH and Farma JM (eds) Codon Publications, Brisbane, pp3-23, 2017.
6. World Cancer Research Fund, American Institute for Cancer Research: Worldwide Cancer Data, Global Cancer Statistics for the most common cancers. WCRF International, London, 2019. <https://www.wcrf.org/dietand-cancer/cancer-trends/worldwide-cancer-data>. Accessed May 22, 2019.
7. Curtin JA, Fridlyand J, Kageshita T, Patel HN, Busam KJ, Kutzner H, Cho KW, Aiba S, Brocker EB, LeBoit PE, *et al*: Distinct sets of genetic alterations in melanoma. *N Engl J Med* 353: 2135-2147, 2005.
8. Palmieri G, Ombra M, Colombino M, Casula M, Sini M, Manca A, Paliogiannis P, Ascierto PA and Cossu A: Multiple molecular pathways in melanomagenesis: Characterization of therapeutic targets. *Front Oncol* 5: 183, 2015.
9. Giehl K: Oncogenic Ras in tumor progression and metastasis. *Biol Chem* 386: 193-205, 2005.
10. Lo MC, Paterson A, Maraka J, Clark R, Goodwill J, Nobes J, Garioch J, Moncrieff M, Rytina E and Igali L: A UK feasibility and validation study of the VE1 monoclonal antibody immunohistochemistry stain for BRAF-V600E mutations in metastatic melanoma. *Br J Cancer* 115: 223-227, 2016.

11. Long GV, Wilmott JS, Capper D, Preusser M, Zhang YX, Thompson JF, Kefford RF, von Deimling A and Scolyer RA: Immunohistochemistry is highly sensitive and specific for the detection of V600E BRAF mutation in melanoma. *Am J Surg Pathol* 37: 61-65, 2013.
12. Colomba E, Helias-Rodzewicz Z, Von Deimling A, Marin C, Terrones N, Pechaud D, Surel S, Côté JF, Peschaud F, Capper D, *et al*: Detection of BRAF p.V600E mutations in melanomas: Comparison of four methods argues for sequential use of immunohistochemistry and pyrosequencing. *J Mol Diagn* 15: 94-100, 2013.
13. Boursault L, Haddad V, Vergier B, Cappellen D, Bellocq JP, Jouary T and Merlio JP: Homogénéité et conservation du statut BRAF entre mélanome primitif et métastases déterminées par immunohistochimie et biologie moléculaire. *Ann Dermatol Vénereol* 140: S396, 2013.
14. Tetzlaff MT, Pattanaprichakul P, Wargo J, Fox PS, Patel KP, Estrella JS, Broaddus RR, Williams MD, Davies MA, Routbort MJ, *et al*: Utility of BRAF V600E immunohistochemistry expression pattern as a surrogate of BRAF mutation status in 154 patients with advanced melanoma. *Hum Pathol* 46: 1101-1110, 2015.
15. Box NF, Vukmer TO and Terzian T: Targeting p53 in melanoma. *Pigment Cell Melanoma Res* 27: 8-10, 2014.
16. Köbel M, Piskorz AM, Lee S, Lui S, LePage C, Marass F, Rosenfeld N, Mes Masson AM and Brenton JD: Optimized p53 immunohistochemistry is an accurate predictor of TP53 mutation in ovarian carcinoma. *J Pathol Clin Res* 2: 247-258, 2016.
17. Kastelein F, Biermann K, Steyerberg EW, Verheij J, Kalisvaart M, Looijenga LH, Stoop HA, Walter L, Kuipers EJ, Spaander MC, *et al*: Aberrant p53 protein expression is associated with an increased risk of neoplastic progression in patients with Barrett's oesophagus. *Gut* 62: 1676-1683, 2013.
18. Rodrigues NR, Rowan A, Smith ME, Kerr IB, Bodmer WF, Gannon JV and Lane DP: p53 mutations in colorectal cancer. *Proc Natl Acad Sci USA* 87: 7555-7559, 1990.
19. Iggo R, Gatter K, Bartek J, Lane D and Harris AL: Increased expression of mutant forms of p53 oncogene in primary lung cancer. *Lancet* 335: 675-679, 1990.
20. Guedes LB, Almutairi F, Haffner MC, Rajoria G, Liu Z, Klimel S, Zoino R, Yousefi K, Sharma R, De Marzo AM, *et al*: Analytic, preanalytic, and clinical validation of p53 IHC for detection of TP53 missense mutation in prostate cancer. *Clin Cancer Res* 23: 4693-4703, 2017.
21. Lepelletier P, Preudhomme C, Vanrumbeke M, Quesnel B, Cosson A and Fenaux P: Detection of p53 mutations in hematological malignancies: Comparison between immunocytochemistry and DNA analysis. *Leukemia* 8: 1342-1349, 1994.
22. Dobes P, Podhorec J, Coufal O, Jureckova A, Petrakova K, Vojtesek B and Hrstka R: Influence of mutation type on prognostic and predictive values of TP53 status in primary breast cancer patients. *Oncol Rep* 32: 1695-1702, 2014.
23. Albino AP, Vidal MJ, McNutt NS, Shea CR, Prieto VG, Nanus DM, Palmer JM and Hayward NK: Mutation and expression of the p53 gene in human malignant melanoma. *Melanoma Res* 4: 35-45, 1994.
24. Akslen LA, Monstad SE, Larsen B, Straume O and OGREID D: Frequent mutations of the p53 gene in cutaneous melanoma of the nodular type. *Int J Cancer* 79: 91-95, 1998.
25. Sparrow LE, English DR, Heenan PJ, Dawkins HJ and Taran J: Prognostic significance of p53 over-expression in thin melanomas. *Melanoma Res* 5: 387-392, 1995.
26. Florenes VA, Oyjord T, Holm R, Skrede M, Borresen AL, Nesland JM and Fodstad O: TP53 allele loss, mutations and expression in malignant melanoma. *Br J Cancer* 69: 253-259, 1994.
27. Weiss J, Heine M, Arden KC, Körner B, Pilch H, Herbst RA and Jung EG: Mutation and expression of TP53 in malignant melanomas. *Recent Results Cancer Res* 139: 137-154, 1995.
28. Soussi T, Legros Y, Lubin R, Ory K and Schlichtholz B: Multifactorial analysis of p53 alterations in human cancer: A review. *Int J Cancer* 57: 1-9, 1994.
29. Hussein MR: The TP53 tumor suppressor gene and melanoma tumorigenesis: Is there a relationship? *Tumor Biol* 25: 200-207, 2004.
30. Le Mercier M, D'Haene N, De Nève N, Blanchard O, Degand C, Rorive S and Salmon I: Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. *Histopathology* 66: 215-224, 2015.
31. D'Haene N, Le Mercier M, De Nève N, Blanchard O, Delaunoy M, El Housni H, Dessars B, Heimann P, Rummelink M, Demetter P, *et al*: Clinical validation of targeted next generation sequencing for colon and lung cancers. *PLoS One* 10: e0138245, 2015.
32. Lebrun L, Milowich D, Le Mercier M, Allard J, Van Eycke YR, Roumeguere T, Decaestecker C, Salmon I and Rorive S: UCA1 overexpression is associated with less aggressive subtypes of bladder cancer. *Oncol Rep* 40: 2497-2506, 2018.
33. Miralor AP, de Abreu FB, Peterson JD, Turner SA, Amos CI, Tsongalis GJ and Yan S: Somatic mutation analysis in melanoma using targeted next generation sequencing. *Exp Mol Pathol* 103: 172-177, 2017.
34. Holderfield M, Deuker MM, McCormick F and McMahon M: Targeting RAF kinases for cancer therapy: BRAF-mutated melanoma and beyond. *Nat Rev Cancer* 4: 455-467, 2014.
35. Coit DG, Thompson JA, Albertini MR, Barker C, Carson WE, Contreras C, Daniels GA, DiMaio D, Fields RC, Fleming MD, *et al*: Cutaneous Melanoma, Version 2.2019, NCCN clinical practice guidelines in oncology. *J Natl Compr Canc Netw* 17: 367-402, 2019.
36. Lee JH, Choi JW and Kim YS: Frequencies of BRAF and NRAS mutations are different in histological types and sites of origin of cutaneous melanoma: A meta-analysis. *Br J Dermatol* 164: 776-784, 2011.
37. Kudchadkar R, Paraiso KH and Smalley KS: Targeting mutant BRAF in melanoma: Current status and future development of combination therapy strategies. *Cancer J* 18: 124-131, 2012.
38. Thiel A, Moza M, Kytola S, Orpana A, Jähkola T, Hernberg M, Virolainen S and Ristimäki A: Prospective immunohistochemical analysis of BRAF V600E mutation in melanoma. *Hum Pathol* 46: 169-175, 2015.
39. Marin C, Beauchet A, Capper D, Zimmermann U, Julie C, Ilie M, Saiag P, von Deimling A, Hofman P and Emile JF: Detection of BRAF p.V600E mutations in melanoma by immunohistochemistry has a good interobserver reproducibility. *Arch Pathol Lab Med* 138: 71-75, 2014.
40. Pearlstein MV, Zedek DC, Ollila DW, Treece A, Gulley ML, Groben PA and Thomas NE: Validation of the VE1 Immunostain for the BRAF V600E mutation in melanoma. *J Cutan Pathol* 41: 724-732, 2014.
41. Schirosi L, Strippoli S, Gaudio F, Graziano G, Popescu O, Guida M, Simone G and Mangia A: Is immunohistochemistry of BRAF V600E useful as a screening tool and during progression disease of melanoma patients? *BMC Cancer* 16: 905, 2016.
42. Cheng L, Lopez-Beltran A, Massari F, MacLennan GT and Montironi R: Molecular testing for BRAF mutations to inform melanoma treatment decisions: A move toward precision medicine. *Mod Pathol* 31: 24-38, 2018.
43. Bisschop C, Ter Elst A, Bosman LJ, Platteel I, Jalving M, van den Berg A, Diepstra A, van Hemel B, Diercks GFH, Hospers GAP and Schuurin E: Rapid BRAF mutation tests in patients with advanced melanoma: Comparison of immunohistochemistry, Droplet Digital PCR, and the Idylla Mutation Platform. *Melanoma Res* 28: 96-104, 2018.
44. Lu M, Miller P and Lu X: Restoring the tumour suppressive function of p53 as a parallel strategy in melanoma therapy. *FEBS Lett* 588: 2616-2621, 2014.
45. Onyshchenko M: The puzzle of predicting response to immune checkpoint blockade. *EBioMedicine* 33: 18-19, 2018.
46. Xiao W, Du N, Huang T, Guo J, Mo X, Yuan T, Chen Y, Ye T, Xu C, Wang W, *et al*: TP53 mutation as potential negative predictor for response of Anti-CTLA-4 therapy in metastatic melanoma. *EBioMedicine* 32: 119-124, 2018.
47. Siroy AE, Boland GM, Milton DR, Roszik J, Frankian S, Malke J, Haydu L, Prieto VG, Tetzlaff M, Ivan D *et al*: Beyond BRAF(V600): Clinical mutation panel testing by next-generation sequencing in advanced melanoma. *J Invest Dermatol* 135: 508-515, 2015.
48. Murniak B and Hortobagyi T: Immunohistochemical correlates of TP53 somatic mutations in cancer. *Oncotarget* 7: 64910-64920, 2016.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.