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BRAF^{V600E} and *NRAS*$^{Q61L/Q61R}$ mutation analysis in metastatic melanoma using immunohistochemistry: a study of 754 cases highlighting potential pitfalls and guidelines for interpretation and reporting

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BRAF^{V600E} and NRAS$^{Q61L/Q61R}$ mutation analysis in metastatic melanoma using immunohistochemistry: a study of 754 cases highlighting potential pitfalls and guidelines for interpretation and reporting

Background and aims: *BRAF* or *NRAS* mutations occur in approximately 60% of cutaneous melanomas, and the identification of such mutations underpins the appropriate selection of patients who may benefit from BRAF and MEK inhibitor targeted therapies. The utility of immunohistochemistry (IHC) to detect *NRAS*^{Q61L} mutations is currently unknown. This study sought to assess the sensitivity and specificity of anti-BRAF^{V600E} (VE1), anti-NRAS^{Q61R} (SP174) and anti-NRAS^{Q61L} (26193) antibodies for mutation detection in a large series of cases.

Methods and results: Mutation status was determined using the OncoCarta assay in 754 cutaneous melanomas. IHC with the anti-BRAF^{V600E} antibody was performed in all cases, and the anti-NRAS^{Q61R} and anti-NRAS^{Q61L} antibodies were assessed in a subset of 302 samples utilizing tissue microarrays. The staining with the anti-BRAF^{V600E} and anti-NRAS^{Q61R} antibodies was diffuse, homogeneous and cytoplasmic. The anti-NRAS^{Q61L} antibody displayed variable intensity staining, ranging from weak to strong in *NRAS*^{Q61L} mutant tumours. The sensitivity and specificity for anti-BRAF^{V600E} was 100 and 99.3%, anti-NRAS^{Q61R} was 100 and 100% and anti-NRAS^{Q61L} was 82.6 and 96.2%, respectively.

Conclusions: The use of IHC is a fast, efficient and cost-effective method to identify single specific mutations in melanoma patients. BRAF^{V600E} and NRAS^{Q61R} antibodies have high sensitivity and specificity; however, the NRAS^{Q61L} antibody appears less sensitive. IHC can help to facilitate the timely, appropriate selection and treatment of metastatic melanoma patients with targeted therapies. Detection of melanoma-associated mutations by IHC may also provide evidence for a diagnosis of melanoma in metastatic undifferentiated neoplasms lacking expression of melanoma antigens.

Keywords: *BRAF*, diagnosis, immunohistochemistry, melanoma, *NRAS*

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Introduction

Clinical trials have demonstrated that selective BRAF inhibitors combined with MEK inhibitors produce high response rates and a prolonged overall survival in advanced stage *BRAF*^{V600} mutant metastatic melanoma patients and are current standard of care.[1,2] These inhibitors are effective only in patients with *BRAF* mutant melanoma and are potentially detrimental to patients with *BRAF* wild-type melanoma, as they induce paradoxical activation of MAPK signalling pathway via RAF dimerization.[3–5] Therefore, the presence of a *BRAF* mutation must be verified prior to initiation of therapy. Patients with *NRAS* mutant melanomas may also respond to targeted therapies with MEK inhibitors and CDK4/6 inhibitors and clinical trials are currently assessing the potential utility of these therapies in this patient cohort.

Mutation testing is undertaken routinely on formalin-fixed paraffin-embedded (FFPE) tumour tissue. Currently, most laboratories use various sequence-based DNA mutation strategies for mutation testing, which are often costly and require specialized equipment. Furthermore, false negative tests may occur, especially if there is minimal tumour available for analysis (such as a small primary tumour or sentinel node metastasis). Monoclonal antibodies to detect mutant BRAF^{V600E} protein have provided a cost-effective, time-efficient and accurate method for detecting *BRAF*^{V600E} mutations.[6–8] Moreover, immunohistochemistry (IHC) is readily available in nearly all pathology laboratories and has the added advantage of allowing visualization of individual tumour cells, e.g. single or small clusters of metastatic tumour cells in lymph nodes or melanoma cells admixed with naevus cells.[9] Anti-NRAS^{Q61R} (SP174; Spring Bioscience, Pleasanton, CA, USA) and anti-NRAS^{Q61L} (26193; NewEast Biosciences, Malvern, PA, USA) antibodies are commercially available for the detection of respective mutant proteins. While two previous studies have reported the high sensitivity and specificity for anti-NRAS^{Q61R} in detecting *NRAS*^{Q61R} mutations,[10,11] as far as we are aware no previously reported study has evaluated the detection of *NRAS*^{Q61L} mutations by IHC. This study assessed the sensitivity and specificity of these three antibodies in the same large set of melanomas.

Materials and methods

STUDY DESIGN AND PATIENT SELECTION

This study was undertaken at Melanoma Institute Australia and the Royal Prince Alfred Hospital, Sydney, Australia with Human Ethics Committee approval (X11-0289, HREC/11/RPAH/444). The study cohort consisted of 754 patients with archival FFPE tumour tissue available for analysis.

MOLECULAR MUTATION TESTING

One core was taken from an FFPE block of melanoma tissue in each case. DNA was extracted using the NucleoSpin FFPE DNA Kit (Macherey Nagel, Duren, Germany), according to the manufacturer's instructions, with an overnight proteinase digestion step. The NanoDrop ND-1000 Spectrophotometer and Qubit fluorometer (ThermoFisher, Scoresby, Vic., Australia) was used to assess the quality and quantity of the extracted DNA. Samples were amplified for 238 variant targets in a 24 multiplex polymerase chain reaction (PCR) using the OncoCarta Panel version 1.0 kit (*ABL1*, *AKT1*, *AKT2*, *BRAF*, *CDK*, *EGFR*, *ERBB2*, *FGFR1*, *FGFR3*, *FLT3*, *JAK2*, *KIT*, *MET*, *HRAS*, *KRAS*, *NRAS*, *PDGFR*, *PIK3CA*, *RET*) and analysed based on the matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF) technology on the Sequenom MassArray platform (Sequenom, San Diego, CA, USA). The specific *BRAF* mutations detectable via this assay are G464R/V/E, G466R, F468C, G469A/E/R/S/V, D594V/G, F595L, G596R, L597Q/R/S/V, T599I and V600E/K/R/L/N. If there was insufficient DNA for OncoCarta analysis, the sample was assayed using the AmoyDx Mutation Detection Kit (Amoy Diagnostics, Xiamen, China). The AmoyDx kit is more sensitive than the OncoCarta panel, but does not allow the *BRAF*^{V600E} mutation to be distinguished from other mutations affecting the same nucleotide base pair (e.g. V600K/R/D/M). The extracted DNA was added to a PCR tube containing the AmoyDx *BRAF* reaction mixture and Taq DNA polymerase, and real-time PCR carried out according to the AmoyDx protocol.

TISSUE MICROARRAY AND IHC

Three tissue microarrays were constructed from a subset of 302 melanoma tumours using the TMArrayer™ (Pathology Devices, Inc., Westminster, MD, USA). A haematoxylin and eosin (H&E) slide was cut for each sample to assess and mark the area with melanoma for coring. IHC was performed on 4-μm-thick sections using an Autostainer Plus (Dako–Agilent Technologies, Glostrup, Denmark) with appropriate positive and negative controls. Sections were baked for 60 min at 60°C in a dehydration

oven and heat-induced epitope retrieved in the PT link (Dako–Agilent Technologies) using EnVision FLEX target retrieval solution for 20 min at 97°C, then cooled to room temperature in Tris-buffered saline and Tween 20 (TBST) wash buffer for 5 min. Slides were incubated with the following antibodies at the following dilutions: anti-BRAF^{V600E} (VE1; Spring Bioscience) 1:50, anti-NRAS^{Q61R} (SP174; Spring Bioscience) 1:200 and anti-NRAS^{Q61L} (26193; NewEast Bioscience) 1:4000. Antibody detection utilized the MACH3™ detection system labelled with horseradish peroxidase and diaminobenzidine (DAB) for visualization, with haematoxylin applied for 5 min as a counterstain prior to cover slipping. Anti-BRAF^{V600E} IHC was performed on whole sections in 452 cases and on tissue microarrays in 302 cases. Anti-NRAS^{Q61R} and anti-NRAS^{Q61L} IHC were performed initially on the 302 cases in tissue microarrays only.

All the IHC slides were evaluated and scored by several independent observers (H.K., W.A.C., S.A.O. and R.A.S.) blinded to the OncoCarta results. In cases where there was a discrepancy between the IHC and OncoCarta results, a third assay was performed using the OncoFocus panel comprising a small number of specific probes assessing mutations found in *BRAF*, *NRAS*, *EGFR* and *KRAS* or the AmoyDx *BRAF*^{V600} mutation detection kit and the IHC was repeated on whole sections of the case.

Results

PATIENTS

A total of 754 cases were available for analysis, 18 samples of which were excluded due to either loss of or damage to the IHC slide. Cases were scored as uninterpretable when the tumour was pigmented heavily with melanin and IHC staining could not be assessed accurately ($n = 10$), and these cases were also excluded from further analysis; thus, 726 cases were included in this study (Table 1). Based on molecular testing, 260 (35.8%) were *BRAF* mutant, 192 (26.4%) *NRAS* mutant and 274 (37.8%) cases were wild-type for both *BRAF* and *NRAS* (Table 2). Of the 260 *BRAF* mutant cases, 188 (72.3%) had a *BRAF*^{V600E} mutation. In the subset of 286 evaluable melanoma specimens that had IHC performed with the anti-NRAS^{Q61L} and anti-NRAS^{Q61R} antibodies on tissue microarrays, the following mutations were detected by molecular testing: 89 were *BRAF*^{V600E} (31.1%) mutant, 22 were *BRAF*^{V600K} (7.7%) mutant, three were *BRAF*^{V600R} (1.0%) mutant, 42 were *NRAS*^{Q61R} (14.7%) mutant, 23 were *NRAS*^{Q61L} (8%) mutant and were 19 *NRAS*^{Q61K} (6.6%) mutant and 88 (30.8%) were wild-type for both *BRAF* and *NRAS*.

ANTI-BRAF^{V600E} ANALYSIS

Seven hundred and twenty-six samples had evaluable anti-BRAF^{V600E} IHC. All positive cases displayed strong diffuse, cytoplasmic staining throughout all tumour cells (Figure 1). The sensitivity and specificity of the anti-BRAF^{V600E} antibody compared to the molecular mutation testing were 100 and 99.1%, respectively. There was a total of 17 discrepancies between the mutation testing with the OncoCarta panel and the IHC with the anti-BRAF^{V600E} antibody. Six cases were IHC-positive for the *BRAF*^{V600E} mutation but failed initial Oncocarta testing, because the tumour content of these samples was below the threshold of detection (<10%). However, molecular retesting with the more sensitive AmoyDx assay

Table 1. Clinicopathological characteristics and percentage *BRAF*^{V600E}+ of the 726 cases

Patient and tumour characteristics	Number (%)	% *BRAF*^{V600E}+
Age (years)		
<40	46 (6.4)	52.2
40–60	242 (33.3)	36.4
61–80	355 (48.9)	20.6
>80	83 (11.4)	4.8
Sex		
Female	249 (34.3)	26.9
Male	477 (65.7)	28.3
Melanoma stage		
I	0 (0.0)	0.0
II	13 (1.8)	7.7
III	336 (46.3)	30.4
IV	377 (51.9)	22.8
Biopsy site		
Primary lesion	13 (1.8)	7.7
Locoregional metastasis	540 (74.4)	28.5
Metastatic metastasis	173 (23.8)	19.7

Table 2. Mutation profiles of the 726 tumours

Gene	Number (%)
BRAF	260 (35.8)
V600E	188 (25.9)
V600K	50 (6.9)
Other	22 (3.0)
NRAS	192 (26.4)
Q61R	81 (11.1)
Q61K	52 (7.2)
Q61L	42 (5.8)
Other	17 (2.3)
Wild-type*	274 (37.8)

*Wild-type for *BRAF* and *NRAS* mutations.

showed the presence of a *BRAF* mutation at codon 600. Although no other mutations in 18 other genes were identified on the Oncocarta panel in these six cases, these results may be unreliable due to low tumour (<10%). In seven of the 17 discrepant cases, restaining with the anti-BRAF^{V600E} antibody suggested that the original IHC was misinterpreted as positive when it demonstrated weak non-specific staining due to the high antibody concentration used. Of these seven cases, three were positive for the *NRAS*^{Q61R}, two for the *NRAS*^{Q61K} and two were wild-type for the 19 genes in the Oncocarta panel of mutations. Four cases remained discrepant between the anti-BRAF^{V600E} IHC and the mutation testing results, which could not be resolved despite retesting with both IHC and molecular testing with the OncoFocus panel. All four cases were IHC positive for the *BRAF*^{V600E} mutation and molecular testing was repeatedly negative. One of these cases also had a concurrent *NRAS*^{Q61R} mutation identified by molecular testing, which was confirmed by IHC; however, the remaining three cases were wild-type for the 19 genes tested in the Oncocarta panel of mutations.

ANTI-NRAS^{Q61R} ANALYSIS

Two hundred and eighty-six of the cases also had evaluable anti-NRAS^{Q61R} and anti-NRAS^{Q61L} IHC performed on them in tissue microarrays. The specificity and the sensitivity with the anti-NRAS^{Q61R} antibody were 100%, as there were no discrepancies between the IHC and the mutation testing results. All positive cases displayed strong diffuse, cytoplasmic staining throughout all tumour cells (Figure 1).

ANTI-NRAS^{Q61L} ANALYSIS

The sensitivity and specificity of the anti-NRAS^{Q61L} antibody were 82.6 and 96.2%, respectively. The intensity of the positive staining in *NRAS*^{Q61L} mutant cases varied from weak to strong. Positive cases generally showed similar intensity of staining in all tumour cells. There were 19 discrepancies (four false negatives and 15 false positives) in the 286 cases between the IHC with the anti-NRAS^{Q61L} antibody and the OncoCarta panel mutation testing results. Upon restaining of the whole tissue section with the anti-NRAS^{Q61L} antibody, in five of the 15 false positive discrepant cases the tumour cells were negative. The remaining 14 cases remained discrepant on repeated IHC with the anti-NRAS^{Q61L} antibody using appropriate positive and negative controls on each section and repeat molecular retesting with the OncoFocus panel, and the reason for the discrepancies could not be resolved. In almost all false positive cases, the intensity of staining was weak to moderate (Figure 2A–C). However, similarly weak staining was observed in a proportion of cases that had molecularly confirmed *NRAS*^{Q61L} mutations (Figure 2D–F).

Discussion

This study assessed the sensitivity and specificity of the anti-BRAF^{V600E} antibody in 726 melanoma specimens and the anti-NRAS^{Q61R} antibody in 286 melanomas which, as far as we are aware, are the largest reported series of melanomas tested with each of these antibodies. Furthermore, we also report the first series of melanomas assessing the utility of the anti-NRAS^{Q61L} antibody. We believe our results have important implications for clinical pathology practice and multidisciplinary melanoma patient care, because they validate the utility of immunohistochemistry for detecting specific melanoma-associated mutations using techniques that are available in nearly all pathology laboratories and can be performed with rapid turnaround times.

The sensitivity and specificity of the anti-BRAF^{V600E} antibody in the 726 cases were 100 and 99.3%, respectively. Discrepancies between the anti-BRAF^{V600E} antibody and molecular testing (n = 17) were able to be resolved in 13 cases. Retesting with the more sensitive PCR-based AmoyDx molecular assay and restaining with the antibody suggested that in approximately

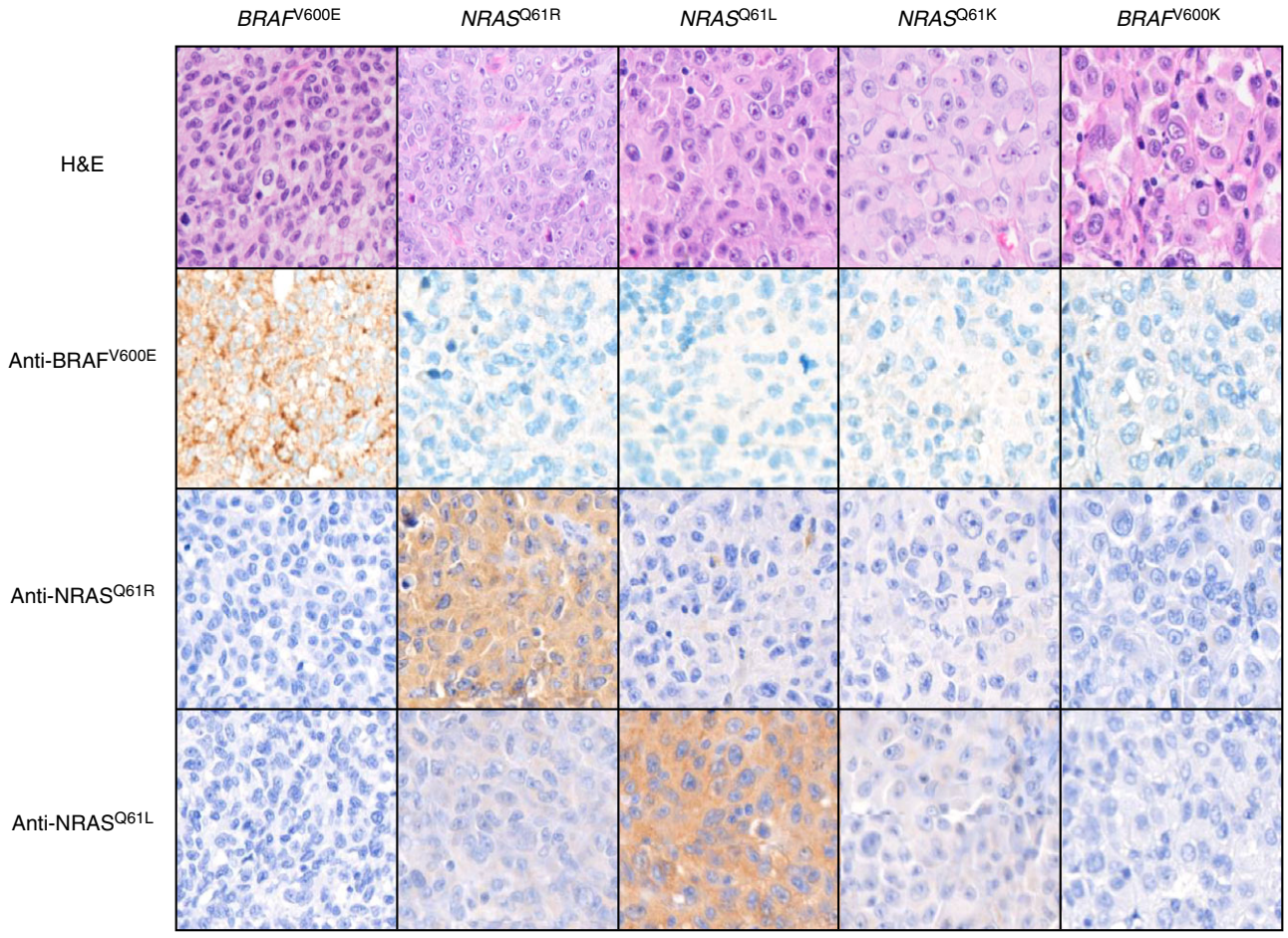

Figure 1. Tissue microarray immunohistochemical analysis of five samples with varying mutation subtypes. All samples were stained with haematoxylin and eosin (H&E), anti-BRAF^{V600E} (VE1) antibody, anti-NRAS^{Q61R} (SP174) antibody and anti-NRAS^{Q61L} antibody (26193).

half the cases the discrepancy was caused by failed molecular testing and in the other half was caused by immunohistochemical misinterpretation caused by weak background positive staining. The original OncoCarta mutation testing may have been negative because the amount of mutant DNA was below the 10% threshold limitation to detect the *BRAF*^{V600E} mutation sequence, which may have been caused by low tumour content or possibly low quality DNA, or misinterpretation. There were four cases that were all IHC-positive and molecular testing negative for which the cause of the discrepancy could not be resolved. The first was a sample from 1997, which could explain the lack of mutations found using the OncoCarta panel, as the nucleic acids could have become fragmented and below the mutant allele frequency threshold required to show confidently that the mutation is present. The second case was reported originally as having a *NRAS*^{Q61R} mutation, yet was also immunoreactive for the anti-BRAF^{V600E} antibody. Retesting with the OncoFocus panel showed only the *NRAS*^{Q61R} mutation and no *BRAF*^{V600E} mutation; however, restaining with both the anti-BRAF^{V600E} and the anti-NRAS^{Q61R} antibody showed positive immunoreactivity with both antibodies. There have been rare case reports of melanomas harbouring mutations in both *BRAF* and *NRAS*.[12] The absence of the *BRAF* mutation in the OncoCarta results could be due to degradation of the nucleic acids from the FFPE sample; however, due to the rarity of this concurrent mutation, a false positive anti-BRAF^{V600E} result appears most likely.

There are some important limitations and potential pitfalls with immunohistochemical evaluation for *BRAF*^{V600E} testing. In our experience, positive staining is usually strong and diffuse throughout all tumour cells in *BRAF*^{V600E} mutant melanomas.[8,9,13] Occasionally, cases may show a weak blush of positive staining, which we recommend be reported as equivocal and require correlation with formal molecular testing. At our institution, *BRAF*^{V600E} testing

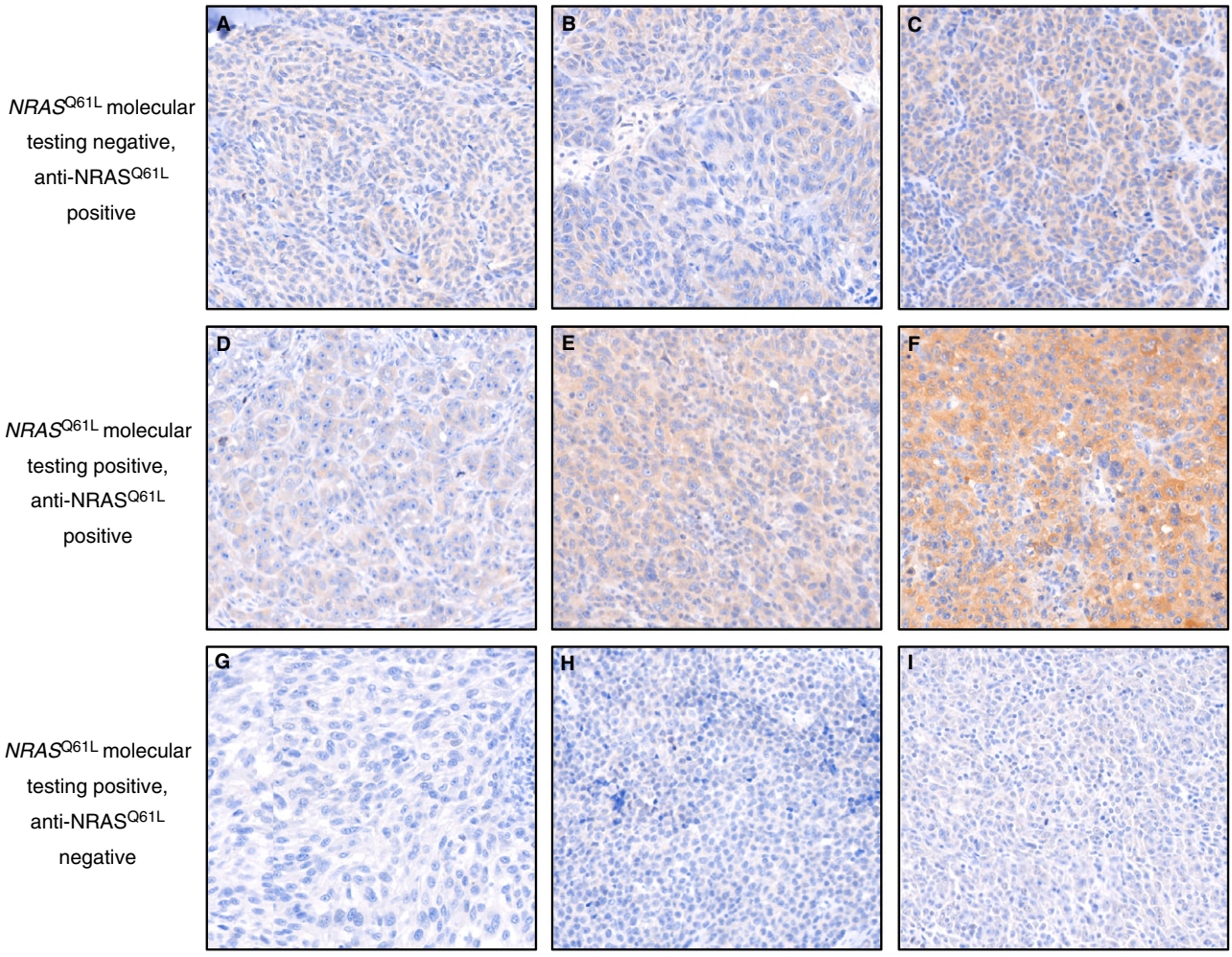

Figure 2. Immunostaining with the anti-NRAS^{Q61L} antibody (26193) was falsely positive in some cases (**A–C**) and falsely negative in others (**G–I**) compared with *NRAS*^{Q61L} mutations by molecular testing. In some of the cases with *NRAS*^{Q61L} mutations by molecular testing that showed positive anti-NRAS^{Q61L} immunohistochemistry staining, the intensity of positivity varied from weak (**D**) to moderate (**E**) to strong (**F**).

with the anti-BRAF^{V600E} antibody is performed on all metastatic melanomas. In addition, to ensure the highest degree of accuracy, any patients requiring formal *BRAF* molecular testing have both IHC and molecular testing performed. It is also important to highlight that the anti-BRAF^{V600E} antibody is specific for *BRAF*^{V600E} mutations, which account for about 70–80% of *BRAF* mutations.[14] Other non-V600E *BRAF* mutations, including *BRAF*^{V600K} mutations, which are also sensitive to targeted therapy with vemurafenib or dabrafenib, are not detected by the anti-BRAF^{V600E} antibody. In contrast to *BRAF*^{V600E} mutations, *BRAF*^{V600K} mutations are more common in older melanoma patients and are associated with higher degrees of solar damage in the adjacent skin.[15] We have analysed a number of commercially available antibodies against BRAF^{V600K} mutant protein, but all have lacked specific binding affinity in our experience.

There were no discrepancies with the anti-NRAS^{Q61R} antibody and the mutation testing results with the OncoCarta panel, which corroborates previous reports of the high sensitivity and specificity of this antibody.[10,11,16] The anti-NRAS^{Q61L} antibody used for the detection of the *NRAS*^{Q61L} mutation had a relatively higher number of discrepancies (n = 14) between the IHC and the OncoCarta mutation testing results, which could not be explained by technical issues or specific cross-reactivity with another antigen; therefore, even though this antibody appears

promising, further work is needed to increase its sensitivity and specificity before its use could be recommended for clinical testing.

The prospect of utilizing a small panel of immunohistochemical antibodies to detect all clinically relevant mutation testing in a rapid and effective manner at the time of pathological diagnosis is appealing. Potentially it would improve the efficient management of advanced stage metastatic melanoma patients and reduce the amount of time between a request for mutation analysis and the confirmation of the result, thereby expediting a patient's treatment options, which may be extremely important in the case of gravely ill patients. However, until more effective antibodies are developed for detecting $BRAF^{V600K}$ and additional *NRAS* mutations, antibody testing will serve only as a screening or adjunct to formal molecular testing. In our experience, a combination of IHC used as a screening tool and parallel or subsequent formal molecular mutation testing provides the highest reliability for capturing the majority of cases with actionable mutations with the highest sensitivity and specificity.[8,17] An additional potential role of IHC for the detection of mutations may be in the diagnostic work-up of undifferentiated malignant tumours. In such instances, detection of melanoma-associated mutations by IHC may provide evidence for a diagnosis of melanoma in neoplasms lacking expression of melanoma antigens such as S100, HMB45, Melan-A and SOX10.

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Conflict of Interest

The authors declare no conflicts of interest.

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