

# Immunohistochemistry is highly sensitive and specific for the detection of *NRASQ61R* mutation in melanoma

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Testing for *NRAS* is now integral part in the assessment of metastatic melanoma patients because there is evidence that *NRAS*-mutated patients may be sensitive to MEK inhibitors, and *RAS* mutation is a common mechanism of acquired resistance during treatment with BRAF inhibitors. This study evaluated the sensitivity and specificity of immunohistochemical analysis using an N-Ras (Q61R) antibody to detect the presence of the *NRASQ61R* mutation in melanoma patients. A total of 98 primary cutaneous melanomas that have undergone examination of *NRAS* mutation were retrieved from a multicentric database. Formalin-fixed and paraffin-embedded melanoma tissues were analyzed for *BRAF* and *NRAS* mutations by independent, blinded observers using both conventional DNA molecular techniques and immunohistochemistry with the novel anti-human N-Ras (Q61R) monoclonal antibody (clone SP174). The antibody showed a sensitivity of 100% (14/14) and a specificity of 100% (83/83) for detecting the presence of an *NRASQ61R* mutation. Of the *NRAS*-mutated cases, none of the non-Q61R cases stained positive with the antibody (0/7). There were three cases with discordant *NRAS* mutational results. Additional molecular analysis confirmed the immunohistochemically obtained *NRAS* result in all cases, suggesting that a multiple analytical approach can be required to reach the correct sample classification. The reported immunohistochemical method is an accurate, rapid, and cost-effective method for detecting *NRASQ61R* mutation in melanoma patients, and represents a valuable supplement to traditional mutation testing. If validated in further studies, genetic testing would only be required for immunohistochemistry-negative patients to detect non-Q61R mutations.

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Ras proteins are crucial crossroads of signaling pathways that link the activation of cell surface receptors with a wide variety of cellular processes

leading to the control of proliferation, apoptosis, and differentiation.<sup>1</sup> When acted upon by specific factors, such as extracellular ligands that bind specific membrane receptors, these proteins cycle between an activated and inactivated form, RAS-GTP and RAS-GDP, respectively.<sup>2</sup> Activation requires dissociation of protein-bound GDP, a process that is accelerated by guanine nucleotide exchange factors. This switch-on process involves the reversible exchange of GDP for GTP. The switch-off process is entirely different and involves

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hydrolysis of GTP to GDP, the guanosine triphosphatase (GTPase) reaction, which is basically irreversible.

Mutations at positions 12, 13, or 61 of the *HRAS*, *NRAS*, and *KRAS* impair the GTPase activity of the carrier RAS proteins and lock them into a constitutively activated state in which they elicit downstream effectors, even in the absence of ligands that bind specific membrane receptors.<sup>3</sup> Currently, mutations in *NRAS*, *KRAS* and *HRAS* are known to be present in 20%, 2%, and 1% of all melanomas, respectively. With regards to *NRAS*, the most common oncogenic change (80–90% of all *NRAS* mutations) reported is a point mutation at position 61, with mutations at positions 12 and 13 occurring less frequently.<sup>4</sup>

In the largest single-institution cohort of melanoma patients characterized for activating *BRAF* and *NRAS* mutations, the most common *NRAS* mutation was represented by the glutamine to arginine substitution at position 61 (*NRASQ61R*).<sup>5</sup>

*NRAS* has so far proven to be intractable to conventional drug discovery, and several different strategies of directly targeting *RAS* have not resulted in effective therapeutics. Nevertheless, there is evidence that some *NRASQ61R*-mutated cell lines are sensitive to MEK inhibition *in vitro*.<sup>6</sup> Recently, an oral MEK inhibitor (MEK162) was tested in patients with metastatic melanoma harboring *BRAF* or *NRAS* mutations with encouraging results in *NRAS*-mutated patients, most of whom harbored *NRASQ61R* mutation.<sup>7</sup> The response rate was reported in 20% of patients and progression-free survival was similar in *BRAF*- and *NRAS*-mutated patients. These data backed the hypothesis that *RAS* may be druggable in melanoma patients and several phase II and III studies are ongoing, most of them including *NRAS*-mutated metastatic melanoma (<https://clinicaltrials.gov/>).

In addition to the recent data on *NRAS* as a potential target, preclinical and clinical data suggest that: (1) in *BRAF*-mutated melanoma patients, a concomitant baseline mutation in the upstream *NRAS* oncogene may result in early lack of clinical benefit to *BRAF* inhibitors; (2) *RAS* mutation is a common mechanism of acquired resistance during treatment with *BRAF* inhibitors.<sup>8</sup> Hence, testing for *NRAS* may be an integral part in the assessment of metastatic melanoma patients. Several molecular techniques have been developed to detect *NRAS* mutation such as conventional Sanger sequencing, pyrosequencing, or expensive and more sophisticated techniques including high-throughput multiplexed mass spectrometry-based platforms or targeted next-generation sequencing technologies.

However, for this purpose, tumor tissues may need to be sent to specialized laboratories for molecular testing, which inevitably results in a delay in defining the patient's optimal management. Immunohistochemical analysis allows visualization of individual antigen-bearing tumor cells, is

routinely performed and, in contrast to the molecular techniques, is more rapid, cheaper, and more accessible for clinical use.

With these considerations in mind, here we report, for the first time in the literature, the sensitivity and specificity of an anti-human N-Ras (Q61R) monoclonal antibody (clone SP174) in melanoma tissues. The encouraging results obtained in the current study on tissue samples from primary melanomas could be extended and validated on metastatic melanoma tissues as, in the era of personalized medicine, *NRAS* mutation status has become a key piece of information in the clinical management of melanoma patients. N-Ras (Q61R) immunohistochemistry could thus represent a screening tool in the assessment of *NRAS* mutant status in melanoma patients. In our view, N-Ras (Q61R) immunostaining seems to be complementary with mutational screening, as re-evaluation of discrepant cases may improve diagnostic accuracy.

## Materials and methods

### Immunohistochemistry on Melanoma Cell Lines

Human melanoma cell lines A375 (harboring wild-type *NRAS*) and SKMel-2 (harboring mutant *NRASQ61R*) were cultured in standard DMEM with 10% FBS. Paraffin embedding of cultured melanoma cells was performed under standard condition. Briefly, 10<sup>7</sup> cells per cell line were trypsinized, washed, and resuspended with 2 ml 4% PFA. Following gentle removal of the supernatant, the pellet was washed two times with PBS and gently resuspended with 600  $\mu$ l Richard-Allan Scientific™ HistoGel™ Specimen Processing Gel (Thermo Scientific). The gelatinized gel blocks were then processed under standard condition for paraffin embedding and the section with 3  $\mu$ m of thickness was stained with the rabbit monoclonal anti-human N-Ras (Q61R), clone SP174 antibody (Spring Biosciences, Pleasanton, CA, USA) (provided by UCS Diagnostic, Morlupo (Roma), Italy). The immunohistochemical staining was processed with automated staining system (Ventana Discovery; Roche).

### Tumor Tissue Samples

A total of 98 patients (median age 73 years, range 16–99 years, 64 males, 34 females) with a diagnosis of primary cutaneous melanoma with Breslow thickness over 4 mm were identified from a retrospectively collected database. Tumor specimens were collected from the Pathological Anatomy Unit, Careggi University Hospital (Florence, Italy), and from the archives of Pathological Anatomy Units belonging to the Tumour Institute of Tuscany (ITT) network of Pathology. Twenty out of the 98 (20%) cases were from the head and neck region, 33 out of 98 (34%) from the trunk, 38 out of 98 (39%) from the

extremities, and 7 out of 98 (7%) from acral sites. Hematoxylin- and eosin-stained sections were reviewed by two pathologists (DM and CU) to confirm the histopathological diagnosis. Representative sections from each lesion were selected for immunohistochemical analysis. From each tumor specimen, sections of formalin-fixed and paraffin-embedded samples were collected. The study was approved by the Institutional Ethical Committee and was performed according to the Declaration of Helsinki of 1975 as revised in 1983.

### DNA Extraction

To obtain genomic DNA from formalin-fixed and paraffin-embedded blocks, 5- $\mu$ m-thick tissue sections underwent proteinase K overnight digestion at 56 °C and extraction was performed using the formalin-fixed and paraffin-embedded Tissue Kit (Qiagen, Milan, Italy). Wild-type and mutated reference DNA were extracted from specific tumor cell lines: HeLa as wild-type control for *BRAF* and *NRAS* analysis, whereas SKMel-28 (homozygous for *BRAFV600E*) and HT1197 (heterozygous for *NRASQ61R*) were used as positive controls. DNA extraction from cell lines was performed using QIAamp DNA Mini Kit (Qiagen).

### Direct Sequencing and High-Resolution Melting Analysis

DNA from formalin-fixed and paraffin-embedded samples were submitted to amplification by using 20 ng of total DNA in a PCR reaction mix of 25  $\mu$ l. Samples were denatured for 9 min at 94 °C, followed by 40 cycles of amplification at 94 °C for 1 min, 58 °C for 1 min, and then 72 °C for 90 s, in a 2700 Thermal Cycler (Life Technologies, Monza, Italy). Total PCR products were purified using the Qiagen PCR Purification Kit (Qiagen). To perform the cycle-sequencing reaction, PCR products were blended with each primer (0.8 mol/l) in a Terminator Ready Reaction Mix containing Big Dye Terminators (Life Technologies) denatured for 1 min at 96 °C, and then submitted to 25 cycles at 96 °C for 10 s, 57 °C for 5 s, and 60 °C for 2 min. A second purification with the DyeEx 2.0 Spin Kit (Qiagen) was performed for big dye removal. A volume of 5 ml of purified cycle-sequencing product was then analyzed using an ABI PRISM 310 Genetic Analyzer (Life Technologies). For the HRMA melting profile, the analysis was performed as reported

previously.<sup>9</sup> Briefly, after PCR, samples were denatured with an initial hold of 5 min at 95 °C and 1 min at 40 °C and analyzed with a melting profile ranging from 75 to 85 °C for *BRAF* and 77 to 87 °C for *NRAS* in RotorGene 6000 (Corbett Research, Sydney, NSW, Australia). Sequences of primers used to perform analysis are reported in Table 1.

### Sequenom Mass Spectrometry Genotyping

Discordant immunohistochemistry-negative and *NRASQ61R* mutation-positive results or discordant immunohistochemistry-positive and *NRAS* wild-type results were retested in DNA newly extracted from formalin-fixed and paraffin-embedded sections, at the Laboratory of Molecular Pathology, UO Pathological Anatomy III, Pisa, Italy, using a different molecular methodology, the Sequenom MassARRAY system (Sequenom), based on matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, together with the 'Myriapod<sup>®</sup> Colon Status' Kit (Diatech Pharmacogenetics, Jesi, Italy).

The 'Myriapod<sup>®</sup> Colon Status' Kit consists of a series of multiplexed assays designed to interrogate the hotspot mutations in four oncogenes: *KRAS*, *BRAF*, *NRAS*, and *PIK3CA*. Genomic DNA amplification and single base pair extension steps (iPLEX<sup>®</sup>) were conducted following the protocol provided by Diatech Pharmacogenetics. Briefly, the initial PCR amplification was performed in a 5  $\mu$ l reaction mixture containing 20 ng of DNA under the following conditions: 95 °C for 2 min, 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s for 45 cycles, and a final extension phase at 72 °C for 5 min. The remaining unincorporated dNTPs were dephosphorylated by adding 2  $\mu$ l of a shrimp alkaline phosphatase (SAP) cocktail. The SAP reaction was then placed in a thermal cycler under the following conditions: 37 °C for 40 min, 85 °C for 5 min, and then held at 4 °C indefinitely. After PCR cleanup, a 2  $\mu$ l of primer extension reaction cocktail (containing extension primer mixture, buffer, enzyme, and mass-modified ddNTPs) is added to the amplification products. Thermal cycling was performed under the following conditions: 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 5 cycles of 52 °C for 5 s, and 80 °C for 5 s, then at 72 °C for 3 min, and was finally held at 4 °C indefinitely. During the iPLEX<sup>®</sup> reaction, the primer is extended by one mass-modified nucleotide depending on the allele and the design of the assay. The primer extension reaction was then desalted by

**Table 1** Sequences of primers used to perform sequencing analysis

	<i>BRAF</i> exon 15 (173 bp)	<i>NRAS</i> exon 3 (162 bp)
Forward	5'-TGCTTGCTCTGATAGGAAAATG-3'	5'-CCCCTTACCCTCCACACC-3'
Reverse	5'-CCACAAAATGGATCCAGACA-3'	5'-TGGCAAATACACAGGAAGC-3'

adding 41  $\mu$ l of water and a cationic resin mixture, placed in a rotating shaker for 15 min and then centrifuged at 2000 r.p.m. for 15 min. Completed genotyping reactions were spotted in nanoliter volumes onto a matrix-arrayed silicon SpectroCHIP with 96 elements using the MassARRAY Nano-dispenser (Sequenom). SpectroCHIP was analyzed using the Sequenom MassARRAY<sup>®</sup> Analyzer 4 spectrometer and the spectra were processed by the MassARRAY Typer Analyzer 4.0 software (Sequenom). All automated system mutation calls were confirmed by manual review of the spectra.

### Immunohistochemistry on Human Melanoma Tissues

As a primary antibody, we used the rabbit monoclonal anti-human N-Ras (Q61R), clone SP174 (Spring Biosciences) (provided by UCS Diagnostic). The sections were dried at 60 °C for 30 min and stained with N-Ras antibody with a dilution of 1:80 on a Ventana BenchMark ULTRA immunostainer (Ventana Medical Systems, Tucson, AZ, USA). The Ventana staining procedure included pretreatment with cell conditioner 1 (pH 8) for 64 min, followed by incubation with antibody at 37 °C for 48 min. Antibody incubation was followed by standard signal amplification with the Ventana amplifier kit, UltraWash, and counterstaining with one drop of hematoxylin for 4 min and one drop of bluing reagent for 4 min. For chromogenic detection, ultra-View Universal RED 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.

BRAF immunostaining was performed in one selected sample using the mouse monoclonal antibody anti-BRAF V600E, clone VE1 (Spring Bioscience, Pleasanton, CA) with a dilution of 1:30 (provided by UCS Diagnostics, Morlupo, Roma, Italy), according to the same protocol as described above. Anti-N-Ras (Q61R) and anti-BRAFV600E immunostaining was performed on the same tissue blocks used for mutational testing. No chromogen was detected when primary antibodies was omitted.

All immunostained slides were evaluated two times by two independent observers (DM and CS) blinded to all clinical, histopathological, and genetic data. The anti-human N-Ras (Q61R), clone SP174 antibody staining was scored as positive when the majority of viable tumor cells showed a distinctly clear cytoplasmic staining. More specifically, 60% of immunostained cells were requested to define a case as positive. The N-Ras antibody staining was scored as negative when there was no staining or weak staining of single interspersed cells. Isolated weak staining in single interspersed cells may be the result of an aspecific chromogen uptake of variably pigmented cells whose true nature (melanoma cells vs cells of histiocytic/macrophage lineage) can be difficult to assess with certainty on immunostained slides.

Discordant immunohistochemistry-negative and *NRASQ61R* mutation-positive results or discordant immunohistochemistry-positive and *NRAS* wild-type results were retested in DNA newly extracted from formalin-fixed and paraffin-embedded sections, at the Laboratory of Molecular Pathology, UO Pathological Anatomy III, Pisa, Italy, using a different molecular methodology (Sequenom mass spectrometry).

### Statistical Analysis

Sensitivity, specificity, and positive and negative predictive values were calculated for the results of immunohistochemistry to predict for *NRASQ61R* mutation. The association between negative immunohistochemistry and mutation status was analyzed using the two-tailed Fisher's exact test.

### Results

SKMel-2 melanoma cell lines carrying *NRASQ61R* mutation immunostained with the N-Ras (Q61R) antibody showed homogeneous and strong cytoplasmic staining, whereas the human melanoma cell line A375 harboring wild-type *NRAS* was negative (Figure 1).

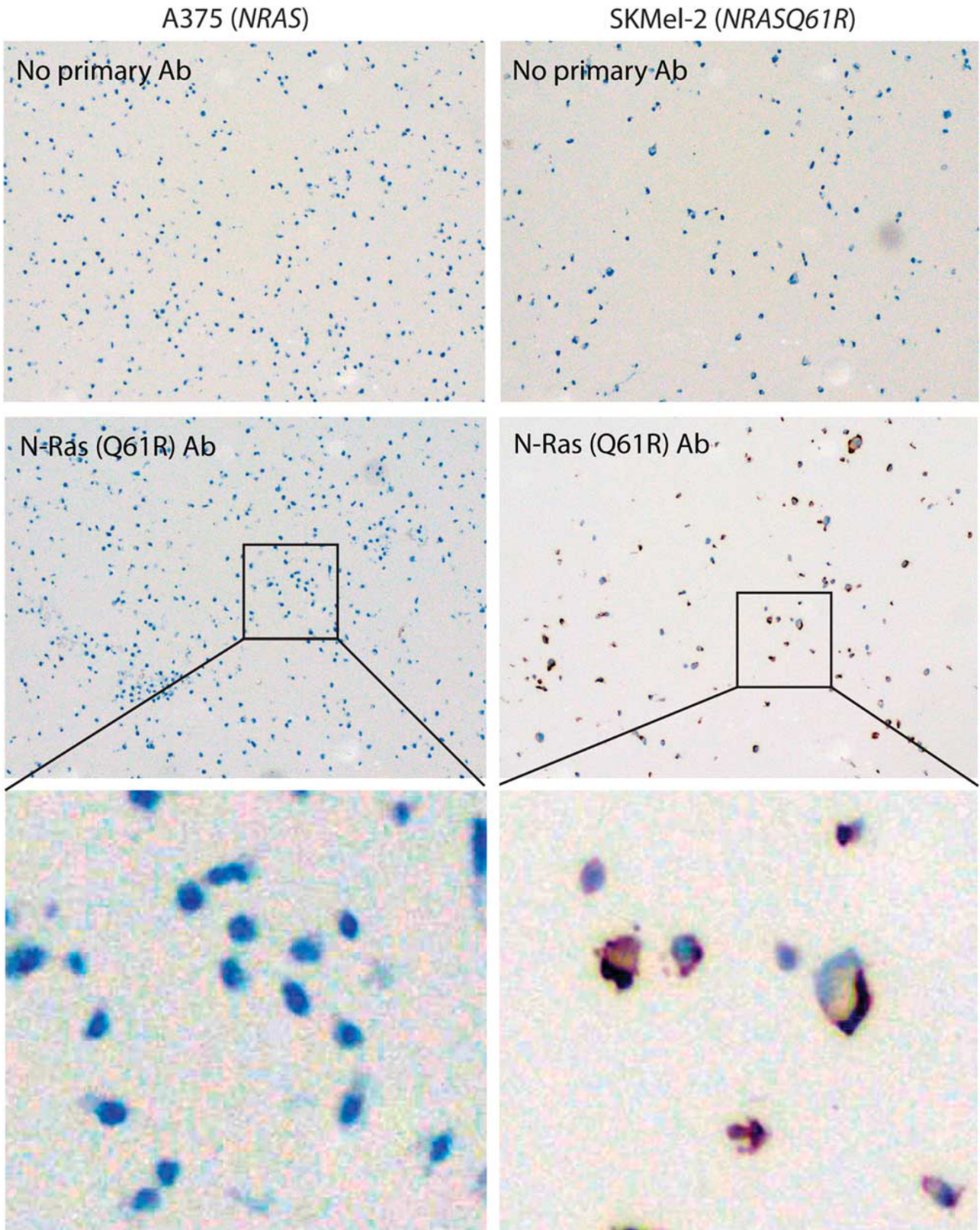
Results of the mutational analysis on human melanoma tissues are reported in Table 2. One case was excluded because it could not be successfully amplified. All the detected mutations have been previously reported and are described in the Catalog of Somatic Mutations in Cancer (COSMIC; <http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/>).

Genotype characterization for *BRAF* exon 15 was obtained for 97/98 samples. Among them, 36/97 (37%) were mutated: 32 samples (33%) showed the *p.V600E* (c.1799T>A) variant, whereas 4 samples (4%) showed the *p.V600K* (c.1798\_1799delinsAA) variant.

Mutations involving codon 61 of the *NRAS* gene were observed in 21/97 samples (22%). In particular, 14 samples (15%) showed the variant *p.Q61R* (c.182A>G), 3 samples (3%) the *p.Q61K* (c.181C>A) mutation, 2 samples (2%) the *p.Q61L* (c.182A>T), and 2 samples (2%) the *p.Q61H* mutation. Overall, *NRASQ61R* represented 67% of all *RAS* mutations.

Interestingly, a concomitant *NRASQ61R* and *BRAFV600E* mutation was found by Sanger DNA sequencing and immunohistochemistry, whereas a *KRASQ61K* mutation was detected in a *BRAF* and *NRAS* wild-type patient.

Of the 97 assessable cases, 20 cases were *NRAS* mutant on initial DNA sequencing (*NRASQ61R* = 15, *NRASQ61K* = 1, *NRASQ61L* = 2, and *NRASQ61H* = 2). Among these cases, two were immunohistochemistry-negative and *NRASQ61R* mutation-positive, whereas another case was immunohistochemistry-positive and *NRASQ61R* mutation-negative. These samples were retested on DNA extracted from subsequent sections of the same block that was used for initial sequencing



**Figure 1** SKMel-2 melanoma cells harboring mutant *NRASQ61R* stained with N-Ras (Q61R) antibody show a homogeneous and strong cytoplasmic staining, whereas human melanoma cell line A375 harboring wild-type *NRAS* was negative.

and N-Ras (Q61R) staining. Two cases were confirmed as mutated also after retesting, notwithstanding that the two methods identified two different sequence variants (*Q61K* instead of *Q61R*). The other discordant case was resequenced and an *NRASQ61R* mutation was detected instead of the initial reported *NRAS* wild-type status (Table 3).

Of the *NRAS* mutant cases, none of the non-*Q61R* stained positively with the N-Ras (Q61R) antibody, and 14 of the *NRASQ61R* mutant tumors stained positively for the N-Ras (Q61R) antibody. Of the 75 *NRAS* wild-type melanomas, 75 were negative for the N-Ras (Q61R) antibody, and none stained positively (Table 4).

Using the mutational status from the retested discordant cases, the sensitivity of the N-Ras (Q61R) antibody was 100% (14/14; 95% confidence interval: 73–99%) and the specificity was 100% (83/83; 95% confidence interval: 94–99%). The positive and negative predictive values were 100% (14/14) and 100% (83/83), respectively (Table 5).

The two independent blinded observers were concordant in the assessment of N-Ras (Q61R) antibody staining in all cases; for one melanoma with high endogenous melanin content, concor-

dance was achieved after re-evaluation at the double-headed microscope. N-Ras (Q61R) staining in melanoma cells appeared as diffusely and homogeneously moderate to strong cytoplasmic staining, with no background (Figures 2 and 3). The distribution of positivity among the positive melanoma cases was as follows: 60–80% of positive cells in 3/14 cases (21%) and 81–100% of positive cells in 11/14 (79%) cases. Intratumoral heterogeneity with N-Ras (Q61R) staining was observed only in one case (Figure 4), whereas in all other cases there was

**Table 2** Frequencies of *BRAF* and *NRAS* mutations in 97 cutaneous melanoma samples

	N (%)
<b>BRAF status</b>	
Wild type	61/97 (63)
V600 mutated	36/97 (37)
<b>BRAFV600 mutated</b>	
<i>BRAFV600E</i>	32/97 (33)
<i>BRAFV600K</i>	4/97 (4)
<b>RAS status</b>	
Wild type	75/97 (77)
<i>NRASQ61</i> mutated	21/97 (22)
<i>KRASQ61K</i> mutated	1/97 (1)
<b>NRASQ61 mutated</b>	
<i>NRASQ61R</i>	14/97 (15)
<i>NRASQ61K</i>	3/97 (3)
<i>NRASQ61L</i>	2/97 (2)
<i>NRASQ61H</i>	2/97 (2)

**Table 3** Discordant cases of genetic *NRAS* mutational analysis and N-Ras (Q61R) immunohistochemistry and reanalysis results

Initial genetic <i>NRAS</i> analysis	N-Ras (Q61R) antibody	Genetic reanalysis	Conclusion
Case 1—Wild type	Positive	<i>Q61R</i> <sup>a,b</sup>	Initial genetic testing false negative
Case 2— <i>Q61R</i>	Negative	<i>Q61K</i> <sup>a</sup>	Initial genetic testing <i>Q61R</i> false positive
Case 3— <i>Q61R</i>	Negative	<i>Q61K</i> <sup>a</sup>	Initial genetic testing <i>Q61R</i> false positive

<sup>a</sup>Retesting using Sequenom mass spectrometry analysis.

<sup>b</sup>Retesting using high-resolution melting analysis sequencing.

**Table 4** Summary of the *NRAS* mutation testing analysis and IHC with anti-human N-Ras (Q61R) monoclonal antibody (clone SP174)

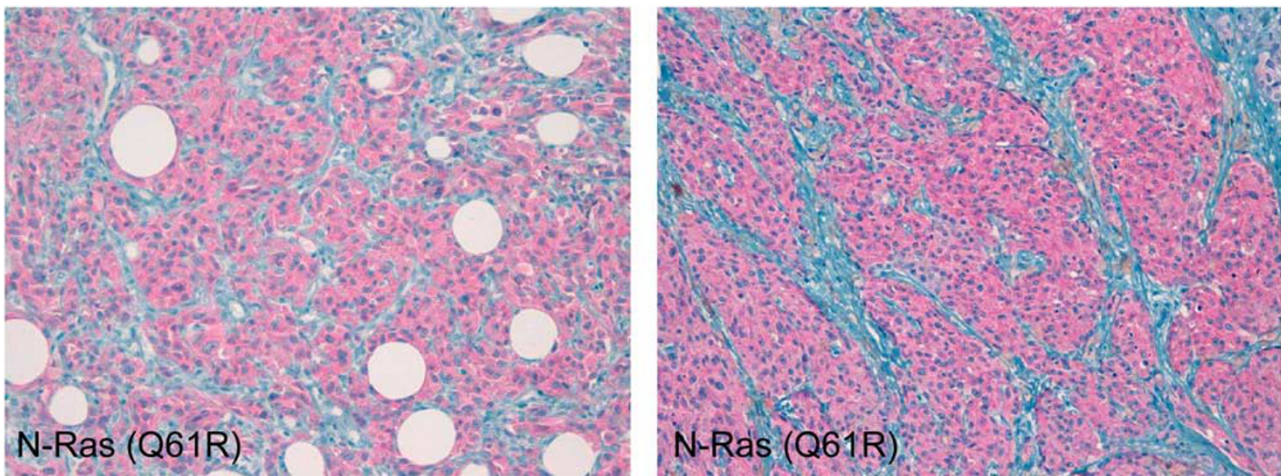
Genotype	NRAS sequencing (n)	IHC N-Ras (Q61R) positive (n)	IHC N-Ras (Q61R) negative (n)
<i>NRASQ61R</i>	14	14	0
<i>NRASQ61K</i>	3	0	3
<i>NRASQ61L</i>	2	0	2
<i>NRASQ61H</i>	2	0	2
<i>NRAS</i> wild type	75	0	75

Abbreviations: IHC, immunohistochemistry.

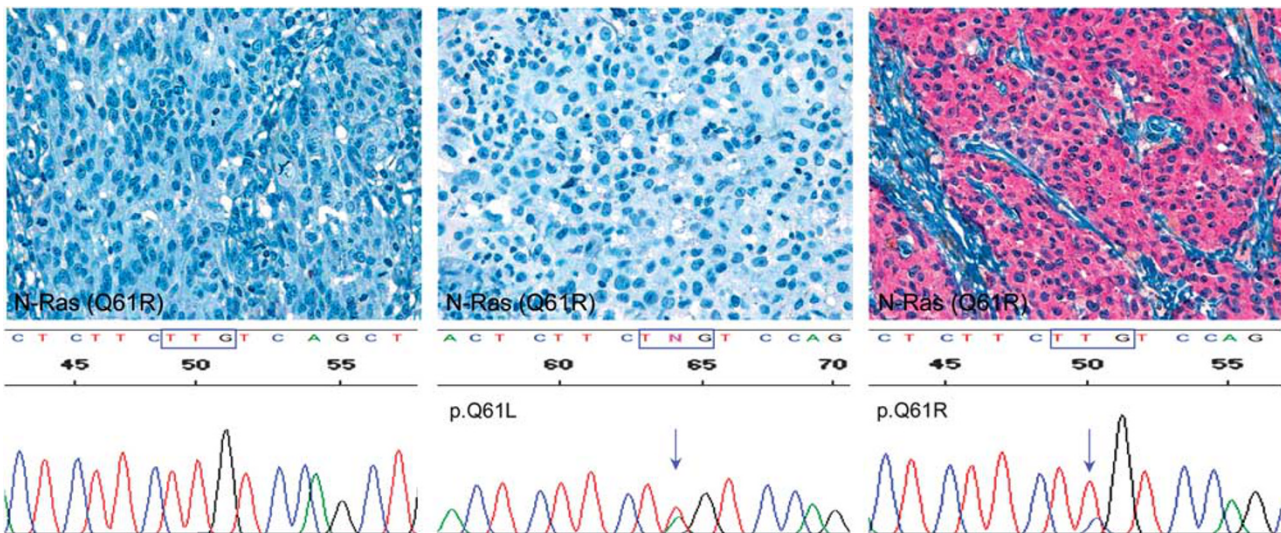
**Table 5** Frequencies of *NRASQ61R* mutation and N-Ras (Q61R) immunohistochemistry (IHC) in melanoma tissues from 97 patients

N	IHC N-Ras (Q61R) negative	IHC N-Ras (Q61R) positive	Total
Row percentage			
Column percentage			
<i>NRASQ61R</i>	83	0	83
wild-type	100.00	0.00	100.00
	100.00	0.00	85.57
<i>NRASQ61R</i> mutated	0	14	14
	0.00	100.00	100.00
	0.00	100.00	14.43
Total	83	14	97
	86	14	100.00
	100.00	100.00	100.00

Abbreviation: IHC, immunohistochemistry.



**Figure 2** N-Ras (Q61R) immunohistochemistry in melanoma cells appears as a diffuse, homogeneous cytoplasmic staining, with no background (original magnification,  $\times 20$ ).



**Figure 3** NRAS wild-type melanoma negative with the N-Ras (Q61R) antibody (left); NRASQ61L-mutated melanoma negative with the N-Ras (Q61R) antibody (middle); and NRASQ61R-mutated melanoma staining positively with the N-Ras (Q61R) antibody (original magnification,  $\times 40$ ). Electropherograms (reverse strand) of sequencing analysis on NRAS exon 3 was performed in the same samples.

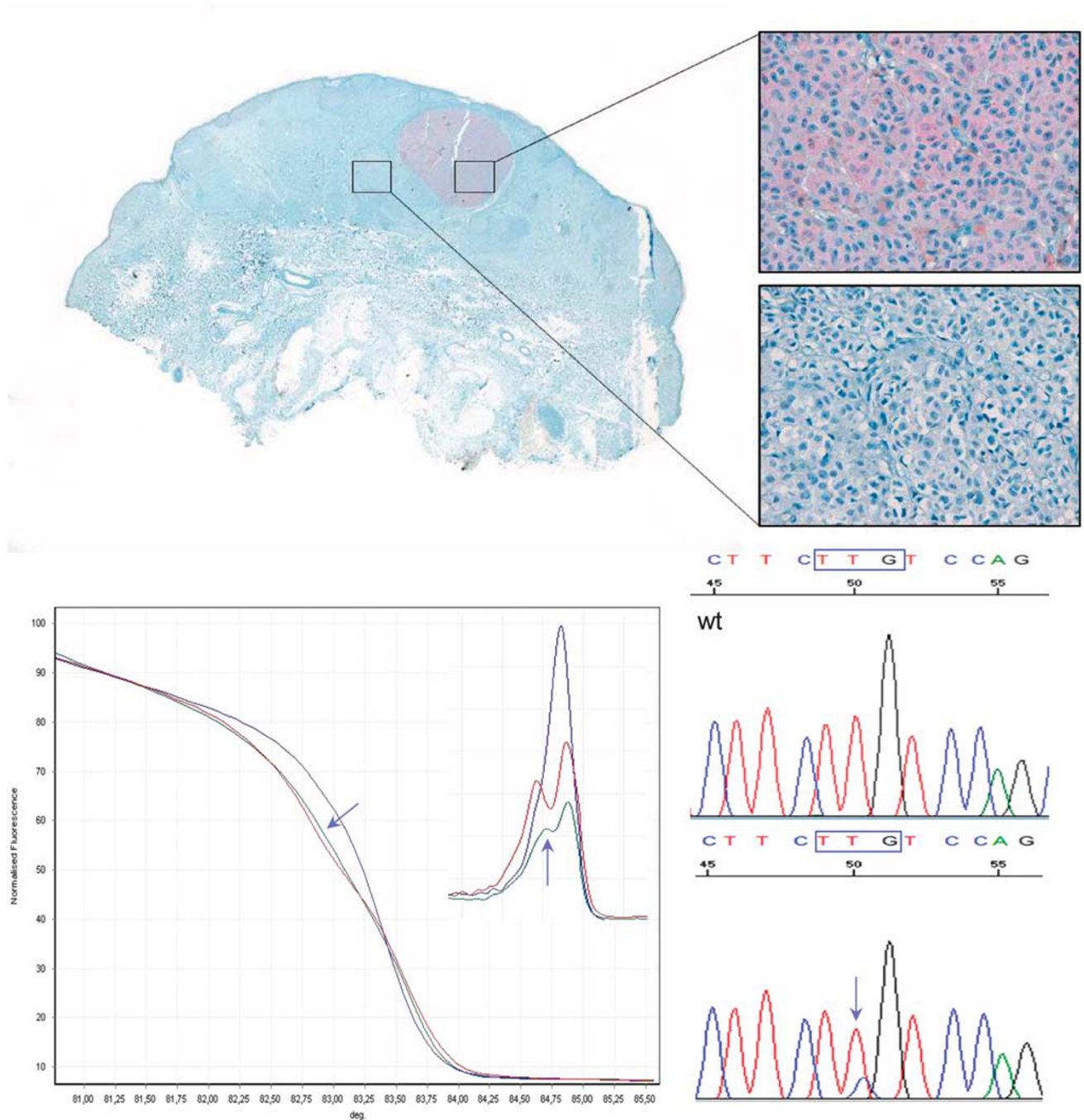
relatively uniform staining throughout the tumor. In one *BRAFV600E*- and *NRASQ61R*-mutated case, immunohistochemical staining with VE1 and N-Ras (Q61R) antibody performed on serial sections showed positivity for the two antibodies in same melanoma cell population (Figure 5).

## Discussion

Testing for *NRAS* is now integral part in the assessment of metastatic melanoma patients for at least three reasons: (1) there is evidence that *NRAS*-mutated patients may be sensitive to MEK inhibi-

tors; (2) in *BRAF*-mutated melanoma patients, a concomitant baseline mutation in the upstream *NRAS* oncogene may result in early lack of clinical benefit to *BRAF* inhibitors; (3) *RAS* mutation is a common mechanism of acquired resistance during treatment with *BRAF* inhibitors.

Furthermore, Johnson *et al*<sup>10</sup> recently reported that patients with *NRAS*-mutated metastatic melanoma achieve increased clinical benefit from immunotherapy compared with those with *BRAF/NRAS* wild-type. These data suggest that *NRAS* mutation status may be a biomarker of response to immunotherapy in metastatic melanoma and that molecularly targeted immunotherapy may be feasible.



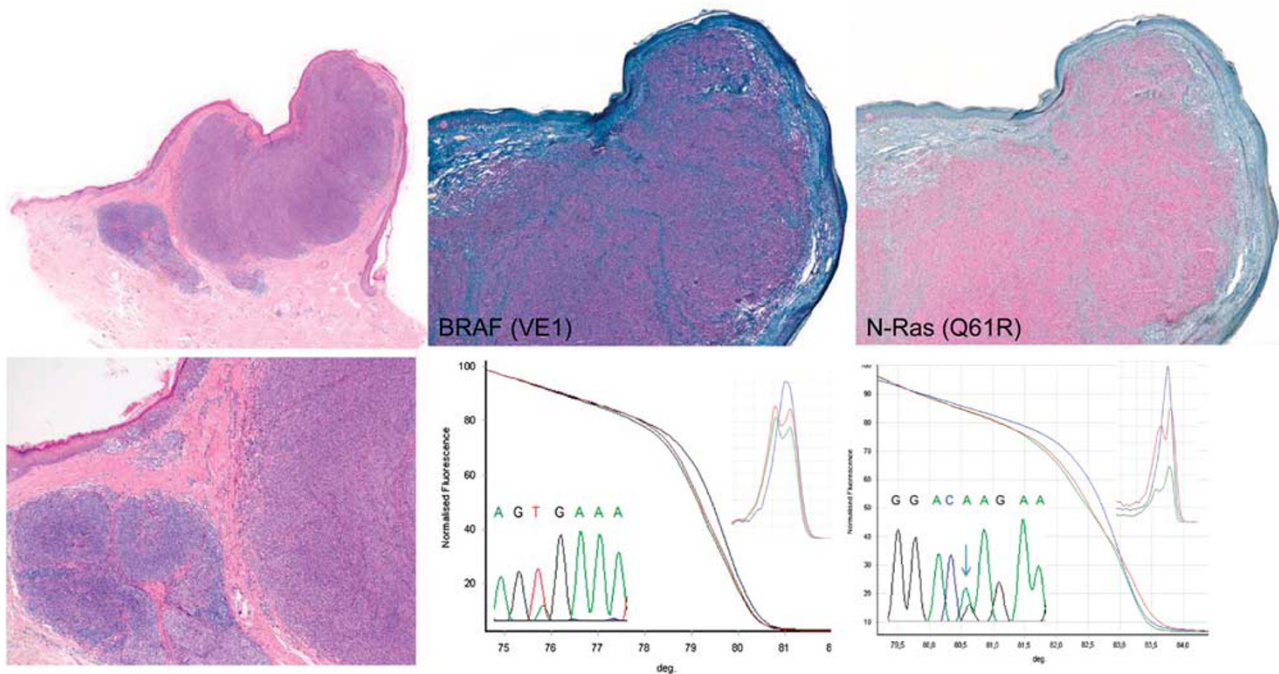
**Figure 4** Intratumoral heterogeneity shown by N-Ras (Q61R) antibody (original magnification,  $\times 2.5$ ; square boxes original magnification,  $\times 40$ ). High-resolution melting analysis of *NRAS* exon 3: wild-type (blue) and heterozygous DNA (red) were used as reference for the analysis of the sample (green). Wild-type and mutated sequence from the same sample are also shown (reverse strand).

Among *RAS* mutations, *NRASQ61R* is the most common in the majority of studies.<sup>11–15</sup> These results have been recently confirmed by a retrospective analysis in a large cohort of patients with advanced melanoma who underwent testing for both *BRAF* (exon 15) and *NRAS* (exons 1 and 2) mutations.<sup>5</sup> Finally, it is important to underline that *NRASQ61R* has been reported to correlate with

resistance to *BRAF* inhibitors in preclinical and clinical data.<sup>8,16</sup>

Because testing for *NRAS* mutations currently requires the use of molecular techniques that are not available in most diagnostic pathology laboratories, testing may require a dispendious laborious process before *NRAS* testing can commence. Furthermore, these molecular techniques are time consuming and





**Figure 5** Immunohistochemical staining for VE1 and N-Ras (Q61R) on serial sections of a human melanoma harboring double mutations *BRAFV600E* and *NRASQ61R* (original magnification, ×5); High-resolution melting analysis and sequencing results (forward strand): data obtained from *BRAF* exon 15 (left) and *NRAS* exon 3 (right) are shown. Left column: hematoxylin and eosin stain—upper original magnification, ×5; lower original magnification, ×10).

expensive to perform and may require to be confirmed by different methodological approaches. On the contrary, mutation testing with immunohistochemistry offers many potential advantages: it is timely, inexpensive, widely available in all pathology departments, and requires minimal tissue.

This study compares an immunohistochemical-based method to identify the *NRASQ61R* mutant protein with PCR and sequencing-based mutation tests in melanoma patients. Our results suggest that the N-Ras (Q61R) antibody is highly sensitive (100%) and specific (100%) for the presence of the *NRASQ61R* mutation. To the best of our knowledge, this is the first time that these results are reported in the literature.

The current study included a consecutive cohort of 98 melanoma cases initially evaluated with direct sequencing and high-resolution melting analysis, and this allowed us detection of all relevant *RAS* mutations. Furthermore, discordant cases were subjected to Sequenom mass spectrometry genotyping, which permitted an accurate analysis of the positive and negative predictive values of the N-Ras (Q61R) antibody.

Initially there were three discordant cases, but after antibody staining and retesting either by sequencing or by Sequenom mass spectrometry, the correct molecular characterization could be obtained. Notwithstanding that the comparisons of different technologies for mutation detection is not the aim of the present paper, our data suggest that a

multiple analytical approach may be required to reach the correct sample classification.

It has been reported that mutations in *RAF* and *RAS* are generally mutually exclusive. Several hypotheses have been advocated for this: *RAS* and *RAF* mutations appear functionally equivalent, or the *RAS/RAF* double mutation seems to be lethal for the cell. The immunohistochemical and molecular analyses revealed a concomitant *RAS* and *RAF* mutation in one patient from our series. The presence of both *NRAS* and *BRAFV600E* mutations in the same lesions is contrary to the current consensus that such mutations are almost always mutually exclusive in melanomas and other tumor types.<sup>17–19</sup> However, Pollock *et al*<sup>20</sup> observed concomitant *NRAS* and *BRAFV600E* mutations in 9% of nevi and suggested this might be because of different clonal nests of cells within these tumors carrying distinct mutations. In our case carrying both *NRASQ61R* and *V600E* mutations, immunohistochemistry with VE1 and N-Ras (Q61R) antibodies allowed direct visual confirmation of the *BRAFV600E*- and *NRAS*-mutated positive status in the same tumor cell population.

The consequences of concurrent mutations in *BRAF* and *RAS* in melanomas have been recently assessed only in terms of impact of these mutations on global gene expression. The definition of mutation-specific gene expression profiles indicate that the two mutations can signal not only through the common MAPK, but even by alternative signaling

pathways, and may confer tumors with distinct biological features.<sup>21</sup> It has been previously pointed out that although activating mutations coexist, they are mutually exclusive at the single-cell level.<sup>22</sup>

In the current study, intratumoral heterogeneity was seen only in one case, whereas intertumoral heterogeneity in the same patient (comparison of immunohistochemistry for *NRAS* mutational status of primary vs paired metastatic samples) was not an aim of the current study and future investigations will assess concordance of *NRAS* protein expression in multiple patient's tumors. Previous immunohistochemical analyses using BRAFV600E antibody have shown that intratumoral heterogeneity of *BRAF* mutation is not generally observed<sup>23–25</sup> and up to 100% concordance between matched primary and metastatic melanomas has been reported.<sup>24</sup> These results support the routine use of immunohistochemistry with the anti-BRAFV600E antibody as an ancillary screening tool to assess the *BRAFV600E* mutation status in melanoma patients<sup>26</sup> for treatment planning.

The strengths of this study are the following: a relative large series of patients evaluated both with immunohistochemistry and molecular analysis; the novelty of detecting the most frequently reported *NRAS* mutation in melanoma through immunohistochemical analysis; and the high sensitivity and specificity of the N-Ras (Q61R) antibody. Furthermore, in the first above-mentioned phase 2 prospective trials investigating MEK162 in *NRAS*-mutated melanoma patients, most of them actually harbored the *NRASQ61R* mutation.<sup>7</sup>

Nevertheless, we are aware that our study has limitations. This is indeed a retrospective series; as a consequence we cannot exclude a selection bias. Furthermore, as we evaluated through immunohistochemistry a relatively limited number of patients with *NRAS* mutation, our findings need to be confirmed in a prospective independent cohort. Furthermore, *NRASQ61R* does not recapitulate all *NRAS*-driven mechanisms of resistance to BRAF inhibitors, as *NRASQ61K* have also been reported to be associated with an acquired resistant phenotype.<sup>27</sup>

The focus of the current study was not on evaluating the frequency of *NRASQ61R* mutations in primary cutaneous melanoma, but on ascertaining the utility of a mutation-specific antibody. Therefore, our cases were not selected specifically to verify mutation rates. In any event, the frequency of *BRAF* and *NRASQ61R* mutations observed in the current study were 37% and 22%, respectively. The frequency of *BRAF* mutations was a little lower than expected<sup>28</sup> and this may be owing to the fact that melanomas studied were not entirely on sites with intermittent sun exposure. The frequency of *NRAS* mutation in melanoma is 15–20% but if only melanomas confined to continuously sun-exposed sites are considered, the frequency rises up significantly.<sup>29</sup>

In conclusion, the N-Ras (Q61R) antibody is highly sensitive and specific for the detection of mutant *NRASQ61R* melanoma in formalin-fixed and paraffin-embedded samples. This immunohistochemical method has several advantages: it is cost effective, uses minimal tissue, and provides a result at the time of pathologic diagnosis. Use of the N-Ras (Q61R) antibody is a valuable supplement to traditional mutation testing, and if validated in further studies, genetic testing would only be required for immunohistochemistry-negative patients to detect non-*Q61R* mutations.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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