Immunohistochemical Detection of *NRAS*Q61R Mutation in Diverse Tumor Types

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ABSTRACT

Objectives: *Testing for* NRAS *mutation at codon Q61 is of therapeutic, prognostic, and diagnostic importance for metastatic melanoma, thyroid carcinoma, and colorectal carcinoma. Immunohistochemistry for* NRAS*Q61R, the most common* NRAS *mutation, offers several practical advantages over current molecular diagnostic techniques.*

Methods: *We investigated the sensitivity and specificity of* NRAS*Q61R in a series of 149 tumors with known* NRAS *genotype (72 malignant melanomas, 13 melanocytic nevi, 28 thyroid carcinomas, 25 gastrointestinal carcinomas, and 11 other malignancies).*

Results: *Thirty-five cases harbored the* NRAS*Q61R mutation (19 malignant melanomas, one melanocytic nevus, 10 thyroid carcinomas, two gastrointestinal carcinomas, and three other malignancies). In this series, the concordance rate between immunohistochemistry and mutational analyses was 100%. The sensitivity and specificity were 100% and 100%, respectively. However, lower staining intensity was observed for thyroid carcinomas in comparison to melanomas and other tumors.*

Conclusions: *Our studies confirmed that immunohistochemistry provides excellent sensitivity and specificity for detecting the* NRAS*Q61R mutation in a variety of tumor types in a clinical setting.*

CME/SAM

Upon completion of this activity you will be able to:
- recognize that *NRAS* mutation is commonly seen in a variety of malignancies, including malignant melanoma, thyroid carcinoma, colorectal carcinoma, and others.
- recognize that immunohistochemistry is a cost-effective means to screen for *NRASQ61R* mutation, which can have therapeutic, prognostic, and diagnostic implications.

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Testing for *NRAS* mutation at codon Q61 is of therapeutic, prognostic, and diagnostic importance for metastatic melanoma, thyroid carcinoma, and colorectal carcinoma. In melanomas, acquired resistance to *BRAF* inhibition can be mediated by *NRAS* mutations, most often Q61R and Q61K.[1] In a recent phase II clinical trial, the MEK inhibitor MEK162 has shown clinical benefit for *NRAS* mutant melanoma, most being *NRAS*Q61R.[2] In addition, a combination of CDK4/6 (cyclin-dependent kinase 4) inhibitor, LEE011, with MEK162 is currently being evaluated in a phase Ib/II clinical trial for *NRAS*-mutated melanoma.[3] On the contrary, in metastatic colorectal cancer, *NRAS* mutations are associated with poor prognosis and lack of response to anti–epidermal growth factor

receptors (EGFRs).[4] Similarly, recent meta-analysis showed that *KRAS*, *NRAS*, *BRAF*, and *PIK3CA* mutations predict resistance to anti-EGFR therapies.[5] *NRAS* codon 61 mutation has recently been reported to be a predictive marker for distant metastasis in follicular thyroid carcinoma.[6,7] It also might serve as a diagnostic marker in thyroid pathology.[8]

*NRAS*Q61R is the most prevalent activating mutation in *NRAS*, and immunohistochemistry for this variant, made recently possible by the availability of a specific antibody, offers several practical advantages over current molecular diagnostic techniques, including more rapid analysis, less expense, greater tissue conservation, and wider laboratory availability. Furthermore, immunohistochemistry may allow for the detection of *NRAS*Q61R in small tumor foci and for the assessment of intratumoral heterogeneity for the *NRAS* genotype, observed in previous studies, without the need for the laborious and costly microdissection required for a molecular-based approach.[9,10] Although a monoclonal antibody has recently been shown to be highly sensitive and specific for the immunohistochemical detection of *NRAS*Q61R in malignant melanomas with this specific mutation,[9,10] a practical comparison of its performance in the clinical setting for various tumor types has not been done. We aim to evaluate its sensitivity and specificity in the detection of *NRAS*Q61R mutation in melanomas, thyroid carcinomas, colorectal carcinomas, and other tumors.

Materials and Methods

Archival, formalin-fixed, and paraffin-embedded materials of tumors submitted for SNaPshot (Applied Biosystems, Waltham, MA) genotyping assay at the Molecular Diagnostic Laboratory, Massachusetts General Hospital, Boston, Massachusetts, between August 2010 and December 2014 were retrieved from the pathology files. Whenever possible, the same tumor block on which molecular testing performed was selected for immunohistochemical studies. Thirteen cases of melanocytic nevi with known *NRAS* genotype have been published previously.[11]

Mutational Analysis

The SNaPshot genotyping assay and a recently developed targeted next-generation sequencing approach have been used for mutation detection in formalin-fixed, paraffin-embedded tumor tissue.[12,13]

Immunohistochemistry

Immunohistochemical studies were performed on 5-µm-thick sections of formalin-fixed, paraffin-embedded tissue in a Bond 3 automated immunostainer (Leica Microsystems, Bannockburn, IL) and primary antibodies against *NRAS*Q61R mutation (clone: SP174, 1:50, Spring Bioscience, Pleasanton, CA). Appropriate positive and negative controls were included. Positive staining was characterized by diffuse cytoplasmic with some membranous staining of the tumor cells. A malignant melanoma with a known *NRAS*Q61R mutation was used as positive control. Diffuse staining in most of the tumor cells was noted in the positive cases. The intensity of staining was graded as strong (3+), moderate (2+), or weak (1+). No significant background staining was seen in the negative cases.

Results

The study included a total of 159 specimens. Of the 149 specimens with known *NRAS* genotype, there were 72 cases of malignant melanoma (20 primary and 52 metastatatic melanomas), 13 melanocytic nevi, 25 cases of gastrointestinal carcinomas (14 primary and 11 metastatic carcinomas), 28 cases of thyroid carcinomas (23 primary and five metastatic carcinomas), six lung carcinomas (three primary, three metastatic), and five miscellaneous tumors (one transformed mycosis fungoides, one plasmacytoma, one plasmacytoid dendritic cell neoplasm, two ovarian serous carcinomas) **Table 1**. In addition, archival materials without known *NRAS* genotype of corresponding primary or metastatic tumors from 10 patients were included to assess intertumoral homogeneity.

Concordance Between Immunohistochemistry and Mutational Analyses

The overall concordance rate between protein expression and mutation was 100% **Table 2**. The sensitivity and specificity were 100% and 100%, respectively.

Nineteen malignant melanomas with *NRAS*Q61R mutation were positive (15 with 3 + and four with 2+), while the remaining 53 melanomas (10 with Q61K, four with Q61L, one with Q61H, one with G12D, and 37 wild type) were all negative **Image 1A** and **Image 1B**. One congenital nevus with *NRAS*Q61R mutation was 2 + positive, while the remaining 12 nevi (one with Q61K, two with G13D, and nine wild type) were negative. Two metastatic colonic adenocarcinomas harboring the *NRAS*Q61R mutation were both positive (3+), and the remaining 23 gastrointestinal carcinomas (two with Q61K, one with G12A, one with G12C, two with G12D, one with G13D, and 16 wild type) were negative **Image 1C** and **Image 1D**. Ten thyroid carcinomas with *NRAS*Q61R mutation were all positive (one with 2+ and nine with 1+) **Image 1E** and **Image 1F**, and the remaining 18 thyroid carcinomas (two with Q61K and 16 wild type) were negative. One transformed mycosis fungoides case, one metastatic lung squamous cell carcinoma, and one metastatic ovarian serous carcinoma mutant with *NRAS*Q61R were all 2+, 2+, and 3 + positive, respectively.

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Table 1
Summary of Studied Cases

Tumor Type	*NRAS* Mutation Type	No. of Cases
Malignant melanoma	Q61R (c.182A > G)	7 primary melanomas, 12 metastatic melanomas
	G61L (c.182A > T)	4 metastatic melanoma
	G61K (c.181C > A)	2 recurrent melanomas, 8 metastatic melanomas
	Q61H (c.183A > T)	1 primary melanoma
	G12D (c.35G > A)	1 nasal melanoma
	Wild type	9 primary melanomas, 28 metastatic melanomas
Melanocytic nevi	Q61R (c.182A > G)	1 congenital nevus
	Q61K (c.181C > A)	1 congenital nevus
	G13D (c.38G > A)	2 congenital nevi
	Wild type	9 nevi
Thyroid carcinoma	Q61R (c.182A > G)	8 carcinomas (5 papillary, 3 follicular); 2 metastatic carcinoma to bone (1 papillary, 1 follicular)
	Q61K (c.181C > A)	1 dedifferentiated carcinoma, 1 papillary carcinoma
	Wild type	13 carcinomas (9 papillary, 4 follicular); 3 metastatic papillary thyroid carcinomas
Gastrointestinal carcinoma	Q61R (c.182A > G)	2 metastatic colonic adenocarcinomas
	Q61K (c.181C > A)	1 colonic adenocarcinoma, 1 metastatatic colonic adenocarcinoma
	G12A (c.35G > C)	1 metastatic colonic adenocarcinoma
	G12C (c.34G > T)	1 colonic adenocarcinoma
	G12D (c.35G > A)	2 metastatic colonic adenocaricnomas
	G13D (c.38G > A)	1 metastatic colonic adenocarcinoma
	Wild type	12 carcinomas (10 colonic, 2 small intestinal); 4 metastatic colonic adenocarcinomas
Hematolymphoid	Q61R (c.182A > G)	1 transformed mycosis fungoides
	Q61K (c.181C > A)	1 plasmacytoma, 1 plasmacytoid dendritic cell neoplasm
Lung carcinoma	Q61R (c.182A > G)	1 metastatic squamous cell carcinoma to lymph node
	Q61K (c.181C > A)	1 metastatic adenocarcinoma
	Q61L (c.182A > T)	3 carcinomas (2 adenocarcinoma, 1 metastatic)
	G12C (c.34G > T)	1 squamous cell carcinoma
Ovarian carcinoma	Q61R (c.182A > G)	1 metastatic ovarian serous carcinoma
	Q61L (c.182A > T)	1 ovarian serous carcinoma

Table 2
Correlation of Monoclonal *NRAS*Q61R Staining With Mutational Results

	*NRAS*Q61R Mutation		
Characteristic	**Positive**	**Negative**	**Total**
IHC, No.			
Positive	35	0	35
Negative	0	114	114
Total	35	114	149
Concordance, No. (%)			
Sensitivity	35 (100)		
Specificity		114 (100)	

Concordance Between Primary and Metastasis of the Same Patient

Additional archival materials of primary or metastatic tumors were available in 10 patients with specimens with known *NRAS* genotype. The mutational analyses were not done on these corresponding samples. With the exception of one case, there was high concordance of *NRAS*Q61R expression in both tumor specimens from the same patient Table 3.

Discussion

KRAS, *BRAF*, or *NRAS* mutations can activate the RAS-RAF-MAPK pathway, which is important for proliferation. Activating *KRAS*, *HRAS*, or *NRAS* mutations at codons 12, 13, or 61 can be seen in one-third of cancers.[14] RAS proteins function as guanosine triphosphatase (GTPase) switches. Mutations at codons 12 and 13 render RAS proteins insensitive to GTPase-activating proteins,[15] and mutations at codon Q61 block the return of RAS to an inactive guanosine diphosphate–bound state.[16] Similar to *BRAF* mutations, *NRAS* mutations are driver mutations on which a tumor is dependent for proliferation.[3] Approximately 15% to 25% of metastatic melanomas possess *NRAS* (neuroblastoma RAS viral oncogene homolog) mutations, and they are localized to codon 61 in 82%, codon 12 in 13%, and codon 13 in 5% of cases.[17] Preference for codon 61 mutations is also observed in thyroid carcinomas.[18,19] *RAS* mutations are the second most common genetic changes in thyroid tumors seen in 10% to 20% of papillary thyroid carcinomas and 40% to 50% of follicular thyroid carcinomas.[19] Mutations of codon 61 can be seen in 67% of *NRAS*-mutated follicular

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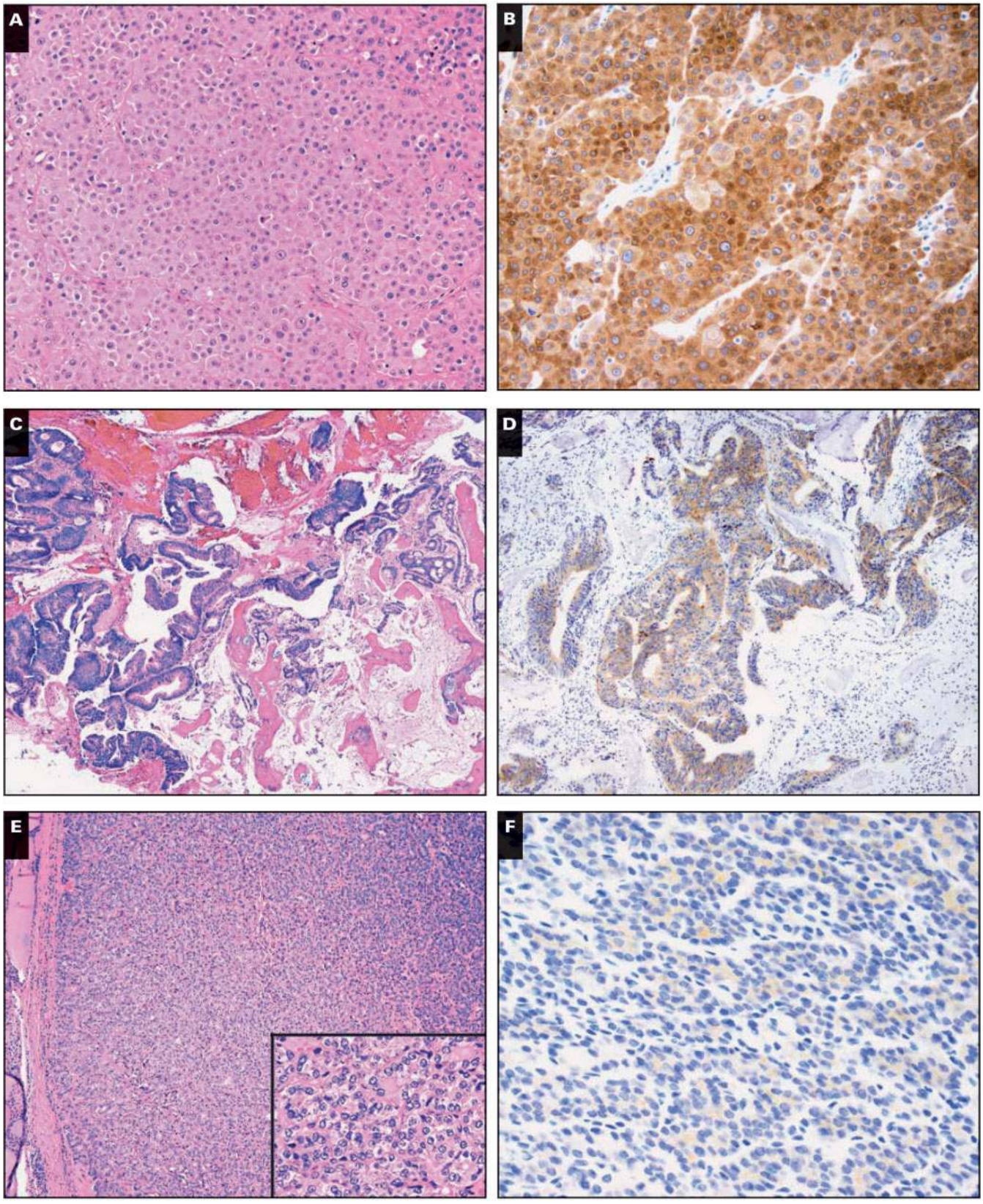

Image 1 Strong and diffuse cytoplasmic expression of *NRAS*Q61R is seen in representative metastatic melanoma in soft tissue (**A**, ×200; **B**, ×200) and metastatic colonic adenocarcinoma to bone (**C**, ×100, **D**, ×100), while only weak cytoplasmic expression is seen in a follicular thyroid carcinoma (**E**, ×100, inset ×400, **F**, ×400).

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Table 3
***NRAS*Q61R Staining in Primary and Corresponding Metastasis From the Same Patient[a]**

Characteristic	With *NRAS* Mutational Analyses	*NRAS*Q61R Staining	Other Tumor in Same Patient Without *NRAS* Mutational Analyses	*NRAS*Q61R Staining
Melanoma with *NRAS*Q61R mutation	Primary	Positive	Primary	Positive
	Primary	Positive	Metastasis	Positive
	Primary	Positive	Metastasis	Positive
	Metastasis	Positive	Metastasis	Positive
	Metastasis	Positive	Primary	Positive
	Metastasis	Positive	Primary	Negative
Melanoma with *NRAS*Q61K mutation	Metastasis	Negative	Metastasis	Negative
	Recurrence	Negative	Metastasis	Negative
	Metastasis	Negative	Metastasis	Negative
Thyroid carcinoma with *NRAS*Q61R mutation	Metastasis	Positive	Primary	Positive

[a]The cases listed in the second column are with known *NRAS* genotyping, while those listed in fourth column did not have *NRAS* genotyping done.

carcinomas.[7] *NRAS*-mutated thyroid cancers are often a follicular variant of papillary thyroid carcinoma.[8,18]

In this study, we did not optimize an individual protocol for each tumor type but rather used one uniform protocol. We observed less staining intensity for thyroid carcinomas in comparison to melanoma cases and gastrointestinal and lung carcinomas. It is unclear why there was different staining intensity noted in different tumor types in our study. It could be due to the variability in protein expression in different tumor types, as noted for VE1 antibody, which better detects *BRAF*V600E mutation in melanoma and papillary thyroid carcinoma than colorectal carcinoma.[20] We showed here that the immunohistochemistry analysis for *NRAS*Q61R is a technically less challenging, more rapid, and cost-effective alternative to molecular testing.

Mutations at codon 61 and codon 12 are commonly seen in melanoma, thyroid cancer, and colorectal carcinoma. *NRAS*Q61R is the most common *NRAS* mutation found in melanoma seen in approximately 35% of *NRAS*-mutated melanomas.[21] It is also found in follicular carcinoma and follicular variant of papillary thyroid carcinoma.[18] Thirty-six cases with seven different *NRAS* variant mutations other than Q61R (Q61L, Q61K, Q61H, G12A, G12C, G12D, and G13D) were included in this study, and all cases were negative for *NRAS*Q61R immunostain. *NRAS*Q61K is a commonly recurring *NRAS* mutation seen in approximately 34% of *NRAS*-mutated melanomas and in hereditary melanoma.[21] Both mutations can be seen in congenital melanocytic nevi.[22] The Q61L and Q61H mutations are seen in approximately 8% and 2% of *NRAS*-mutated melanomas, respectively. The G12D mutation is the third most common *NRAS* mutation, which is a recurring mutation in colorectal carcinoma[23] and melanoma.[17]

High concordance between matched primary and metastatic melanomas has been reported for BRAFV600E antibody.[24] Similarly, intertumoral homogeneity for melanoma and thyroid carcinoma was noted for the *NRAS*Q61R antibody in this study (Table 3). Although future confirmation with additional cases will be valuable, there appears to be no need for additional biopsy provided there is a specimen with adequate tumor cellularity. These findings are supportive of the fact that driver mutations, including oncogenic *NRAS* and *BRAF* mutations, are typically somatically preserved during progression.

As the role of targeted therapy and therapy-guiding *NRAS* mutational analysis expands to include a spectrum of malignancies beyond melanoma, *NRAS*Q61R immunostain offers timely clinical diagnosis of *NRAS*Q61R expression in malignancies of various sites and tumor types. Our results confirmed that a monoclonal antibody provides excellent sensitivity and specificity for detecting the *NRAS*Q61R mutation in a variety of tumor types, for primary as well as metastatic tumors.

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