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Clinical Cancer Research

Clinical Correlates of *NRAS* and *BRAF* Mutations in Primary Human Melanoma

Julie A. Ellerhorst[1], Victoria R. Greene[1], Suhendan Ekmekcioglu[1], Carla L. Warneke[2], Marcella M. Johnson[2], Carolyn P. Cooke[1], Li-E Wang[3], Victor G. Prieto[4], Jeffrey E. Gershenwald[5], Qingyi Wei[3], and Elizabeth A. Grimm[1]

Abstract

Purpose: *NRAS* and *BRAF* mutations are common in cutaneous melanomas, although rarely detected mutually in the same tumor. Distinct clinical correlates of these mutations have not been described, despite *in vitro* data suggesting enhanced oncogenic effects. This study was designed to test the hypothesis that primary human cutaneous melanomas harboring mutations in *NRAS* or *BRAF* display a more aggressive clinical phenotype than tumors wild type at both loci.

Experimental Design: Microdissection of 223 primary melanomas was carried out, followed by determination of the *NRAS* and *BRAF* mutational status. Genotypic findings were correlated with features known to influence tumor behavior including age, gender, Breslow depth, Clark level, mitotic rate, the presence of ulceration, and American Joint Committee on Cancer (AJCC) staging.

Results: Breslow depth and Clark level varied significantly among the genotypes, with *NRAS* mutants showing the deepest levels and wild-type tumors the least depth. Ulceration also differed significantly among the genotypes, with *BRAF* mutants demonstrating the highest rate. In addition, tumors with mutated *NRAS* were more likely to be located on the extremities. Patients whose tumors carried either mutation presented with more advanced AJCC stages compared with patients with wild-type tumors, and specifically, were more likely to have stage III disease at diagnosis. Overall survival did not differ among the 3 groups.

Conclusions: Distinct clinical phenotypes exist for melanomas bearing *NRAS* and *BRAF* mutations, whether considered together or separately, and are associated with features known to predict aggressive tumor behavior. The impact of these mutations is most evident at earlier stages of disease progression.
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Introduction

Cutaneous melanomas have long been known to harbor activating mutations of the *NRAS* gene in 15% to 20% of primary tumors (1). Although definitive clinical correlates of mutated *NRAS* have not been established in the melanoma patient population, at least 1 study has suggested aggressive behavior of this melanoma subset (2). The most common site of mutation is codon 61 in exon 2, although exon 1 mutations are occasionally found (1–3). The codon 61 mutations are heterogeneous, with C181A (Q61K) and A182G (Q61R) being found most frequently. There is *in vitro* evidence to suggest that codon 61 mutations result in prolongation of the GTP-bound state of N-Ras, leading to enhanced signaling through growth promoting pathways such as the mitogen-activated protein kinase (MAPK) pathway (4).

In this context, considerable excitement was generated some years ago when activating mutations of *BRAF*, which encodes the kinase immediately downstream of N-Ras, were reported in 60% of cultured melanoma cell lines and 50% of primary human tumors (5, 6). These mutations for the most part consist of T1799A (V600E) in exon 15 and are rarely found in melanomas bearing *NRAS* mutations. Contrary to expectations, clinical studies have been largely unsuccessful in demonstrating a clear phenotype associated with mutated *BRAF* (7–10). Adding to the debate surrounding the clinical relevance of *BRAF* mutations in primary melanoma is the observation that these mutations occur in benign nevi at a similar or greater frequency (11).

In an attempt to further clarify the impact of mutated *NRAS* and *BRAF* on clinical melanoma behavior, we

Authors' Affiliations: Departments of [1]Experimental Therapeutics, [2]Biostatistics, [3]Epidemiology, [4]Pathology and Dermatology, and [5]Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

Corresponding Author: Julie Ellerhorst, Department of Experimental Therapeutics, Unit 362, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd. Houston, TX 77030. Phone: 713-792-8990; Fax: 713-563-3531; E-mail: jaellerh@mdanderson.org.

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Translational Relevance

In this study, we present evidence for aggressive behavior of cutaneous melanomas bearing the common mutations of *NRAS* or *BRAF* when compared with tumors wild type at both loci. The translational significance of these findings are 2-fold. First, our data provide clinical support for drug development targeting the mutated forms of these important oncogenic signaling molecules. Second, with the rising frequency of *NRAS* and *BRAF* mutational analysis in the clinical setting, our findings support a more conservative approach to wild-type primary tumors, particularly in the case of patients at risk for complications of sentinel node biopsy or general anesthesia, or patients generally reluctant to undergo invasive procedures.

determined the mutational status of 223 primary tumors and correlated these findings with pathologic and clinical data. Here, we report that *NRAS*- and *BRAF*-mutated tumors are more invasive than tumors wild type at both loci. Furthermore, patients with tumors carrying either mutation are more likely to present with stage III disease.

Materials and Methods

Study population

The study was approved by The University of Texas M. D. Anderson Cancer Center (MDACC) Institutional Review Board and conducted according to the Health Insurance Portability and Accountability Act (HIPAA) guidelines. A written informed consent was obtained from all subjects. Tumor samples consisted of consecutive entries into the MDACC Melanoma Informatics, Tissue Resource, and Pathology Core (Melanoma Tumor Bank) as part of a larger MDACC Melanoma Specialized Program of Research Excellence (SPORE) project.

DNA extraction and amplification

For each primary tumor, three 5 μm thick, formalin-fixed, paraffin-embedded tissue sections were prepared for laser capture microdissection (LCM) as previously described (12). Microdissection of tumor cells was performed by a board-certified pathologist (VRG) using the PixCell II Laser Capture Microdissection System (Arcturus). Dissected cells were incubated for 72 hours at 37°C in 50 to 100 μL of lysis buffer (10 mmol/L of Tris-HCl, pH = 8.0; 1% Tween-20; 1 mmol/L of EDTA; and 0.04% proteinase K). After a 5-minute high-speed centrifugation, the sample was heated to 95°C for 8 minutes to inactivate the proteinase K. PCR was performed using the GeneAmp Gold PCR Reagent Kit (Applied Biosystems). Primers and conditions for *NRAS* exon 2 and *BRAF* exon 15 amplification (Sigma Genosys) are listed below (5, 13):

NRAS exon 2: Forward: 5′-CCCCTTACCCTCCACAC-3′
Reverse: 5′-AGGTTAATATCCGCAAATGAC-3′
95°C 45 seconds, 55°C 45 seconds, 72°C 60 seconds; 40 cycles; magnesium chloride 1.5 mmol/L

BRAF exon 15 Forward: 5′-TCATAATGCTTGCTCTGA TAGGA-3′
Reverse: 5′-GGCCAAAAATTTAATCAGTGGA-3′
94°C 30 seconds, 57°C 60 seconds, 72°C 60 seconds; 40 cycles; magnesium chloride 1.5 mmol/L

PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Sequencing of the PCR products in both directions was performed by the MDACC DNA Core Facility using an ABI Prism 3100 DNA Genetic Analyzer (Applied Biosystems) using Big Dye v.3.1 dye terminator chemistry (Applied Biosystems). Chromatogram results were interpreted independently by 2 reviewers (JAE and VRG). All mutations were identified on both strands.

Statistical analysis

Associations with mutation types were evaluated using the 3 genotypes (mutated *NRAS*, mutated *BRAF*, and both wild type), as well as mutational status collapsed into 2 categories (either mutation vs. no mutation). Fisher's exact test was used to examine associations between mutation status and the following factors: patient gender, Breslow thickness $\leq$ 1 mm, Clark level, ulceration, and American Joint Committee on Cancer (AJCC) staging. The associations between genotype and ordinal variables (patient age, Breslow depth, and mitotic rate) were determined using the Kruskal–Wallis test. Overall survival was computed from the date of pathologic diagnosis until the date of death. Patients alive at the end of the study period were censored at the date of last follow-up. Survival curves were constructed using the Kaplan–Meier method and the log-rank test was used to evaluate equality across strata. Associations between independent variables and survival were further investigated using Cox proportional hazards regression models. All P values were 2-sided and P values < 0.05 were considered statistically significant. Analyses were conducted using SAS for Windows (release 9.1, SAS Institute).

Results

Patient population

A total of 297 primary melanomas were microdissected, representing consecutive entries into the MDACC Melanoma Tumor Bank. Of this initial set, sequences for both *BRAF* exon 15 and *NRAS* exon 2 were successfully determined in 223 cases. The remaining 74 samples were excluded from analysis for the following reasons: one or both exons could not be adequately amplified or

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sequenced (39 cases); the material remaining in the tissue block did not show invasive melanoma (21 cases); the tumor was too small for microdissection (14 cases).

On the basis of the results of PCR and sequencing, each of the 223 cases was assigned one of the following genotypic designations: mutated *NRAS* (MN), 31 patients (13.9%); mutated *BRAF* (MB), 109 patients (48.9%); wild type at both loci (WW), 80 patients (35.9%); and mutated at both loci (MM), 3 patients, (1.3%). For analytical purposes, the 3 MM cases were examined separately, such that the final analysis included 220 subjects. The study population consisted of 118 males and 102 females, with a median age of 49 years (range = 18–77 years). The distribution of histologic types included superficial spreading 70%, nodular 19%, lentigo maligna 4%, and unclassified 7%. The rates of specific mutations are shown in Table 1.

Table 1. Summary of *BRAF* and *NRAS* mutations

	DNA mutation	Amino acid mutation	*n* (%)
BRAF ($n = 109$)	T1799A	V600E	93 (85.3)
	GT1798AA	V600K	9 (8.3)
	A1801G	K601E	2 (1.8)
	TG1799AA	V600E	2 (1.8)
	T1790A	L597Q	1 (0.9)
	T1785A	F595L	1 (0.9)
	T1799A, C1834G	V600E, Q612E	1 (0.9)
NRAS ($n = 31$)	C181A	Q61K	15 (48.4)
	A182G	Q61R	11 (35.5)
	A182T	Q61L	3 (9.7)
	AA182TG	Q61L	1 (3.2)
	C181A, A183G	Q61K	1 (3.2)

Association of mutational status with tumor histopathology

To test the hypothesis that mutation-bearing tumors display a more aggressive clinical phenotype, various relevant patient characteristics and histologic features were compared among the 3 genotypes. These included age, gender, Breslow depth, Clark level, mitotic rate, and the presence of ulceration (Table 2). Results show that the median Breslow depth varied significantly among the 3 genotypes, with MN tumors showing the deepest invasion (1.40 mm) and WW the least invasion (0.93 mm), whereas MB tumors were intermediate (1.28 mm; $P = 0.006$). The same pattern was seen if Breslow depth was examined at a cutoff of 1 mm ($P = 0.021$). Clark levels followed suit, with mutated and WW tumors showing the most and least aggressive patterns, respectively ($P < 0.001$). Ulceration also differed significantly among the genotypes, with MB tumors demonstrating the highest rate, whereas rates for MN and WW were considerably lower and similar to each other ($P = 0.045$). The 3 genotypes did not differ in terms of gender, age, or mitotic index.

Association of mutational status with patient staging and outcomes

All patients were assigned a pathologic stage according to 2009 AJCC criteria (14). As shown in Table 3, there was a trend for patients with either mutation to present with a higher stage than those with wild type tumors ($P = 0.104$). If the MN and MB categories were collapsed, these findings reached significance ($P = 0.027$). In particular, 52 subjects presented with stage III disease and patients in this subset were more likely to have mutated tumors ($P = 0.031$ for MN or MB vs. WW). As further seen in Table 3, the majority

Table 2. Association of mutational genotype with histopathologic findings

	MN	MB	WW	*P*
n	31	109	80	
Median age, y (range)	51 (19–75)	48 (19–77)	49.5 (18–73)	n.s.
Gender, % female	48	45	48	n.s.
Median Breslow, mm (range)	1.40 (0.49–16.00)	1.28 (0.22–11.00)	0.93 (0.11–10.00)	0.006
Breslow $\leq$ 1 mm, %	9 (29.0)	45 (41.7)	45 (56.3)	0.021
Clark level, %				
2	1 (3.2)	12 (11.0)	21 (26.3)	
3	15 (48.4)	42 (38.5)	38 (47.5)	
4	13 (41.9)	55 (50.5)	21 (26.3)	
5	2 (6.5)	0 (0.0)	0 (0.0)	<0.001
Median mitotic figures/mm^2 (range)	1 (0–20)	2 (0–18)	1 (0–43)	n.s.
Ulceration present, %	3 (9.7)	24 (22.4)	8 (10.1)	0.045

Abbreviation: n.s., not significant.

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Table 3. Stage at presentation by genotype, with stage III subcategories

	AJCC stage			Stage III subcategory		
	I	II	III	IIIA	IIIB	IIIC
MN	18 (58.1)	5 (16.1)	8 (25.8)	4 (50.0)	2 (25.0)	2 (25.0)
MB	57 (52.3)	20 (18.4)	32 (29.4)	17 (53.1)	4 (12.5)	11 (34.4)
WW	57 (71.3)	11 (13.8)	12 (15.0)	7 (58.3)	4 (33.3)	1 (8.3)

NOTE: Values given are number (%).

of stage IIIC tumors, that is those with larger volume nodal disease, carried one or the other mutation (findings not significant). In spite of this, there was no difference in survival among the stage III patients when stratified according to the presence or absence of mutation (Fig. 1). Furthermore, with a median follow-up of 39 months, overall survival did not differ between the 3 mutational groups as a whole or stratified by stage at presentation (data not shown).

Tumor localization by mutational status

It has been noted previously that *NRAS* mutated tumors display a propensity for developing on the upper extremities (10). A similar finding was seen in our patient population, with 90% of MN tumors located on either extremity and only 6% on the trunk (Fig. 2). In contrast, MB tumors were localized to the extremities in 41% of cases, with a similar proportion (46%) found on the trunk. The site distribution of WW tumors was similar to that of MB tumors. Differences in location among the 3 genotypes were statistically significant ($P < 0.0001$).

Primary tumors with uncommon mutational status

Three tumors carried mutations in both *BRAF* exon 15 and *NRAS* exon 2, designated MM (Table 4). The ages of these 3 patients (58, 58, and 57 years) were somewhat greater than the median age of 49 years for the other groups. Also, interesting was the presence of tandem nucleotide mutations in *BRAF* exon 15 in 2 of the 3 tumors. Otherwise, the MM tumors were not remarkable for any distinct features and fell across the spectrum of the other 3

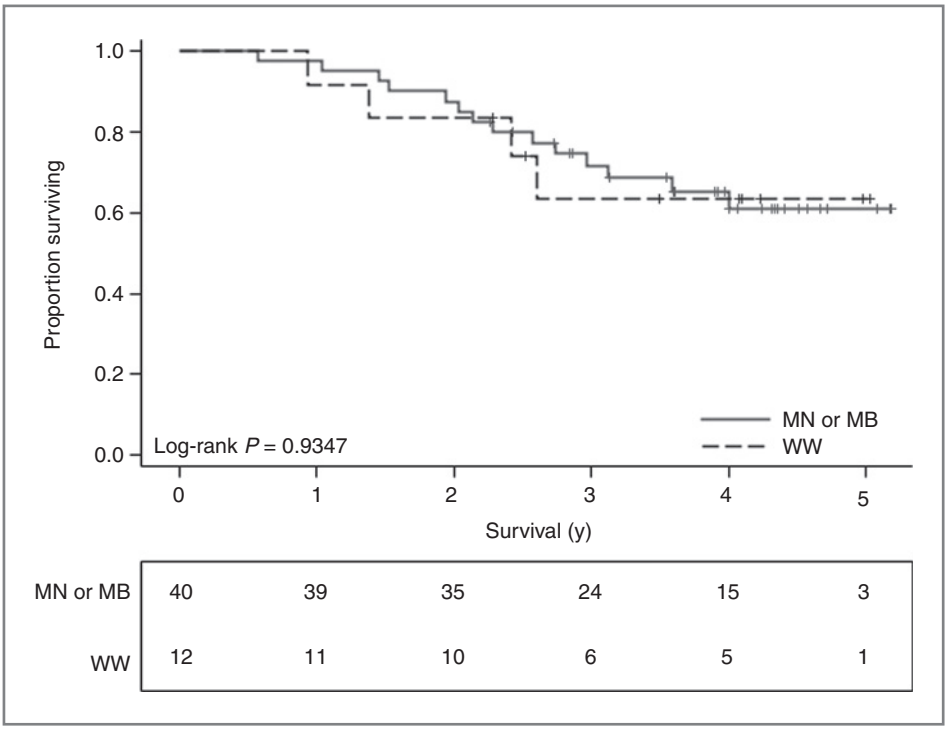

Figure 1. Survival of patients presenting with stage III disease according to the presence or absence of mutation. Patients with tumors carrying either *NRAS* or *BRAF* mutations are combined into a single category.

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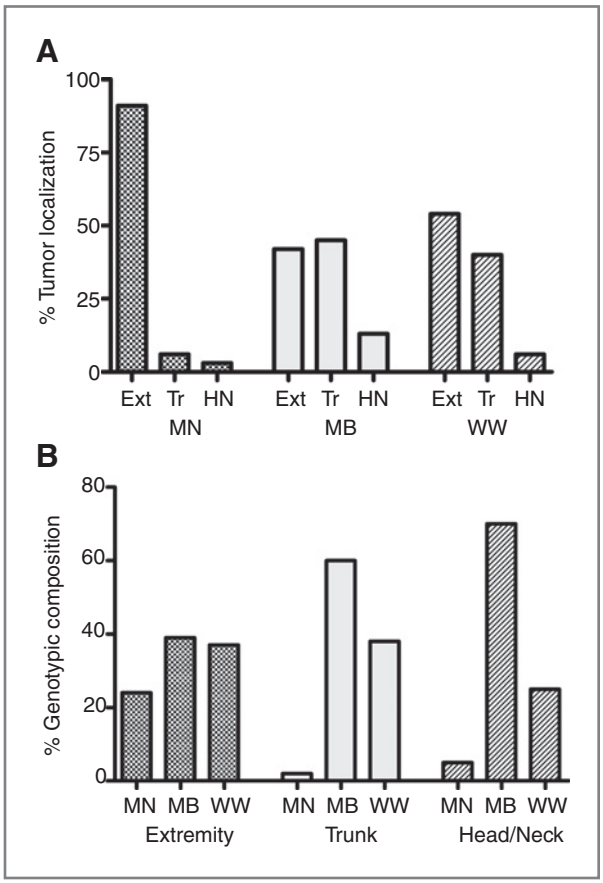

Figure 2. Influence of genotype on tumor localization. Anatomic location of tumors for each genotype is shown in A. Conversely, B shows the distribution of genotypes for each general locale. "Extremity" (Ext) includes hand, arm, shoulder, foot, and leg. "Trunk" (Tr) includes back, chest, abdomen, and buttocks. Scalp, face, ears, and neck are included in "Head and Neck" (HN).

genotypes. One MM patient was found to have regional nodal metastasis on presentation and underwent a successful lymphadenectomy. All 3 patients remain alive and disease-free at 40, 44, and 49 months from diagnosis.

Another uncommon mutational finding was the exclusive presence of mutated DNA, whether *NRAS* or *BRAF*, as indicated by a solitary mutation peak on the sequencing chromatogram. This can occur as a result of identical mutations on both gene copies or a mutation on 1 copy and deletion of that stretch of DNA on the other. This finding was observed in the tumors of 5 patients, 2 with mutated *NRAS* and 3 with mutated *BRAF* (Table 4). Notably, all 5 tumors displayed aggressive features with a median Breslow depth of 3.8 mm and a median mitotic rate of 5 figures/mm^2. Three of these patients developed regional metastases, but all remain alive and disease-free with a median follow-up of 49.5 months.

Discussion

Since the recognition of the high frequency of *BRAF* mutations in cutaneous melanoma, this anomaly has become a favored target for drug design. However, justification for such directed therapy has largely been based on *in vitro* studies indicating that the *BRAF* V600E mutation is activating and drives the MAPK pathway. The data that we now present provide clinical justification for the therapeutic targeting of mutated *B-Raf*, as well as mutated *N-Ras*, in the treatment of melanoma. Our findings demonstrate a correlation of *NRAS* and/or *BRAF* mutations with other factors well known to negatively influence prognosis, such as invasion and ulceration, and are consistent with our previous report that *NRAS* and *BRAF* mutations are acquired as melanoma cells progress from the radial to the vertical growth phase (12). In keeping with these observations, patients with mutated tumors are more likely to present with regional metastases. It is interesting to note, however, that in our study population to date, survival does not differ between stage III patients whose primary tumors do or do not carry mutations, even though the mutated tumors tended to produce larger volume nodal disease.

A similar attempt to establish clinical correlates of *NRAS* and *BRAF* mutations in melanoma has been published by Edlundh-Rose et al. (10). This group examined 294 tumors,

Table 4. Uncommon genotypes

	NRAS	*BRAF*	Age, y	Gender	Breslow depth, mm	Clark level	Mitotic index, figures/mm^2	Ulceration
Double mutant	A182G	TG1799AA	58	M	0.85	3	1	No
	C181A	T1799A	58	M	1.46	4	1	No
	A182G	GT1798AA	57	F	1.2	4	3	Yes
Only mutated DNA	C181A	Wild type	49	F	3.9	5	5	No
	A182G	Wild type	51	M	2.2	4	2	No
	Wild type	T1799A	35	F	3.1	3	3	No
	Wild type	GT1798AA	77	M	3.8	4	9	No
	Wild type	T1799A	57	M	5	4	16	Yes

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the majority of which were metastases, and reported the characteristics of the primary tumors from which they were derived. Although this approach selected for tumors that eventually metastasized and assumed genotypic agreement between primaries and metastases, many of their findings were consistent with ours, particularly the invasiveness of (presumed) *NRAS*-mutated primary tumors and lack of survival differences based on genotype. Taken together, these data suggest that the effects of *NRAS* and *BRAF* mutations may be limited to early disease stages and that other factors are more influential after regional metastases have occurred. It is also possible that the mutation pattern in our study population differs between the primary and metastatic tumors, as we have not sequenced the exons in question in metastases from these patients. However, the literature would suggest that the mutational status is maintained throughout the various stages of disease progression (15).

The high frequency of ulceration in *BRAF*-mutated tumors is notable, reaching a rate of 22% compared with around 10% in *NRAS*-mutated and wild-type tumors. Because *BRAF* and *NRAS* are components of the same signaling pathway, it is difficult to reconcile the differences in ulceration rates on the basis of downstream MAPK effectors. Implicit here is that the mechanism of melanoma ulceration, although poorly understood, may be linked to factors unique to *BRAF* mutation and may ultimately shed light on *BRAF*-specific molecular processes.

The difference in tumor localization based on genotype is compelling, and here, *NRAS* mutants distinguish themselves from the 2 other genotypes with their propensity for the extremities. *BRAF*-mutated tumors have been reported to arise less frequently in skin with chronic sun exposure and actinic damage (16). Although less well documented, melanomas with *NRAS* mutations are reported as more common on skin with continuous sun exposure (3, 10). Our data generally support these previous reports in terms of sites more likely to be sun-exposed (extremities) or sun-protected (trunk). To further address the suggested association of mutations and sun exposure, we are presently examining the tumor sections for solar elastosis, an accepted marker of chronic sun damage. Alternatively, other differences in the external environment or even differences that occur in melanocytes as they migrate to and develop in central versus peripheral regions of the body may influence the risk of one or another mutation.

In conclusion, our data provide convincing evidence for distinct clinical phenotypes of melanomas bearing *NRAS* and *BRAF* mutations, whether considered together or separately, and largely point to an inferior patient outcome. Conversely, patients with tumors wild type at both loci might be expected to exhibit a less aggressive form of this disease. Although melanoma has reliable histopathologic predictors of tumor behavior, genotypic data may assist in decision making for patients with borderline cases, particularly those at risk for complications of sentinel node biopsy or general anesthesia, or patients generally reluctant to undergo invasive procedures. Our ultimate goal is to strengthen and expand these correlative findings as we continue to follow this patient cohort.

Disclosure of Potential Conflicts of Interest

Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health.

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Correction

Correction: Clinical Correlates of *NRAS* and *BRAF* Mutations in Primary Human Melanoma

In this article (Clin Cancer Res 2011;17:229–35), which was published in the January 15, 2011 issue of *Clinical Cancer Research* (1), the alignment of Table 4 is incorrect. The correct table is shown below.

Table 4. Uncommon genotypes

	NRAS	*BRAF*	Age, y	Gender	Breslow, mm	Clark	Mitotic index, figures/mm^2	Ulceration
Double mutant	A182G	TG1799AA	58	M	0.85	3	1	No
	C181A	T1799A	58	M	1.46	4	1	No
	A182G	GT1798AA	57	F	1.2	4	3	Yes
Only mutated DNA	C181A	Wild type	49	F	3.9	5	5	No
	A182G	Wild type	51	M	2.2	4	2	No
	Wild type	T1799A	35	F	3.1	3	3	No
	Wild type	GT1798AA	77	M	3.8	4	9	No
	Wild type	T1799A	57	M	5	4	16	Yes

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