Examination of Mutations in BRAF, NRAS, and PTEN in Primary Cutaneous Melanoma

Vikas K. Goel[1,2,5], Alexander J.F. Lazar[2,3,5,6], Carla L. Warneke[4], Mark S. Redston[2,3] and Frank G. Haluska[1,2]

Frequent somatic mutation of v-raf murine sarcoma viral oncogene homolog B (BRAF), a downstream effector of the rat sarcoma oncogene (RAS) signaling pathway, is described in melanoma and other tumors. Our analysis of melanoma cell lines suggests that activating mutations in BRAF can occur simultaneously with inactivation of phosphatase and tensin homolog (PTEN), but neuroblastoma RAS (NRAS) mutations are not coincident. We determined the concurrent prevalence of mutations in BRAF and NRAS, and alteration of PTEN expression in 69 primary cutaneous melanomas. BRAF mutations were seen in 57% of cases. NRAS was mutated in 17% of samples, exclusively in exon 2. Two cases showed concurrent BRAF and NRAS mutations. Using immunohistochemistry, PTEN protein expression was lost or greatly reduced in 19% of tumors. Seven tumors with reduced PTEN yielded DNA amenable to sequencing, and three also showed mutation in BRAF but none in NRAS. In all, 11 (85%) of 13 tumors showing reduced PTEN expression were greater than 3.5 mm thick, and the association of increasing Breslow thickness and loss or reduction of PTEN expression was statistically significant ($P<0.0001$). Mutations in NRAS were not coincident with reduced PTEN expression, and the concurrent mutation of NRAS and BRAF was rare.

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INTRODUCTION

Cutaneous melanoma is an aggressive malignancy with complex genetic etiology and advanced disease is generally resistant to medical treatment (Haluska and Multani, 1999). In other tumor types, the success of small-molecule kinase inhibitors has underscored the relationship between somatic tumor-specific mutations and therapy, and the promise of developing new targeted therapies for the treatment of melanoma has focused intense interest on understanding the genetic contributors to melanomagenesis. Evidence shows that the tumor suppressor genes CDKN2A (p16) and phosphatase and tensin homolog (PTEN), along with the oncogene neuroblastoma rat sarcoma oncogene (NRAS), are involved in melanoma biology (Haluska and Hodi, 1998; Wu *et al.*, 2003; Daniotti *et al.*, 2004). More recently, mutations in v-raf murine sarcoma viral oncogene homolog B (BRAF), a serine–threonine kinase and potential oncogene, have been implicated in the majority of cases of melanoma (Davies *et al.*, 2002).

While mutations in BRAF are noted in up to 80% of primary cutaneous melanoma samples, BRAF is also mutated in the majority of benign melanocytic nevi (Brose *et al.*, 2002; Kumar *et al.*, 2003; Maldonado *et al.*, 2003; Sasaki *et al.*, 2004; Shinozaki *et al.*, 2004; Thomas *et al.*, 2004). Most melanocytic nevi do not progress to melanoma (Omholt *et al.*, 2003; Pollock *et al.*, 2003; Uribe *et al.*, 2003; Yazdi *et al.*, 2003; Saldanha *et al.*, 2004). Furthermore, the prognostic significance of BRAF mutational status is somewhat controversial, but most studies do not show a significant link with outcome (Kumar *et al.*, 2003; Chang *et al.*, 2004; Shinozaki *et al.*, 2004). If BRAF mutation is indeed required for tumorigenesis, additional changes must accumulate for tumor progression and we need to understand mutations that occur in conjunction with BRAF.

Our previous analysis of melanoma cell lines and the work of Davies and others show that cell lines that harbor BRAF mutations do not have NRAS mutations (Tsao *et al.*, 2000; Davies *et al.*, 2002). Although complete reciprocity is not universal, these findings suggest that activation is required at only one point in the rat sarcoma oncogene (RAS)/V-raf-1 murine leukemia viral oncogene homolog (RAF)/mitogen-activated protein kinase (MAPK) pathway to activate the downstream targets and initiate cell proliferation and/or

[1]*Department of Medicine, Division of Hematology/Oncology, Massachusetts General Hospital, Melanoma Center, Boston, Massachusetts, USA;* [2]*Harvard Medical School, Boston, Massachusetts, USA;* [3]*Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts, USA and* [4]*Department of Biostatistics and Applied Mathematics, UT-MD Anderson Cancer Center, Houston, Texas, USA*

[5]*These authors contributed equally to the work*

[6]*Current address: Departments of Pathology and Dermatology, Sections of Dermatopathology and Soft Tissue/Sarcoma Pathology, UT-M.D. Anderson Cancer Center, Houston, Texas, USA.*

Correspondence: Dr Frank G. Haluska, Department of Medicine, Division of Hematology/Oncology, GRJ 1021, Massachusetts General Hospital, Melanoma Center, 55 Fruit Street, Boston, Massachusetts, USA. E-mail: Haluska.frank@mgh.harvard.edu

Abbreviations: AKT, v-akt murine thymoma viral oncogene homolog; BRAF, v-raf murine sarcoma viral oncogene homolog B; PTEN, phosphatase and tensin homolog; RAF, v-raf-1 murine leukemia viral oncogene homolog; NRAS, neuroblastoma rat sarcoma oncogene

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tumorigenesis (Kumar *et al.*, 2003; Pollock *et al.*, 2003). These data are borne out in studies of excised melanocytic nevi and primary and metastatic melanoma samples as well.

The tumor suppressor PTEN was found to be mutated in melanoma cell lines at a rate of 30–50% (Guldberg *et al.*, 1997; Tsao *et al.*, 1998; Whiteman *et al.*, 2002), but rates were lower in primary cutaneous melanoma in some studies (Poetsch *et al.*, 2001; Pollock *et al.*, 2003). Loss of PTEN activity, through deletion, mutation, or reduced expression, results in signaling through PIP3, activation of Akt, and interference with apoptosis (reviewed by Wu *et al.*, 2003).

Previously, we observed that, as is true for NRAS and BRAF, NRAS mutations and PTEN inactivation are reciprocal in at least a subset of melanoma cell lines (Tsao *et al.*, 2000). One would expect not to observe concurrent mutation of NRAS and PTEN if they act in series in the PI3K/Akt signaling pathway. We propose that this is the case in melanoma, that mutations of the PTEN/AKT pathway render NRAS mutation redundant, and that NRAS mutation and PTEN loss will be distributed reciprocally. This hypothesis, generated from cell line data, has not been tested in primary melanoma samples.

We examined 69 micro-dissected, formalin-fixed primary cutaneous melanomas that were enriched for thick melanomas in an effort to further examine the roles of BRAF, NRAS, and PTEN in NRAS in primary cutaneous melanoma progression. We evaluated the mutational status of NRAS and BRAF by DNA analysis and PTEN expression by immunohistochemistry.

RESULTS

A total of 69 formalin-fixed paraffin-embedded primary melanoma samples were examined. Limited demographic and histologic data, tumor site, and BRAF, NRAS, and Pten status for each sample are presented in Table 1. The patient population included 41 females (59%) and 28 males (41%). Ages ranged from 16 to 91 years, with a mean age of 58.3 years (median, 58 years). In all, 21 tumors were in the head and neck region, eight were from the upper extremities, 21 from the lower extremities, 16 were from the back/trunk, and three from mucosal sites. The Breslow thickness ranged from 0.4 to 16.0 mm, with a median of 1.70 mm (mean of 3.78 mm). The majority of cases were of the superficial spreading type ($n=45$; 65%), with the remaining being nodular ($n=7$; 10%), acral lentiginous ($n=5$; 7%), lentigo maligna/desmoplastic ($n=6$; 9%), mucosal ($n=3$; 4%), or other/unclassified ($n=3$; 4%).

Altogether, 16 samples did not yield amplifiable DNA for at least one of the PCR analyses: seven failed BRAF PCR alone, four failed both BRAF and NRAS PCR, and five failed NRAS PCR alone. These samples showed a trend ($P=0.1065$) towards being thicker melanomas (mean thickness 5.20 mm; median 3.85 mm).

Amplification of BRAF by PCR was successful in 58 samples and 33 (57%) revealed the BRAFV600E mutation. Of the 33 B-RAFV600E mutations, 22 (67%) were seen in tumors less than or equal to 2.0 mm in thickness. BRAF mutational status was significantly associated with tumor thickness for all melanoma types ($P=0.0273$), but was not significantly associated if only superficial spreading type is considered ($P=0.6873$). BRAF mutational status was significantly associated with younger patient age in the entire population ($P=0.0212$, $r^2=0.0912$), but this association was not present when only superficial spreading type was considered ($P=0.3526$).

Exon 2 of NRAS was amplified in 60 samples and 10 activating mutations (17%) at amino-acid position 61 were noted. No mutations in exon 1 of NRAS were seen in the 51 samples where amplification was successful. In all, 53 samples were assessable for both BRAF and NRAS exon 2; among these, 38 or 72% carried a BRAF and/or NRAS mutation. Activating mutations in NRAS showed no discernable correlation with tumor thickness ($P=0.6640$) or any of the other variables, with the exception of age ($P=0.0238$). Older patient age was associated with mutations.

Of the 33 tumors with mutations in BRAF, 30 were also assessable for NRAS and only two showed concurrent mutation of NRAS. In the 53 tumors assessable with regard to both genes, BRAF and NRAS were significantly associated ($P=0.0138$) as 93% of those tumors with mutant BRAF had wild-type NRAS2 versus 65% of those with wild-type BRAF who had wild-type NRAS2. In general, BRAF and NRAS2 mutations were mutually exclusive.

Immunohistochemistry revealed that Pten was completely absent (three cases) or reduced by greater than 50% (10 cases) in 13 (19%) of the 69 tumors. In all, 11 of the 13 tumors with reduced/absent Pten expression by immunohistochemistry occurred in tumors greater than 3.5 mm in thickness; loss or reduction of Pten expression was significantly associated with increased tumor thickness in the entire population (Spearman $r=-0.46$, $P<0.0001$) and this association held when only superficial spreading tumors were considered (Spearman $r=-0.33$, $P=0.0274$). None of the 10 tumors with NRAS mutations showed reduced expression of Pten. Of the 13 tumors with reduced/absent Pten expression, seven could be amplified for BRAF and three of these harbored BRAF mutations. The inability to amplify BRAF in six of the 13 Pten reduced/loss cases did not allow further analysis of relationships.

DISCUSSION

We observed that there was an overlap between Pten loss/reduction and BRAF mutation, although it was not possible to comment on the significance of the relationship as only seven of the 13 cases with abnormal Pten expression yielded amplifiable material for BRAF analysis. One might be concerned that the loss of Pten expression and the concordant lack of BRAF DNA of sufficiently high quality to amplify might be due to tissue degradation; however, in all but two the DNA was amplifiable for the NRAS gene. This argues that the loss of Pten expression was in fact biological, and not an artifact. In addition, these cases all showed intact staining of PTEN in the endothelial cells of tumor-associated vessels. However, we cannot exclude poor DNA quality as explanation for BRAF PCR failure.

We examined the relationship between alteration in these three genes in melanoma cell lines (Tsao *et al.*, 2000). We found that, in cell lines, concurrent mutation of BRAF and loss of PTEN, with alteration of the MAPK and PI3K/Akt

Table 1. Primary cases of invasive melanoma with clinical parameters and relationships between pathway mutations and PTEN loss

Sample	Gender	Age	Sites	Breslow	Type/Clark	PTEN	BRAF	NRAS
Genotype: BRAF mutation only								
44	M	71	Back	0.44	SS/III	+++	MUT	wt
50	F	54	Back	0.4	SS/II	++	MUT	wt
46	M	50	Back	0.44	SS/III	++	MUT	wt
57	F	46	Shoulder	0.5	SS/II	++	MUT	wt
62	M	47	Back	0.5	SS/II	+++	MUT	wt
36	F	51	Forearm	0.62	SS/III	++	MUT	wt
2	F	26	Thigh	0.6	SS/III	++	MUT	wt
35	F	54	Leg	0.6	SS/III	++	MUT	wt
51	M	47	Thigh	0.66	SS/IV	++	MUT	wt
38	M	32	Cheek	0.68	SS/II	++	MUT	wt
29	F	52	Leg	0.8	SS/IV	++	MUT	wt
41	F	57	Thigh	0.8	SS/IV	+++	MUT	wt
42	F	29	Mons pubis	0.88	SS/IV	++	MUT	wt
32	F	37	Arm	0.99	SS/IV	++	MUT	wt
4	M	78	Cheek	1.1	SS/IV	++	MUT	wt
53	F	86	Leg	1.2	SS/IV	++	MUT	wt
61	F	76	Arm	1.7	SS/IV	++	MUT	wt
7	M	63	Shoulder	2	N/IV	++	MUT	wt
34	F	43	Ankle	2.2	SS/IV	+++	MUT	wt
24	M	45	Flank	2.32	SS/IV	+++	MUT	wt
13	M	71	Abdomen	2.5	SS/IV	++	MUT	wt
66	F	42	Neck	3	D/IV	++	MUT	wt
10	F	64	Foot	4	AL/IV	++	MUT	wt
21	F	43	Finger	4.15	D/IV	++	MUT	wt
26	F	21	Back	12	SS/V	++	MUT	wt
Genotype: NRAS mutation only								
58	F	53	Forearm	0.4	SS/IV	+++	wt	MUT
5	M	76	Calf	0.66	SS/IV	++	wt	MUT
8	M	76	Leg	0.66	LM/D V	++	wt	MUT
25	F	82	Heel	5	N/IV	++	wt	MUT
14	F	43	Scalp	7	U(mb) V	++	wt	MUT
23	M	72	Shoulder	7.3	N/IV	++	wt	MUT
22	M	91	Ear	10	UV	++	wt	MUT
12	M	81	Forehead	15	LM/D V	++	wt	MUT
Genotype: BRAFand NRAS mutation								
31	F	55	Neck	0.46	SS/III	+++	MUT	MUT
40	F	80	Back	0.85	SS/IV	++	MUT	MUT
Genotype: BRAF and NRAS wt								
69	F	25	Leg	0.4	SS/II	++	wt	wt
60	F	72	Arm	0.55	SS/III	++	wt	wt

Table 1 continued on following page

Table 1. continued

Sample	Gender	Age	Sites	Breslow	Type/Clark	PTEN	BRAF	NRAS
37	M	91	Neck	0.66	SS/IV	+++	wt	wt
6	F	58	Breast	0.7	SS/IV	++	wt	wt
54	F	48	Back	1.3	SS/IV	+++	wt	wt
59	F	68	Heel	1.3	AL III	++	wt	wt
67	M	69	Finger	3.6	AL IV	++	wt	wt
68	F	16	Ear	3.7	SS/IV	+++	wt	wt
63	M	80	Forehead	6	LM/D V	++	wt	wt
3	F	45	Foot	6.2	AL IV	++	wt	wt
9	M	76	Ear	10	D V	++	wt	wt
Sample	**Gender**	**Age**	**Sites**	**Breslow**	**Type/Other/ Clark**	**PTEN**	**BRAF**	**NRAS**
Genotype: PTEN loss and evaluable BRAF and NRAS								
11	F	73	Forehead	0.9	SS/III	+	wt	wt
17	F	71	Clitoris	4.3	M/NA	–	wt	wt
28	F	82	Cheek	4.5	N/IV	+	wt	wt
52	M	58	Back	5	SS/V	+	MUT	wt
20	M	50	Scalp	7.5	U/V	+	MUT	wt
45	F	79	Ankle	15	SS/V	–	MUT	wt
15	F	90	Vagina	13.5	M/NA	–	wt	wt
No amplification of BRAF and/or NRAS (16/69 (23%) mean Breslow=5.2 mm)								
1	M	58	Back	9	N/V	+	na	na
16	F	79	Clitoris	12	M/N/A	++	na	na
18	F	65	Foot	14	N/IV	+	na	na
19	M	30	Toe	2.67	AL/IV	++	na	wt
27	M	29	Back	6.2	SS/IV	+	na	na
30	M	49	Scalp	3.85	SS/IV	–	na	wt
33	F	36	Calf	0.75	SS/IV	++	na	wt
39	M	81	Arm	1.55	N/III	++	MUT	na
43	M	74	Leg	1.55	SS/IV	+++	na	wt
47	F	58	Cheek	8	SS/V	+	na	wt
48	F	57	Back	0.5	SS/III	++	MUT	na
49	F	78	Calf	1	SS/III	+	na	wt
55	F	60	Cheek	16	LM/D V	++	wt	na
56	M	38	Knee	0.5	SS/IV	++	na	wt
64	F	45	Ear	2.4	SS/IV	++	MUT	na
65	M	43	Back	3.2	N/III	++	wt	na

Sample: case number; gender: F, female; M, male; age, age in years; site: site of tumor; type, melanoma subtype; AL, acral lentiginous type; D, desmoplastic type; LM/D, lentigomaligna/desmoplastic type; M, mucosal type; N, nodular type; SS, superficial spreading type; U, unclassified type; U(mb), malignant blue nevus; Clark, Clark level; Breslow (mm): Breslow thickness expressed in mm; PTEN, result of immunohistochemistry for PTEN; BRAF, results of mutational screen for BRAF at V600E; NRAS2, results of mutational screening at NRAS exon 2; MUT: mutation; wt, wild-type; na, not amplified.

pathways, respectively, recapitulate the same downstream biochemical effects of NRAS mutation. Although hampered by the lack of amplification in a small subset of our samples, our data indicate that concurrent alteration of both pathways, in the absence of NRAS mutations, occurs in primary tumors, as we demonstrated previously. This is an important confirmation of the significance of the results from melanoma cell lines, most of which are derived from metastatic

melanoma samples and have passed through many generations *in vitro*.

We observed a highly significant relationship between increasing melanoma thickness and the loss or reduced expression of Pten. The data seem to suggest a potential relationship between thin melanomas and BRAF mutation, but the observation is confounded by the fact that most of the thicker melanomas were of subtypes where BRAF mutations are known to be less prevalent, such as acral lentiginous, desmoplastic, and mucosal melanomas (Sasaki *et al.*, 2004; Davison *et al.*, 2005). In this study, only superficial spreading type melanoma ($n = 45$, 65%) was present in numbers that allowed for examination of BRAF status within a single histology. When this was done, there was no relationship between Breslow thickness and mutation. This is in keeping with the findings of others. However, the relationship between loss of or reduced expression of Pten and thicker melanomas remained highly significant within this single subtype.

Data that provide some understanding as to the sequence of these multiple events are accumulating. BRAF mutation, based on its prevalence in studies of otherwise benign melanocytic nevi, is likely an early event. Parallel PTEN/AKT pathway involvement may accrue later. This view is supported by four lines of evidence. First, early PTEN loss, as occurs in Cowden and related inherited cancer syndromes, does not confer an increased risk for the development of melanoma. Second, Tsao *et al.* (2003) did not report any significant loss of PTEN expression in benign melanocytic tumors. Third, our data here suggest a low rate of PTEN loss in thin primary melanomas (3/36 samples carrying a BRAF mutation have PTEN loss), and a correlation with increasing Breslow thickness. Fourth, our cell line data (Tsao *et al.*, 1998, 2004), in which PTEN loss approximates 30%, may be explained if PTEN loss is more frequent in the metastatic melanomas used to initiate the cell lines. We cannot exclude possible selection for PTEN loss by tissue culture itself. But taken together, these data suggest PTEN loss, or, perhaps more generally, AKT pathway abrogation occurs later in melanoma tumorigenesis. Its contribution to the biological behavior of the tumor is not yet understood.

In these primary samples, PTEN loss reduction (13 cases) or NRAS activation (10 cases) was observed in 23/69 or 33% of tumors. These data underscore the importance of considering the concurrent presence of abnormalities in both MAPK and Akt pathways for melanoma tumorigenesis. If PTEN loss does accumulate with progression, metastatic melanoma will be expected to exhibit higher rates of AKT pathway abnormalities.

Finally, these genetic findings may have implications for melanoma therapy, even though our examination here is limited to primary tumors. Inhibitors of each of these pathways have singly been tested in the preclinical setting. MAPK activation in a melanoma cell line was abrogated by the MAPK/ERK kinase inhibitor PD98059, as well as specific orally available MAPK inhibitor CI 1040 (Collisson *et al.*, 2003; Satyamoorthy *et al.*, 2003). Similarly, treatment of melanoma cell lines with high pAKT levels using PI3-K inhibitors such as LY294002 or wortmannin has been reported (Dhawan *et al.*, 2002). In the clinic, the inhibitor of BRAF, BAY 43-9006, has been tested in phase I trials with little single-agent activity (Strumberg *et al.*, 2005). The data reported here suggest that alteration of the BRAF-MAPK and PTEN-AKT occurs frequently, possibly in sequence but often in concert. It is possible that successful targeted therapy strategies will need to inhibit both pathways simultaneously to be successful.

MATERIALS AND METHODS

Sample selection

The case files of Brigham and Women's Hospital, Boston, MA, spanning 1992–2001 were reviewed for hematoxylin-and-eosin-stained sections representative of advanced primary cases of invasive melanoma. We set out to identify all cases with Breslow thickness greater than 4 mm with paraffin-embedded tissue available from the time interval. Our goal was to ascertain that all available advanced primaries, which are rare, were examined in order to compare early/thin and late/thick primary tumors. Thus, a subset of this study is a 10-year consecutive series of all lesions greater than or equal to 4 mm in Breslow depth. Subsequently, representative lesions with thickness less than 4 mm and with available paraffin blocks were randomly chosen in a proportional manner from the same time period. Cases of melanoma *in situ* were excluded.

The described studies using human tissue samples were performed in adherence with the Declaration of Helsinki principles and under the review and approval of the Partners Human Research Committee, Assurance #FWA00003136. The tumors were classified by a dermatopathologist (A.J.F.L.) as superficial spreading, nodular, acral lentiginous, or lentigo maligna according to established histopathologic standards. If the invasive component was primarily desmoplastic, this was also noted. One case of so-called malignant cellular blue nevus (confirmed by the presence of a regional lymph node metastasis) was included in the study.

Immunohistochemistry for PTEN

For each case, 4-μm formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin. A section from a representative paraffin block in each case was also studied by immunohistochemistry for the Pten protein (monoclonal 6H2.1, Cascade Biosciences, Winchester, MA) using a modification of a previously established protocol (Mutter *et al.*, 2001). Briefly, the slides were deparaffinized and treated with an alcohol gradient to absolute ethanol. Endogenous peroxidase activity was blocked with a solution of 1% hydrogen peroxide and 65% ethanol. Antigen retrieval was performed in Target Retrieval Solution (DAKO Carpinteria, CA) in a microwave for 30 minutes at 78°C. After cooling for 30 minutes in fresh Target Retrieval Solution, primary antibody was applied at 1:750 dilution and incubated at 4°C overnight. Anti-mouse IgG with the DAKO Envision+ detection system (horseradish peroxidase) was employed as per the manufacturer's directions. If the tumor contained abundant melanin that interfered with interpretation, the DAKO LSAB+ system (alkaline phosphatase) was substituted. Light hematoxylin was used as counterstain. Separate positive and negative controls were performed in parallel. Endothelial cells were an important internal positive control and were present with robust staining in all 69 cases examined (see Figure 1). Pten immunohistochemistry was interpreted independently by two pathologists (AJFL and MR) who were blinded to the BRAF, RAS mutational status of the cases. A

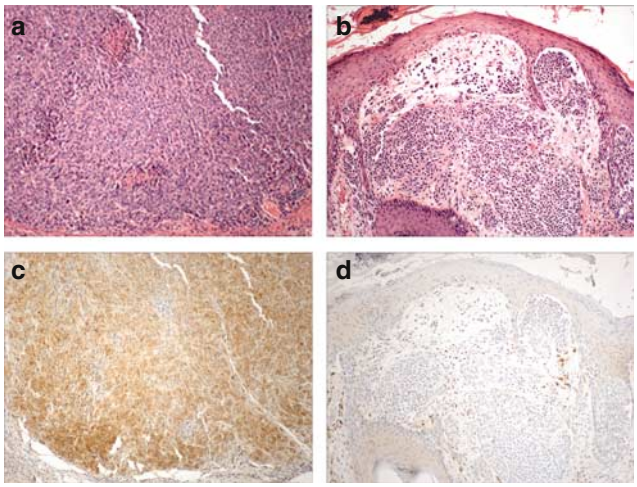

Figure 1. Hematoxylin-and-eosin-stained sections (panels a and c) with respective immunohistochemistry for Pten (panels b and d), showing intact staining for Pten (panel b) and virtually a complete loss of Pten (panel d) in the same fields of the two cases, respectively. Endothelial cells in vessels serve as the internal positive control.

previously described system for scoring the staining by comparison to adjacent endothelial cells in tumor-associated vasculature was used (Zhou *et al.*, 2000). Scoring included the following categories: increased (+++), equivalent (++), significantly reduced (+), and absent (−). Significantly reduced was defined as less than 50% of levels in adjacent endothelial cells. A few tumors showed broad areas of reduced or absent expression in an apparently clonal distribution, and these were scored as reduced (+). These areas of reduced expression comprised approximately half of the tumors in the few cases where it was noted.

DNA isolation and mutation detection for NRAS and BRAF

Genomic DNA was extracted as described previously (Tsao *et al.*, 1998), with minor modifications. Briefly, paraffin was removed from two or three 10-μm-thick unstained slides from each case by xylene immersion, washed with isopropanol, and rehydrated with graded ethanol and water mixtures. The slides were then lightly stained with hematoxylin (Gill's formulation #2; Fischer Scientific, Hanover Park, IL) to aid microdissection and washed in 10 mM Tris, pH = 8, and 1 mM EDTA buffer. The melanoma tumor was then manually microdissected from the stained slides using scalpel blades under microscopic guidance and incubated with proteinase K in a 100–150 μl aliquot overnight at 55°C. Based on this direct visualization during microdissection, we estimate that all samples consisted of greater than 80% melanoma cells. The proteinase K was inactivated by heating the sample to 100°C for 5 minutes. To the crude DNA extract, 100 μl of the protein extraction (Puregene®, Gentra Sytsems, Minneapolis, MN) reagent was added, incubated on ice for 1 hour, and centrifuged to sediment the insoluble fraction. The supernatant was transferred to a new eppendorf tube and supplemented with glycogen and 100% ethanol and stored at −80°C for 48 hours to precipitate the DNA. The DNA was recovered after centrifugation at 4°C and the pellet was washed once with ice-cold 70% ethanol. The DNA pellet was air-dried, re-suspended in 70–80 μl Tris buffer (pH 8.0) and used for subsequent PCR amplification. The primer sets used for the PCR amplification were as follows:

BRAF, 15F 1 5′-TCATAATGCTTGCTCTGATAGGA-3′
BRAF, X15R 23 5′-GGCCAAAAATTTAATCAGTGGA-3′
This pair produces an amplicon of approximately 240 bp.
RmutantFOR, 23, 5′-CTAAACTCTTCATAATGCTTGCTC-3′
RmutantREV (new primer), 5′-CCAGACAACTGTTCAAACTGATG GGAC-3′
This primer pair yields a PCR amplicon of about 176 bp.

For NRAS exon 1 and exon 2 sequencing, primers and conditions were used as described previously (Omholt *et al.*, 2003).

NRAS-1F, 5′-CAG GTT CTT GCT GGT GTG AAA TGA CTG AG-3′
NRAS-1R, 5′-CTA CCA CTG GGC CTC ACC TCT ATG G-3′
The amplicon size is 148 bp.
NRAS, 2F 5′-GTT ATA GAT GGT GAA ACC TG-3′
NRAS, 2R 5′-ATA CAC AGA GGA AGC CTT CG-3′
The amplicon size is about 115 bp.

For all PCR reactions, 0.25–2.5 μl of extracted DNA was used with the following PCR conditions:

Initial denaturation at 96°C for 10 minutes, followed by 45 cycles of 96°C denaturation for 40 seconds, annealing at 51°C for 90 seconds, extension at 72°C for 90 seconds, and a final extension at 72°C for 10 minutes.

After PCR amplification, the samples were applied to a 1.2% agarose gel and eluted and purified using QIAquick Gel Extraction Kit (QIAGEN, Valencia, CA) as per the manufacturer's instructions. Sequencing was performed using an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) at the Massachusetts General Hospital DNA sequencing core facility (Boston, MA).

Statistical analysis

Simple univariate descriptive statistics were utilized to describe the population. For bivariate relationships, proportions were compared using Fisher's exact test. Associations between age and categorical variables were determined using one-way analysis of variance, and associations between Breslow thickness and categorical variables were compared using the Wilcoxon rank-sum test normal approximation with continuity correction, or the Kruskal–Wallis test. Correlations were determined using Spearman's correlation coefficient. All tests are two-sided, with a 5% significance level.

CONFLICT OF INTEREST

The author states no conflict of interest.

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