NRAS and *BRAF* Mutations Arise Early during Melanoma Pathogenesis and Are Preserved throughout Tumor Progression

Katarina Omholt, Anton Platz, Lena Kanter, Ulrik Ringborg, and Johan Hansson

Cancer Centre Karolinska, Department of Oncology-Pathology, Radiumhemmet, Karolinska Hospital and Institute, Stockholm, Sweden

ABSTRACT

Purpose: **Recently, it was reported that *BRAF* mutations are frequent in melanoma. Previously, we analyzed a large series of paired primary and metastatic melanomas for *NRAS* codon 61 mutations and showed that they arise early and are preserved during tumor progression. Here, we have screened the same tumor samples for *BRAF* mutations.**

Experimental Design: **Primary melanomas ($n = 71$) and corresponding metastases ($n = 88$) from 71 patients were screened for *BRAF* exon 11 and exon 15 mutations using single-strand conformational polymorphism and nucleotide sequence analysis**

Results: ***BRAF*** **mutations were found in 42 of 71 patients (59%). Thirty-seven patients had mutations that lead to a Val599Glu change, whereas mutations resulting in Gly468Ser, Val599Arg, Val599Lys, and Lys600Glu changes were detected in one patient each. Furthermore, one patient had a 6-bp insertion between codons 598 and 599, encoding two threonine residues. In most cases, paired primary and metastatic lesions had the same *BRAF* genotype (*i.e.*, mutations present in the primary tumors were preserved in the corresponding metastases, and mutations did not arise at the metastatic stage if they were not present in the primary lesion). Using laser-capture microdissection, *BRAF* mutations were found in the radial growth phase of the primary lesions. *BRAF* mutations occurred exclusively in tumors that were wild type for *NRAS*, and in total, 89% of the patients analyzed (63 of 71) had mutations in either of these two genes.**

Conclusions: **The Ras–Raf–mitogen-activated protein kinase/extracellular signal-regulated kinase–extracellular signal-regulated kinase signaling pathway is activated in the vast majority of melanomas. Activation occurs through either *NRAS* or *BRAF* mutations, both of which arise early during melanoma pathogenesis and are preserved throughout tumor progression.**

INTRODUCTION

It has long been known that activating *NRAS* codon 61 mutations occur in up to 30% of all cutaneous melanoma cases (1–7). Recently, Davies *et al.* (8) reported that *BRAF* mutations occur at a high frequency in malignant melanoma, whereas in several other tumor types the mutation frequency was found to be lower. *BRAF* mutations were found in 20 of 34 melanoma cell lines (59%), 12 of 15 short-term cultures (80%), and six of nine melanoma tumors (67%; 8). Other studies have subsequently confirmed these results (9–12). By far, the most common alteration observed is Val599Glu, found in exon 15 and affecting the kinase domain of B-Raf. A limited number of mutations have also been found in exon 11. Thus, in melanoma, activation of the Ras–Raf–mitogen-activated protein kinase/extracellular signal-regulated (ERK) kinase (MEK)-ERK signaling pathway occurs both through *RAS* and *RAF* mutations.

Raf is a serine/threonine kinase that functions downstream of the Ras GTPase protein in the mitogen-activated protein kinase signaling pathway (13). When activated, Raf phosphorylates MEK, which in turn phosphorylates ERK. Activated ERK then phosphorylates different cytoplasmic and nuclear targets, mediating a cellular response. There are three different mammalian Raf proteins: Raf-1, A-Raf, and B-Raf. Whereas Raf-1 is ubiquitously expressed, B-Raf expression is found mainly in neural tissues and testis (14). The Raf proteins also differ somewhat in the way they are activated. For example, whereas Raf-1 depends on Src phosphorylation for activation, B-Raf does not, and of the three Raf proteins, B-Raf has the highest basal kinase activity (15, 16). The residues Thr598 and Ser601 of B-Raf constitute two phosphorylation sites that are required for Ras-induced activation of B-Raf (17), and these are conserved in the other Raf proteins as well as through evolution. When these residues are changed to acidic residues, B-Raf becomes constitutively active (17).

We have previously analyzed a large series of paired primary and metastatic cutaneous melanomas for *NRAS* codon 61 mutations (7). We found that these mutations arise at an early stage during melanoma development, because they were detected in the radial growth phase (RGP) of the primary lesions, and that they are preserved throughout tumor progression. Here, the same tumor samples that were included in our previous study have been screened for *BRAF* exon 11 and exon 15 mutations, to determine the role of *BRAF* mutations in melanoma tumor initiation and progression. The *BRAF* mutations have also been correlated to clinical data including patient overall survival.

Received 4/7/03; revised 9/8/03; accepted 9/8/03.

Grant support: The Cancer Society of Stockholm, The King Gustav V Jubilee Fund, The Karolinska Institute Research Funds, and The Swedish Radiation Protection Institute.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Johan Hansson, CCK R8:03, Karolinska Hospital, S-171 76 Stockholm, Sweden. Fax: 46-8-517-74-245; E-mail: Johan.Hansson@onkpat.ki.se.

MATERIALS AND METHODS

Tumor Samples. Melanoma tumors from 71 patients who were followed up at the Department of Oncology, Karolinska Hospital, were included in this study. In all patients, the disease had progressed to the metastatic stage, and for 51 patients, both primary tumors ($n = 52$) and matched metastases ($n = 82$) were available for analysis. In 18 patients ($n = 19$) and in 2 patients ($n = 6$), only primary tumors and metastases, respectively, were available for analysis. All specimens were obtained as surgical excision biopsies that had been formalin fixed and paraffin embedded. Of the 71 primary tumors, 40 were superficial spreading melanomas, 28 were nodular melanomas, and 1 was a lentigo maligna melanoma. In two primary tumors, the histogenetic type was not classifiable. The median primary tumor thickness was 2.6 mm (range, 0.5–25), and the levels of tumor invasion according to Clark were II in 2 tumors, III in 28 tumors, IV in 32 tumors, V in 7 tumors, and unknown in 2 tumors. Of the 88 metastases, 50 involved lymph nodes, 37 involved skin and s.c. tissue, and the site of one metastasis was unknown. Of the 53 patients from whom metastases were available for analysis, a single metastasis was analyzed in 36 patients. In the remaining 17 patients, multiple metastases were analyzed: two metastases in 8 patients, three in 3 patients, four in 4 patients, and five and six metastases were each analyzed in a single patient.

DNA Extraction. Tumor tissue was dissected manually from 20-μm sections using parallel H&E-stained sections to localize tumor cell areas. The dissected tissue was treated with proteinase K, as described previously (7), and DNA was extracted using the Wizard DNA clean-up system (Promega, Madison, WI). A limited number of the metastases, which had lymphocytic infiltrates that were difficult to avoid by manual dissection, were subjected to laser-capture microdissection (LCM) using a PixCell LCM system (Arcturus Engineering, Mountain View, CA). In these cases, tumor cells were dissected from three to four consecutive 5-μm sections, and DNA was extracted according to the manufacturer's instructions. Likewise, approximately one quarter of the primary tumors was subjected to LCM, dissecting out only vertical growth phase (VGP) tumor cells. Superficial spreading melanoma lesions that were found to have *BRAF* mutations were evaluated histologically by a pathologist (L. K.), and intraepidermal RGP nests were identified. Tumor cells from these intraepidermal RGP nests were also dissected by LCM.

Mutational Analyses. *BRAF* exons 11 and 15 were amplified by PCR using primers described by Davies *et al.* (8). Fifty nanograms of genomic DNA were amplified in the presence of [α-^{32}P]dCTP using standard PCR conditions. When LCM had been used to isolate tumor cells from the primary tumors, seminested PCR was used for amplification. In these cases, the reverse primers 5′-TGACTTGTCACAATGTCACCA-3′ (*BRAF* exon 11) and 5′-AATCAGTGGAAAAATAGCCTCA-3′ (*BRAF* exon 15) were used. Single-strand conformational polymorphism (SSCP) was performed as described previously (7). For *BRAF* exon 11, SSCP was performed in the presence of glycerol at 5°C and for *BRAF* exon 15 in the absence of glycerol at 5°C. For *BRAF* exon 15, the human melanoma cell line A375 was used as a positive control because it contains the Val599Glu mutation (8). Mutations were confirmed by two independent PCR-SSCP analyses. Nucleotide sequence analyses were performed using the BigDye Terminator Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence analyses were performed in both directions.

Table 1 *BRAF* mutations in paired primary and metastatic melanoma lesions

Primary tumors ($n = 70$)		Corresponding metastases ($n = 88$)	
Number	*BRAF*	Number	*BRAF*
24[a]	Val599Glu	34[b]	Val599Glu[c]
12	Val599Glu	[d]	
2	Wild type	4[e]	Val599Glu
		1	Val599Glu
		1	Gly468Ser
1	Val599Arg		
1	Val599Lys	2	Val599Lys
1	Lys600Glu		
1	6-bp ins[f]	1	6-bp ins
23	Wild type	40[g]	Wild type
5[h]	Wild type		
		5[i]	Wild type

[a] The tumors originated from 23 patients; one patient had two primary tumors.
[b] Three patients each had four metastases, and two patients each had two metastases.
[c] One metastasis from a patient with two metastases did not have the Val599Glu mutation.
[d] Tumor samples were not available for analysis.
[e] One patient had three metastases.
[f] The mutation consisted of a 6-bp insertion between codons 598 and 599, coding for two Thr residues. In addition, there was a silent ACA to ACT mutation at codon 598.
[g] Five patients each had two metastases, two had three metastases, one had four metastases, and one patient had six metastases.
[h] The tumors originated from four patients; one patient had two primary tumors.
[i] The metastases originated from a single patient.

Statistical Analyses. To evaluate possible relationships between *BRAF* and *NRAS* mutations and various clinical parameters, either the χ^2 test or one-way ANOVA test was used. The impact of mutations on overall survival was determined using the life table method and tested with Wilcoxon-Gehan's statistics. $P \leq 0.05$ was regarded as statistically significant.

RESULTS

Mutational Analyses. Primary and/or metastatic melanoma lesions from a total of 71 patients were screened for *BRAF* exon 15 mutations using SSCP and nucleotide sequence analysis. Exon 15 mutations were found in 41 of 71 patients (58%) (Table 1 and Fig. 1). Of these 41 patients, 37 (90%) had a GTG to GAG transversion at codon 599, leading to a valine to glutamic acid exchange at this position. One patient had a mutation in the third position of codon 598 as well as in the first two positions of codon 599 (AGT to GAG). This mutation changes the valine in position 599 to an arginine, whereas codon 598 remains unchanged. One patient had a tandem GTG to AAG mutation at codon 599, altering the valine at this position to a

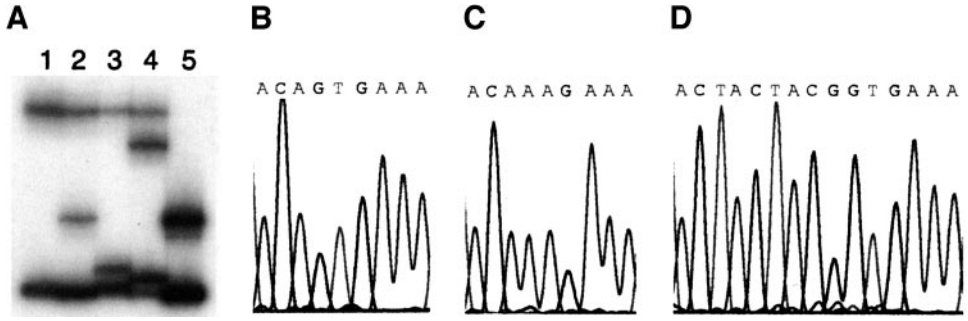

Fig. 1 Mutational analysis of *BRAF* exon 15 in cutaneous malignant melanoma. *A*, SSCP analysis showing aberrant bands of different mutation types. *Lane 1*, example of a metastasis wild type for *BRAF* exon 15; *Lane 2*, example of a metastasis with the Val599Glu change; *Lane 3*, example of a metastasis with the Val599Lys change; *Lane 4*, metastasis with the 6-bp insertion between codons 598 and 599, coding for two threonine residues; *Lane 5*, melanoma cell line A375, used as a positive control (Val599Glu). *B*, wild type nucleotide sequence of *BRAF* codons 598–560. *C*, nucleotide sequence of the codon 599Lys mutation. *D*, nucleotide sequence of the 6-bp insertion between codons 598 and 599. In addition, codon 598 is changed from ACA to ACT.

lysine. Another patient had an AAA to GAA transition at codon 600, altering the lysine at this position to a glutamic acid. Moreover, one patient had a 6-bp insertion (ACTACG) between codons 598 and 599, coding for two threonine residues. In addition, a silent mutation at codon 598 (ACA to ACT) was observed in this patient.

Tumor samples that were wild type for *BRAF* exon 15 and for which DNA was still available were analyzed further for *BRAF* exon 11 mutations, because this is the only exon apart from exon 15 in which mutations have been reported to occur (8). Of 25 primary tumors and 43 metastases analyzed, one metastasis was found mutated. This mutation consisted of a GGA to AGC change at codon 468, altering the glycine at this position to a serine.

In 51 patients, both primary tumors and metastases were analyzed. All of these patients, except two, showed the same *BRAF* genotype in their primary lesion as in their corresponding metastatic lesion(s) (Table 1). That is, if the primary tumor contained a mutation, the same mutation was also present in the corresponding metastatic lesion(s). Furthermore, if the primary tumor was wild type for *BRAF*, no mutations arose by the metastatic stage. An exception was two patients whose primary tumors were wild type for *BRAF* but corresponding metastases ($n = 4$) all contained the Val599Glu mutation.

From 17 patients, more than one metastasis was obtained, and it was, thus, possible to perform mutational analyses on separate metastases in these individuals. In most cases, multiple metastases from the same patient had the same *BRAF* genotype. For example, there were three patients with four metastases each, all of which had the Val599Glu mutation. Only in one patient, who had two metastases available for analysis, did the metastases show different *BRAF* genotypes: one had the Val599Glu change whereas one was wild type (Table 1).

To better determine at what stage in the primary lesions the *BRAF* mutations occur, intraepidermal RGP tumor cells from eight mutated superficial spreading melanoma lesions were isolated by LCM and subjected to mutational analysis (Fig. 2). In all eight cases, the *BRAF* mutation that had been detected in the VGP was also present in the corresponding RGP (Table 2).

We have previously analyzed the tumors included in the present study for *NRAS* codon 61 mutations (7), and the results of that screening are included in Table 3. Interestingly, *BRAF* mutations occurred exclusively in tumors with a wild type *NRAS* exon 2 genotype. Altogether, 89% of the patients analyzed (63 of 71) had tumors with either *NRAS* codon 61 or *BRAF* mutations. Metastases that were wild type for both *NRAS* exon 2 and *BRAF* were screened further for *NRAS* exon 1 mutations, to determine whether they harbored any codon 12 and codon 13 mutations, but no such mutations were found (data not shown).

Statistical Analyses. *BRAF* and *NRAS* mutations showed no significant correlation to any clinical parameter, including gender, age at diagnosis, clinical stage at diagnosis, histogenetic type, level of tumor invasion according to Clark, tumor thick-

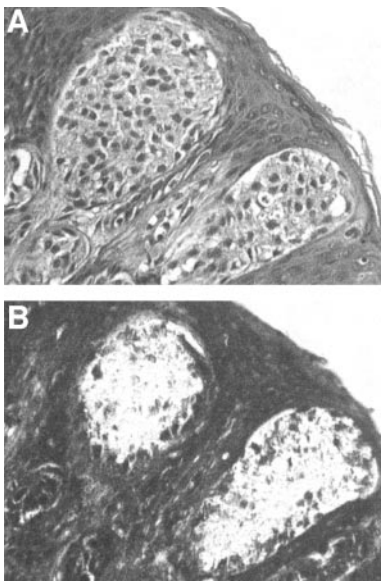

Fig. 2 Laser-capture microdissection (LCM) of melanoma radial growth phase (RGP). *A*, H&E staining of a superficial spreading melanoma lesion showing intraepidermal RGP nests. *B*, RGP tumor cells were dissected by LCM and subjected to *BRAF* mutational analysis. The *panel* shows the section after dissection.

Table 2 *BRAF* mutations in different stages of melanoma tumor progression

No. of primary tumors	RGP	VGP[a]	Metastasis
7	Val599Glu	Val599Glu	Val599Glu[b]
1	Val599Arg	Val599Arg	[c]

[a] Four of the primary tumors were subjected to laser-capture microdissection and consisted of a pure vertical growth phase. The remaining four tumors were manually dissected and, therefore, also included the radial growth phase.

[b] Metastases were available for analysis from six of the seven primary tumors.

[c] No metastases were available for analysis.

ness, ulceration, and site of first recurrence (Table 4). In a univariate analysis, there was no relationship between *BRAF* and *NRAS* mutations and overall survival (Table 5), with median survival times from time of diagnosis of 41 months in patients with *BRAF*-mutated tumors, 47 months in patients with *NRAS*-mutated tumors, and 51 months in patients with tumors being wild type for both these genes.

DISCUSSION

In this study, we report a high frequency of *BRAF* mutations in a series of paired primary and metastatic melanomas, which we have previously analyzed for *NRAS* codon 61 mutations (7). *BRAF* mutations were found in 59% of the patients analyzed (42 of 71), with Val599Glu accounting for 88% of all mutations. The other observed alterations included Gly468Ser, Val599Arg, Val599Lys, Lys600Glu, and a 6-bp insertion between codons 598 and 599, coding for two threonine residues.

The Val599Glu change is also the most frequent alteration observed by others (8–12). Likewise, Gly468Ser, Val599Arg, Val599Lys, and Lys600Glu changes have been described previously in cutaneous melanoma tumors (8–12), although the Gly468Ser and Val599Arg alterations detected in our samples result from different mutations than those reported previously. In contrast, the 6-bp insertion constitutes a novel alteration. The different codon 599 and codon 600 changes and the double threonine insertion most likely interfere with the phosphorylation sites Thr598 and Ser601. Indeed, Val599Glu-mutated B-Raf has been shown to have a higher kinase activity than wild type B-Raf (8), and both the Val599Glu (8, 11) and Val599Lys (11) changes render B-Raf more potent in inducing transformation. Gly468 represents the third glycine residue in a glycine-rich motif that is found in protein kinases and is involved in the binding of ATP (18). Gly463Val and Gly468Ala (*i.e.*, alterations affecting the glycine-rich motif) have been shown to have similar functional effects as Val599Glu (8). With the exception of Val599Glu (8, 11) and Val599Lys (11), none of the other mutations described here have been analyzed functionally, and their precise effects are, therefore, unknown. However, because of their similarities to mutations with known effects, they are probably also of importance for melanoma development.

As mentioned above, the tumors included in the present study have been screened previously for *NRAS* codon 61 mutations (7). In total, 30% of the patients analyzed (21 of 71) had *NRAS* mutations, of which Gln61Lys and Gln61Arg were the two most common mutations. The combined results of the two screenings show that *NRAS* and *BRAF* mutations are mutually exclusive and that as many as 89% of the patients analyzed (63 of 71) had tumors that were mutated in either of these genes. Similar results (*i.e.*, that *RAS* and *BRAF* mutations never seem to coexist in the same lesion) have also been reported in other tumor types, including colorectal, ovarian, and papillary thyroid carcinomas (19–21). These findings support the hypothesis that *RAS* and *BRAF* mutations are complementary and may have similar effects during tumor development.

The screening of *BRAF* mutations in paired primary and metastatic melanoma lesions has made it possible to determine at what stage during melanoma pathogenesis these mutations occur. We found that *BRAF* mutations present in primary lesions were always preserved in the corresponding metastatic lesions and that *BRAF* mutations generally did not arise at the metastatic stage. Together, these results indicate that *BRAF* mutations occur at an early stage during melanoma pathogenesis rather than being associated with metastasis initiation. However, the *BRAF* mutations that were found in the metastatic lesions of two patients were not detected in the corresponding primary lesions, suggesting that they had occurred during tumor progression. In one of these two patients, the same *BRAF* mutation was detected in three separate metastases. This strongly indicates that they had a common clonal origin and that the mutation most likely had occurred at an early stage. Therefore, it is possible that the mutations were present in the primary lesions of these patients, but probably only in minor subclones that were too small for a mutation to be detected. Altogether, our finding that separate metastases from the same individual had the same *BRAF* genotype not only demonstrate a clonal relationship between the different metastases but also indicate that *BRAF* mutations are unlikely to represent late events during melanoma tumorigenesis. Although the *BRAF* mutations do not seem to be important for metastasis initiation, the finding that they are preserved throughout tumor progression suggests that they may still influence tumor maintenance. In support of this idea, studies in a mouse model system have shown that activated *RAS* is required for melanoma maintenance (22).

Melanoma progression occurs through well-defined steps. In RGP melanomas, tumor growth is confined to the epidermis (intraepidermal RGP) and the most superficial dermis (microinvasive RGP), whereas in VGP melanomas,

Table 3 *NRAS* and *BRAF* mutations in melanoma patients

No. of patients (n = 71)	*NRAS*	*BRAF*
37	wt[a]	Val599Glu
1	wt	Gly468Ser
1	wt	Val599Arg
1	wt	Val599Lys
1	wt	Lys600Glu
1	wt	6-bp ins
10	Gly61Lys	wt
9	Gln61Arg	wt
1	Gln61His	wt
1	Gln61Leu	wt
8	wt	wt

[a] wt, wild type.

Table 4 Association of *BRAF* and *NRAS* mutations with various clinical parameters

Clinical factor	Genotype			*P*
	BRAF mut ($n = 42$)	*NRAS* mut ($n = 21$)	Wild type ($n = 8$)	
Gender				
Male	25 (56.8%)	13 (29.5%)	6 (13.6%)	
Female	17 (63.0%)	8 (29.6%)	2 (7.4%)	0.776[a]
Age at diagnosis, yr (mean ± SD)	55.3 ± 16.5	64.4 ± 15.9	54.9 ± 24.1	0.136[b]
Clinical stage at diagnosis				
I–II	38 (58.5%)	19 (29.2%)	8 (12.3%)	
III	2 (50.0%)	2 (50.0%)		
IV	1 (100%)			0.770[a]
Histogenetic type				
SSM[c]	23 (56.1%)	13 (31.7%)	5 (12.2%)	
NM	17 (63.0%)	8 (29.6%)	2 (7.4%)	
LMM	1 (100%)			0.895[a]
Level of tumor invasion				
II	1 (50.0%)	1 (50.0%)		
III	22 (75.9%)	4 (13.8%)	3 (10.3%)	
IV	16 (51.6%)	11 (35.5%)	4 (12.9%)	
V	2 (28.6%)	5 (71.4%)		0.075[a]
Tumor thickness, mm (mean ± SD)	3.5 ± 2.6	4.6 ± 5.4	3.3 ± 2.8	0.518[b]
Ulceration				
Yes	24 (64.9%)	9 (24.3%)	4 (10.8%)	
No	17 (53.1%)	12 (37.5%)	3 (9.4%)	0.478[a]
Site of first recurrence				
Regional lymph node metastasis	29 (63.0%)	11 (23.9%)	6 (13.0%)	
Regional skin metastasis	4 (30.8%)	7 (53.8%)	2 (15.4%)	
Distant metastasis	8 (72.7%)	3 (27.3%)		0.138[a]

[a] χ^2 exact test, two sided.
[b] One-way ANOVA with F-test.
[c] SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma.

tumor cells expand in the dermis (23). In contrast to the more advanced VGP melanomas, RGP melanomas do not form metastases and are efficiently cured by surgery alone (24). We (7) and others (25, 26) have previously shown that *NRAS* mutations are present in the RGP of primary melanoma lesions as well as in tumor-associated nevi and that they are preserved in corresponding VGP and metastatic lesions. Here, we show that *BRAF* mutations are also present in the RGP. Likewise, the *BRAF* mutations are preserved in the corresponding VGP and metastatic lesions. These results suggest that the *BRAF* mutations do arise at an early stage during melanoma pathogenesis and that they are not involved in the transition of RGP to VGP melanoma. In addition, these results show that *NRAS* and *BRAF* mutations occur at the same stage during melanoma pathogenesis, both representing early events that are preserved throughout progression. Our finding that *BRAF* mutations are present in the RGP of primary melanoma lesions is consistent with a previous study showing that *BRAF* mutations occur at a high frequency in nevi (9). However, our results differ from a recent study by Dong *et al.* (11), in which it was suggested that *BRAF* mutations correlate with melanoma progression rather than initiation. This suggestion was based on the fact that the frequency of *BRAF* mutations was significantly lower in RGP melanomas compared with VGP melanomas. An explanation for the discrepancy between our study and the study by Dong *et al.* (11) may be that the studies were performed in slightly different ways. For instance, we analyzed the RGP of advanced VGP melanoma lesions that all had progressed to the metastatic stage, whereas Dong *et al.* (11) instead analyzed RGP melanomas. In our study, no such early melanoma lesions were included. Also, the fact that we have used different screening methods may have contributed to the different results [*i.e.*, Dong *et al.* (11) used direct sequencing, whereas we used the more sensitive SSCP technique (7, 27)].

Table 5 Univariate analysis of overall survival from time of diagnosis

	No. of patients	Death	χ^2	*P*
Genotype			0.240	0.887[a]
BRAF mut	41	36 (88%)		
NRAS mut	21	14 (67%)		
Wild type	8	7 (88%)		

[a] Wilcoxon-Gehan test.

In conclusion, by showing that *BRAF* mutations occur at high frequency in a large clinical cohort of melanoma tumors, we confirm the high incidence of *BRAF* mutations in melanoma reported previously by others (8–12). Our finding that *NRAS* and *BRAF* mutations occur in the vast majority of cutaneous melanomas, and that they are present in early RGP lesions and preserved throughout tumor progression, indicates that *NRAS* and *BRAF* are attractive targets for therapeutic interventions.

ACKNOWLEDGMENTS

We thank Doris Kröckel for technical assistance and Bo Nilsson for statistical analyses.

REFERENCES

1. van′t Veer, L. J., Burgering, B. M. T., Versteeg, R., Boot, A. J. M., Ruiter, D. J., Osanto, S., Schrier, P. I., and Bos, J. L. N-ras mutations in human cutaneous melanoma from sun-exposed body sites. Mol. Cell. Biol., *9:* 3114–3116, 1989.

2. Albino, A. P., Nanus, D. M., Mentle, I. R., Cordon-Cardo, C., McNutt, N. S., Bressler, J., and Andreeff, M. Analysis of ras oncogenes in malignant melanoma and precursor lesions: correlation of point mutations with differentiation phenotype. Oncogene, *4:* 1363–1374, 1989.

3. Ball, N. J., Yohn, J. J., Morelli, J. G., Norris, D. A., Golitz, L. E., and Hoeffler, J. P. Ras mutations in human melanoma: a marker of malignant progression. J. Invest. Dermatol., *102:* 285–290, 1994.

4. Platz, A., Sevigny, P., Norberg, T., Ring, P., Lagerlöf, B., and Ringborg, U. Genes involved in cell cycle G_1 checkpoint control are frequently mutated in human melanoma metastases. Br. J. Cancer, *74:* 936–941, 1996.

5. van Elsas, A., Zerp, S. F., van der Flier, S., Krüse, K. M., Aarnoudse, C., Hayward, N. K., Ruiter, D. J., and Schrier, P. I. Relevance of ultraviolet-induced N-ras oncogene point mutations in development of primary human cutaneous melanoma. Am. J. Pathol., *149:* 883–893, 1996.

6. Jiveskog, S., Ragnarsson-Olding, B., Platz, A., and Ringborg, U. N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. J. Invest. Dermatol., *111:* 757–761, 1998.

7. Omholt, K., Karsberg, S., Platz, A., Kanter, L., Ringborg, U., and Hansson, J. Screening of N-ras codon 61 mutations in paired primary and metastatic cutaneous melanomas: mutations occur early and persist throughout tumor progression. Clin. Cancer Res., *8:* 3468–3474, 2002.

8. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Woffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cossu, A., Flanagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. Mutations of the BRAF gene in human cancer. Nature (Lond.), *417:* 949–954, 2002.

9. Pollock, P. M., Harper, U. L., Hansen, K. S., Yudt, L. M., Stark, M., Robbins, C. M., Moses, T. Y., Hostetter, G., Wagner, U., Kakareka, J., Salem, G., Pohida, T., Heenan, P., Duray, P., Kallioniemi, O., Hayward, N. K., Trent, J. M., and Meltzer, P. S. High frequency of BRAF mutations in nevi. Nat. Genet., *33:* 19–20, 2003.

10. Brose, M. S., Volpe, P., Feldman, M., Kumar, M., Rishi, I., Gerrero, R., Einhorn, E., Herlyn, M., Minna, J., Nicholson, A., Roth, J. A., Albelda, S. M., Davies, H., Cox, C., Brignell, G., Stephens, P., Futreal, P. A., Wooster, R., Stratton, M. R., and Weber, B. L. BRAF and RAS mutations in human lung cancer and melanoma. Cancer Res., *62:* 6997–7000, 2002.

11. Dong, J., Phelps, R. G., Qiao, R., Yao, S., Benard, O., Ronai, Z., and Aaronson, S. A. BRAF oncogenic mutations correlate with progression rather than initiation of human melanoma. Cancer Res., *63:* 3883–3885, 2003.

12. Gorden, A., Osman, I., Gai, W., He, D., Huang, W., Davidson, A., Houghton, A. N., Busam, K., and Polsky, D. Analysis of BRAF and N-RAS mutations in metastatic melanoma tissues. Cancer Res., *63:* 3955–3957, 2003.

13. Peyssonnaux, C., and Eychene, A. The Raf/MEK/ERK pathway: new concepts of activation. Biol. Cell, *93:* 53–62, 2001.

14. Barnier, J. V., Papin, C., Eychene, A., Lecoq, O., and Calothy, G. The mouse B-raf gene encodes multiple protein isoforms with tissue-specific expression. J. Biol. Chem., *270:* 23381–23389, 1995.

15. Marais, R., Light, Y., Paterson, H. F., Mason, C. S., and Marshall, C. J. Differential regulation of Raf-1, A-Raf, and B-Raf by oncogenic ras and tyrosine kinases. J. Biol. Chem., *272:* 4378–4383, 1997.

16. Mason, C. S., Springer, C. J., Cooper, R. G., Superti-Furga, G., Marshall, C. J., and Marais, R. Serine and tyrosine phosphorylations cooperate in Raf-1, but not B-Raf activation. EMBO J., *18:* 2137–2148, 1999.

17. Zhang, B. H., and Guan, K. L. Activation of B-Raf kinase requires phosphorylation of the conserved residues Thr598 and Ser601. EMBO J., *19:* 5429–5439, 2000.

18. Hanks, S. K., and Hunter, T. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J., *9:* 576–596, 1995.

19. Rajagopalan, H., Bardelli, A., Lengauer, C., Kinzler, K. W., Vogelstein, B., and Velculescu, V. E. Tumorigenesis: RAF/RAS oncogenes and mismatch-repair status. Nature (Lond.), *418:* 934, 2002.

20. Singer, G., Oldt, R., III, Cohen, Y., Wang, B. G., Sidransky, D., Kurman, R. J., and Shih, I. M. Mutations in BRAF and KRAS characterize the development of low-grade ovarian serous carcinoma. J. Natl. Cancer Inst., *95:* 484–486, 2003.

21. Kimura, E. T., Nikiforova, M. N., Zhu, Z., Knauf, J. A., Nikiforov, Y. E., and Fagin, J. A. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res., *63:* 1454–1457, 2003.

22. Chin, L., Tam, A., Pomerantz, J., Wong, M., Holash, J., Bardeesy, N., Shen, Q., O'Hagan, R., Pantginis, J., Zhou, H., Horner, J. W., II, Cordon-Cardo, C., Yancopoulos, G. D., and DePinho, R. A. Essential role for oncogenic Ras in tumour maintenance. Nature (Lond.), *400:* 468–472, 1999.

23. Clark, W. H., Jr., Elder, D. E., and Van Horn, M. The biologic forms of malignant melanoma. Hum. Pathol., *17:* 443–450, 1986.

24. Clark, W. H., Jr., Elder, D. E., Guerry, D., Braitman, L. E., Trock, B. J., Schultz, D., Synnestvedt, M., and Halpern, A. C. Model predicting survival in stage I melanoma based on tumor progression. J. Natl. Cancer Inst., *81:* 1893–1904, 1989.

25. Demunter, A., Ahmadian, M. R., Libbrecht, L., Stas, M., Baens, M., Scheffzek, K., Degreef, H., De Wolf-Peeters, C., and van den Oord, J. J. A novel N-ras mutation in malignant melanoma is associated with excellent prognosis. Cancer Res., *61:* 4916–4922, 2001.

26. Demunter, A., Stas, M., Degreef, H., De Wolf-Peeters, C., and van den Oord, J. J. Analysis of N- and K-ras mutations in the distinctive tumor progression phases of melanoma. J. Invest. Dermatol., *117:* 1483–1489, 2001.

27. Nataraj, A. J., Olivos-Glander, I., Kusukawa, N., and Highsmith, W. E., Jr. Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis, *20:* 1177–1185, 1999.

Clinical Cancer Research

NRAS and *BRAF* Mutations Arise Early during Melanoma Pathogenesis and Are Preserved throughout Tumor Progression

Katarina Omholt, Anton Platz, Lena Kanter, et al.

Clin Cancer Res 2003;9:6483-6488.