Screening of N-*ras* Codon 61 Mutations in Paired Primary and Metastatic Cutaneous Melanomas: Mutations Occur Early and Persist throughout Tumor Progression[1]

Katarina Omholt,[2] Sofia Karsberg,[2] Anton Platz, Lena Kanter, Ulrik Ringborg, and Johan Hansson[3]

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

ABSTRACT

Purpose: **Mutations in the *ras* genes often occur during tumorigenesis. In malignant melanoma, the most common *ras* alterations are N-*ras* codon 61 mutations. This study was aimed to measure the frequency of such mutations in a large series of paired primary and metastatic melanomas to determine their role in melanoma initiation and progression.**

Experimental Design: **Seventy-four primary melanomas and 88 metastases originating from 54 of the primary tumors were screened for N-*ras* codon 61 mutations using single-strand conformation polymorphism and nucleotide sequence analyses.**

Results: **Twenty-one of the 74 primary tumors (28%) had activating N-*ras* codon 61 mutations. From 20 of the mutated primary tumors, a total of 34 metastases were analyzed, and all but one showed the same mutation as the primary tumor from which they originated. The remaining 53 primary tumors and corresponding metastases ($n = 54$) were wild-type for N-*ras* codon 61. Analysis of the different growth phases of the mutated primary tumors showed that the mutations were already present in the radial growth phase. Mutations were also detected in tumor-associated nevi. N-*ras* codon 61 mutations were associated with a higher Clark level of invasion ($P = 0.012$) and a lower age at diagnosis ($P = 0.042$) but did not affect survival ($P = 0.671$).**

Conclusions: **This study shows that N-*ras* codon 61 mutations occur early in primary melanomas rather than in the metastatic stage and that once the mutations have occurred, they persist throughout tumor progression. This suggests that activated N-*ras* may be an attractive target for therapy in the subset of melanoma patients carrying such mutations.**

INTRODUCTION

Cutaneous malignant melanoma is the most fatal of skin cancers. The successive steps in tumor progression of cutaneous melanoma have been described (1). It has been demonstrated that superficial spreading melanomas, which may arise from dysplastic nevi, display an early radial growth phase where tumor cells are confined to the epidermis and lack invasive capacity. This may be followed by a vertical growth phase characterized by invasion of melanoma cells into the dermis. These cells have also acquired metastatic capacity. Metastatic melanoma is frequently resistant to radio- and chemotherapy and, consequently, has a high mortality rate. The disease seems to be genetically heterogeneous, and mutations in a variety of genes have been reported. Thus far, some of the most frequently found alterations are *ras*-activating mutations.

The human *ras* proto-oncogenes (H-*ras*, K-*ras*, and N-*ras*) encode cell membrane-associated proteins that are involved in transduction of extracellular growth and differentiation signals. The ras proteins have GTPase activity, and as such, they cycle between an inactive GDP-bound form and an active GTP-bound form. Once activated, ras stimulates several different effector pathways, one of which is the raf-MEK-ERK pathway (2).

Mutations in the *ras* genes frequently occur during tumorigenesis. Different tumor types are often associated with mutations in a distinct *ras* gene. Thus, high frequencies of K-*ras* alterations have been found in carcinomas of the pancreas, colon, and lung, whereas N-*ras* mutations are frequent in myeloid leukemias (3). The codons at which most activating *ras* mutations occur include codons 12, 13, and 61. Mutations in these codons reduce the intrinsic GTPase activity of the ras proteins and also make them insensitive to GTPase-activating proteins (4–6). As a result, mutated ras is locked in the GTP-bound state and continuously activates its downstream effector targets.

In sporadic malignant melanoma, the frequencies of *ras* mutations reported in the literature vary between 5 and 36% (7–18). The discrepancy in mutation frequency may partly be attributable to the use of different screening techniques but may also reflect differences in the composition of melanoma tumors included in the studies. Indeed, *ras* mutations have been found more frequently in melanomas from chronically sun-exposed body sites (8, 11, 16, 17) and have also been associated with nodular melanomas in some studies (14, 16). In melanoma, the vast majority of activating *ras* mutations has been identified in the N-*ras* gene, whereas mutations in the H-*ras* and K-*ras* genes are rare. Only a single study has reported a frequent occurrence of K-*ras* mutations in melanoma (10). The most frequent N-*ras* mutations in melanoma are codon 61 mutations, whereas codon

Received 3/27/02; revised 7/22/02; accepted 7/23/02.
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.
[1] Supported in part by grants from The Cancer Society of Stockholm, The King Gustav V Jubilee Fund, The Karolinska Institute Research Funds, and The Swedish Radiation Protection Institute.
[2] These authors contributed equally to this work.
[3] To whom requests for reprints should be addressed, at Department of Oncology-Pathology, Radiumhemmet, Karolinska Hospital, S-171 76 Stockholm, Sweden. Fax: 46-8-5177-6290.

12 and 13 mutations are less common. Recently, a novel mutation in N-*ras* codon 18 has been reported in a subset of primary melanomas (18).

Although it is well documented that N-*ras* codon 61 mutations are the most common *ras* alterations in malignant melanoma, there are no studies where large series of paired primary and metastatic lesions have been analyzed. Therefore, to evaluate the role of N-*ras* codon 61 mutations in melanoma initiation and progression, we have screened a large set of paired primary and metastatic melanomas. For this study, we identified primary and metastatic tumors from 53 patients with cutaneous melanoma. Primary tumors from an additional 19 patients whose metastases were not available for analyses were also included in the study. Our purpose was to measure the frequency of activating N-*ras* codon 61 mutations and to correlate mutations in the primary tumors to those in the corresponding metastases. N-*ras* mutations were also related to histopathological and clinical parameters, including patient survival.

PATIENTS AND METHODS

Tumor Samples. Seventy-four primary cutaneous malignant melanomas from 72 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 from 53 of the patients, a total of 88 metastases were available for analysis. All specimens were obtained as surgical excision biopsies, which had been formalin fixed and paraffin embedded. Forty-two of the primary tumors were SSMs,[4] 29 were nodular melanomas, and 1 was a lentigo maligna melanoma. In 2 primary tumors, the histogenic type was not classifiable. The median tumor thickness was 2.55 mm (range, 0.5–25 mm), and the levels of tumor invasion according to Clark were II in 3 tumors, III in 29 tumors, IV in 33 tumors, V in 7 tumors, and unknown in 2 tumors. Of the metastases, 50 involved lymph nodes, and 37 involved skin and s.c. tissue, whereas in 1 metastasis, the site was unknown. Of the 53 patients where both primary tumors and metastases were analyzed, a single metastasis was analyzed in 36 cases. In the remaining 17 patients, multiple metastases were analyzed: 2 metastases in 8 cases, 3 in 3 cases, 4 in 4 cases, and 5 and 6 metastases were each analyzed in a single patient.

DNA Extraction. Tumor tissue was manually dissected from 20-μm sections of the paraffin blocks using parallel H&E-stained sections to localize tumor cell areas. Dissected tissue was deparaffinized in xylene and incubated in PK2 buffer [50 μg/ml proteinase K (Boehringer Mannheim, Germany), 10 mM Tris (pH 8.3), 1 mM EDTA, and 0.5% Tween 20] at 56°C overnight. After inactivation of proteinase K at 95°C for 10 min, DNA was extracted using the Wizard DNA clean-up system (Promega Corp., Madison, WI). Metastases showing lymphocytic infiltrates difficult to avoid by manual dissection were subjected to laser capture microdissection using a PixCell LCM system (Arcturus Engineering, Mountain View, CA). Likewise, in addition to manual dissection, a limited number of the primary tumors were laser capture microdissected to separate the radial and vertical growth phases as well as tumor-associated nevi. In these cases, tumor tissue was dissected from three to four consecutive 5-μm sections, and DNA was extracted according to the manufacturer's instructions.

Table 1 Primers used for PCR amplification of exon 2 of the N-*ras* gene

Primer	Sequence (5′→3′)	Product length
Forward	GATTCTTACAGAAAACAAGTG	157 bp
Reverse	ATGACTTGCTATTATTGATGG	
Nested forward	CAAGTGGTTATAGATGGTGA	118 bp
Nested reverse	ATACACAGAGGAAGCCTTCG	

Mutational Analysis. A 118-bp fragment of exon 2 of the N-*ras* gene was amplified by nested PCR. Table 1 shows the sequences of the primers used. In the first PCR, 25 ng of genomic DNA were amplified in a 10-μl mixture containing 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 5 pmol of each primer, and 0.25 unit of AmpliTaq polymerase (Applied Biosystems, Foster City, CA). Samples were denatured at 94°C for 4 min; amplified for 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 30 s; and finally extended at 72°C for 7 min. The PCR products were diluted 1:20, and 1 μl of the dilution was used for the nested PCR. In the nested PCR, 3 μCi of [α-^{32}P]dCTP was included. The conditions for the second PCR were the same as for the first PCR, except that the annealing temperature was 60°C, and amplification was performed for 20 cycles.

For SSCP analysis, the PCR products were denatured in denaturing buffer (95% formamide, 10 mM NaOH, 0.05% bromphenol blue, and 0.05% xylene cyanol) at 92°C for 10 min. Electrophoresis was carried out on 7.5% nondenaturing acrylamide gels with 10% glycerol at 18°C. Shifted bands were cut out from the gels, reamplified, and purified using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). Nucleotide sequence analyses were carried out using the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). Sequence analyses were performed in both directions, using the nested primers as sequencing primers. Mutations were confirmed by two independent PCR/SSCP analyses.

To determine the lowest proportion of mutated cells that is required for SSCP to detect an N-*ras* codon 61 mutation, dilution series of wt DNA from the melanoma cell line 397 and DNA from either the melanoma cell line 224 (codon 61 Arg mutation) or the fibrosarcoma cell line HT-1080 (codon 61 Lys mutation) were analyzed. We found that 10% of mutated cells are enough for detection of mutations (data not shown).

Statistical Analysis. To evaluate possible relationships between N-*ras* codon 61 mutations and histopathological or clinical parameters, either the χ^2 test or Student's *t* test was used. Survival analyses were carried out using the life table method and Cox regression analysis. $P \leq 0.05$ was regarded as statistically significant.

[4] The abbreviations used are: SSM, superficial spreading melanoma; SSCP, single-strand conformation polymorphism; wt, wild-type; rgp, radial growth phase; vgp, vertical growth phase.

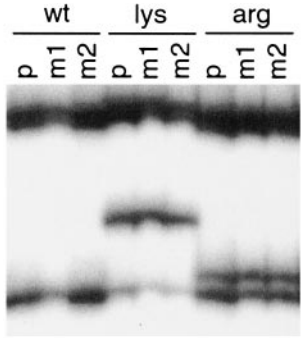

Fig. 1 Examples of SSCP analysis of N-*ras* exon 2 in primary malignant melanomas and their corresponding metastases. At *left*, wt bands of a primary melanoma (*p*) and its two metastases (*m1* and *m2*) are shown. In the *middle*, band shifts resulting from codon 61 Lys mutations in a primary tumor and its two metastases are shown. The reduced intensity of the wt bands indicates a loss of the wt allele in both the primary tumor and the metastases. At *right*, band shifts resulting from codon 61 Arg mutations in a primary tumor and its two metastases are shown.

Table 2 Summary of N-*ras* codon 61 mutational screening in paired primary and metastatic melanomas

N-*ras* codon 61 primary tumors	No. of primary tumors	No. of corresponding metastases/ primary tumor	N-*ras* codon 61 metastases
CAA(Gln)-AAA(Lys)	6	1	CAA(Gln)-AAA(Lys)
	3	2	CAA(Gln)-AAA(Lys)
CAA(Gln)-CGA(Arg)	4	1	CAA(Gln)-CGA(Arg)
	1	2	CAA(Gln)-CGA(Arg)
	1	3	CAA(Gln)-CGA(Arg)
	1	4	CAA(Gln)-CGA(Arg)
	1	6	CAA(Gln)-CGA(Arg)
	1	1	wt
CAA(Gln)-CAT(His)	1	1	CAA(Gln)-CAT(His)
CAA(Gln)-TTA(Leu)	1	1	CAA(Gln)-TTA(Leu)
wt	24[a]	1	wt
	4	2	wt
	2	3	wt
	3	4	wt
	1	5	wt

[a] These primary tumors originated from 23 patients; 1 patient had two primary tumors.

RESULTS

Seventy-four primary malignant melanomas and 88 metastases, originating from 54 of the primary tumors, were screened for mutations in exon 2 of the N-*ras* gene using SSCP and nucleotide sequence analyses. Twenty-one of the 74 primary tumors (28%) and 33 of the 88 metastases (37.5%) showed band shifts indicative of mutations on the SSCP-gels (Fig. 1). DNA sequence analysis confirmed the presence of mutations in all of these cases and revealed that they were all located in codon 61 of the N-*ras* gene.

Of the 21 mutated primary tumors, 10 (48%) had CAA(Gln)-AAA(Lys) mutations, 9 (43%) CAA(Gln)-CGA(Arg) mutations, 1 (4.5%) a CAA(Gln)-CAT(His) mutation, and 1 (4.5%) a tandem CAA(Gln)-TTA(Leu) mutation. From 20 of the mutated primary tumors, a total of 34 metastases were available for analysis, and all but one metastasis showed the same mutation as the primary tumor from which they originated (Table 2). The 33 mutated metastases included 12 metastases with the AAA(Lys) mutation, 19 with the CGA(Arg) mutation, 1 with the CAT(His), and 1 with the TTA(Leu) mutation. From 7 of the patients with mutated primary tumors, 2–6 separate metastatic tumors were analyzed. It is worth noting that in all of these patients with multiple metastases, every single metastasis had the same mutation as detected in the corresponding primary tumor.

To better determine at what stage in the primary lesions the N-*ras* codon 61 mutations occur, 10 of the 21 mutated primary tumors were further analyzed (Table 3 and Fig. 2). Using a laser capture microscope, the rgp and vgp, respectively, were dissected and subjected to mutational analyses. The analyses showed that the N-*ras* codon 61 mutations could be detected both in the rgp and vgp. In two cases, the primary tumors were associated with adjacent nevi (a dysplastic and compound nevus, respectively). In both cases, the N-*ras* mutation could already be detected in these lesions.

Fifty-three of the 74 primary tumors (72%) were wt for N-*ras* exon 2. From 34 of these primary tumors, a total of 54 metastases were available for analysis, and they all showed a wt genotype (Table 2). In many metastases, lymphocytic infiltrates were present. However, manual dissection, or in some cases laser capture microdissection, was used to obtain material enriched for tumor cells, and in all metastases showing a wt genotype, the presence of lymphocytes was reduced to ≤10%. Therefore, a non-tumor cell population should not have prevented the detection of possible mutations in the melanoma cells, but rather that the metastases are truly wt.

In 1 patient, the N-*ras* mutation found in the primary tumor was not detectable in the metastatic lesion. Instead, this metastasis showed a wt genotype (Table 2). This metastasis was heavily infiltrated by lymphocytes, which were impossible to eliminate by any dissection method. However, the proportion of tumor cells was still ≥10%, and a possible mutation should therefore have been detected. Thus, overall the N-*ras* exon 2 genotype of the primary tumor was retained in 87 of the 88 metastases (99%), and multiple metastases in the same patient always shared the same N-*ras* exon 2 genotype.

Table 3 Summary of N-*ras* codon 61 mutations in the different growth phases of 10 primary melanomas and in 2 melanoma-associated nevi

Histogenic type of primary tumor (*n*)	N-*ras* codon 61 mutations		
	Nevus	rgp	vgp
SSM (5)		Lys	Lys
SSM (1)		His	His
SSM (1)		Arg	Arg
SSM (1)	Lys[a]	Lys	Lys
SSM (1)	Arg[b]	ND[c]	Arg
NM (1)		Arg[d]	Arg

[a] A compound nevus.
[b] A dysplastic nevus.
[c] ND, not determined.
[d] Not a true rgp but rather a radial component.

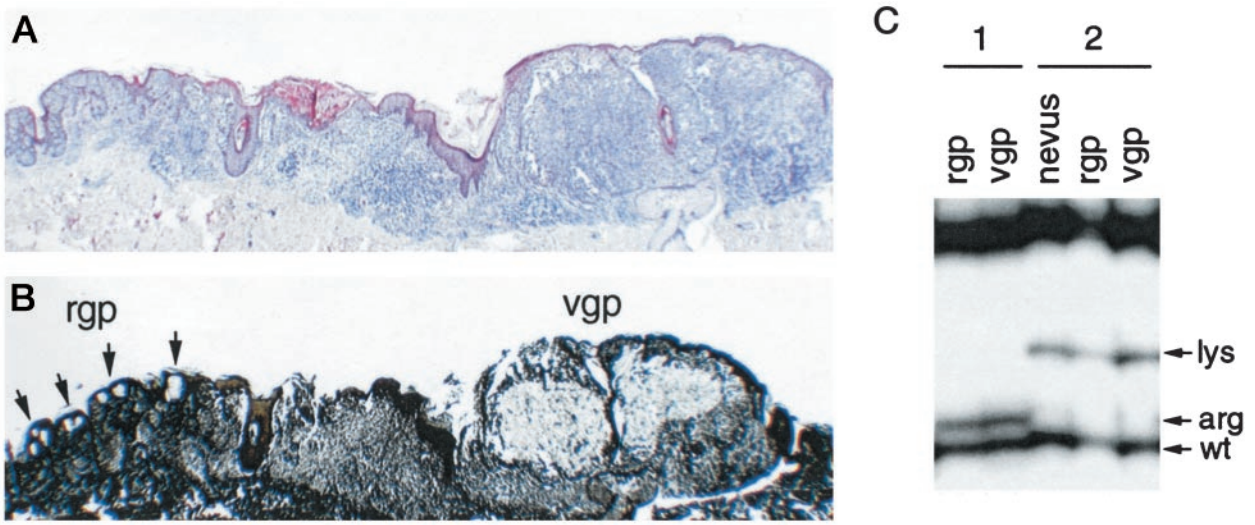

Fig. 2 N-*ras* codon 61 mutations in the different growth phases of primary melanomas. *A*, H&E staining of a primary SSM tumor. *B*, the rgp and vgp areas were dissected using a laser capture microscope. The panel shows the slide after microdissection. *C*, SSCP analysis. *Lanes 1*, Arg band shifts of the rgp and vgp from the tumor shown in *A* and *B*. *Lanes 2*, DNA extracts from a second primary SSM tumor, showing Lys band shifts in an associated compound nevus as well as in the rgp and vgp.

For mutated tumor samples, the SSCP analysis provides information about the relative abundance of wt and mutant alleles. The majority of patients displayed balanced band intensities for the wt and mutant alleles. However, 3 of the 21 patients with mutated tumors showed a marked decrease of the wt allele in comparison with the mutant allele. In two of these patients, the wt allele was lost in both the primary tumor as well as in the metastases (Fig. 1), whereas in 1 patient the wt loss was only obvious in the metastasis.

From Table 4, it can be seen that the Clark level of tumor invasion and age at diagnosis were the only clinical parameters that significantly differed between patients with tumors that were wt for N-*ras* codon 61 and those with tumors that were mutated. N-*ras* codon 61 mutations were associated with a higher Clark level of invasion ($P = 0.012$) and a lower age at diagnosis ($P = 0.042$). None of the other parameters analyzed, including gender, histogenic type, ulceration, and tumor thickness differed between the two patient groups.

The univariate survival analyses showed that N-*ras* codon 61 mutations had no impact on patient overall survival in our patient material (Table 5). From Table 5, it can further be seen that neither gender, ulceration, nor age at diagnosis had an impact on survival. In contrast, the univariate analyses showed that clinical stage at diagnosis, histogenic type, level of tumor invasion according to Clark, and tumor thickness according to Breslow all affected overall survival. In the multivariate analysis, tumor thickness and clinical stage were the only independent prognostic factors with respect to overall survival (Table 6).

Table 4 Relationship between N-*ras* codon 61 mutations and clinical parameters in primary melanoma tumors

Clinical parameter	N-*ras* codon 61 wt	N-*ras* codon 61 Mutant	*P*
Gender			
Male	33 (63.5%)	13 (61.9%)	
Female	19 (36.5%)	8 (38.1%)	0.990[a]
Age at diagnosis, yr (mean ± SD)	55.3 ± 17.5	64.4 ± 15.9	0.042[b]
Histogenic type			
SSM	29 (56.9%)	13 (61.9%)	
NM	21 (41.2%)	8 (38.1%)	
LMM	1 (2.0%)		0.856[a]
Level of invasion			
II	2 (3.9%)	1 (4.8%)	
III	25 (49.0%)	4 (19.0%)	
IV	22 (43.1%)	11 (52.4%)	
V	2 (3.9%)	5 (23.8%)	0.012[c]
Ulceration			
Yes	21 (41.2%)	13 (61.9%)	
No	30 (58.8%)	8 (38.1%)	0.127[a]
Tumor thickness, mm (mean ± SD)	3.4 ± 2.6	4.6 ± 5.4	0.211[b]

[a] χ^2 exact test.
[b] Student's *t* test.
[c] χ^2 exact test for trend.

DISCUSSION

In this study, a large series of paired primary and metastatic melanomas has been analyzed for N-*ras* codon 61 mutations to obtain a clearer picture of the role of these mutations in melanoma tumor initiation and progression. The study was limited to exon 2 of the N-*ras* gene, because several earlier studies pointed to N-*ras* codon 61 mutations as the most common *ras* alterations in human melanomas (7–9, 11–17).

We found that N-*ras* codon 61 mutations first appeared in the primary tumors rather than in the metastases, indicating that they are early events in melanoma tumor development. In total, 21 of 74 primary tumors (28%) had activating N-*ras* codon 61 mutations. Substitution of glutamine by lysine or arginine were

Table 5 Univariate analysis of overall survival from time of diagnosis in relation to patient and tumor characteristics

A. Categorical parameters

Parameter	*n*	Death	%	χ^2	*P*
Clinical stage at diagnosis				6.8	0.033
I–II	68	53	77.9		
III	4	4	100		
IV	1	1	100		
Gender				0.7	0.396
Male	45	38	84.4		
Female	27	20	74.1		
Histogenic type				6.6	0.038
SSM	42	34	80.9		
NM	29	22	75.9		
LMM	1	1	100		
Level of invasion				15.8	0.001
II	3	1	33.3		
III	29	20	69.0		
IV	33	30	90.9		
V	7	6	85.7		
Ulceration				3.5	0.062
Yes	34	25	73.5		
No	38	32	84.2		
N-*ras* codon 61				0.2	0.671
Mutant	21	14	66.7		
wt	52	44	84.6		

B. Continuous background parameters

Parameter	RH[a]	CI[b]	*P*
Age (10)[c]	1.15	0.98–1.35	0.071
Tumor thickness (mm)[d]	1.07	1.03–1.12	0.003

[a] RH, relative hazard.
[b] CI, confidence interval (95%).
[c] Per 10-yr increment.
[d] Per mm increment.

Table 6 Multivariate Cox regression (stepwise) analysis of overall survival showing relative hazard (RH), degrees of freedom (df), and 95% confidence intervals (CI)

Parameter	RH	df	CI	*P*
Tumor thickness, mm	1.07[a]	1	1.03–1.12	0.005
Stage		2		<0.001
I–II *vs.* III	3.73	1	1.29–10.8	0.015
I–II *vs.* IV	78.7	1	4.89–	0.002

[a] Per mm increment. For competing nonsignificant factors, see Table 5.

the two most common alterations, detected in 10 and 9 primary tumors, respectively. Substitution of glutamine by histidine or leucine each occurred in one primary tumor. Thus, the frequency of N-*ras* mutations in our study does not diverge from previous reports on melanoma tumors. Similarly, the lysine and arginine mutations, which predominated in our patients, belong to the most common codon 61 alterations. However, the CAA-(Gln)-TTA(Leu) tandem mutation identified in 1 primary tumor and its corresponding metastasis has, to our knowledge, not been detected previously in melanomas.

To better determine at what stage in the primary lesions the N-*ras* codon 61 mutations occur, the different growth phases of the mutated primary melanomas were analyzed. We could show that the mutations are present already in the rgp and are preserved in the vgp. Furthermore, in 2 cases, the primary tumor was associated with a nevus (a dysplastic and compound nevus, respectively), and in both cases the mutation was present already at this stage. The demonstration of N-*ras* mutations in melanoma-associated nevi indicates that such mutations are already present in precursor lesions and may be among the initial genetic alterations leading to melanoma development. Similar results, *i.e.*, detection of *ras* mutations in the rgp of primary melanomas as well as in associated nevi, has been reported recently by Demunter *et al.* (18, 19).

UV light has been proven to induce activation of the N-*ras* gene (20). The mutations that were detected in the present study may all be explained by UV-induced DNA lesions; the CAA(Gln)-AAA(Lys) mutation may arise from mispairing of adenosine with 8-oxo-deoxyguanosine, whereas the other mutations all occurred opposite dipyrimidine sequences. Together, our results indicate that the activating N-*ras* codon 61 mutations occur early during melanoma development as a result of UV irradiation.

This study shows clearly that N-*ras* codon 61 mutations are preserved throughout melanoma tumor progression, once they have occurred in the primary lesions. This was demonstrated by the fact that metastases belonging to 19 of the 20 mutated primary tumors showed the same mutation as the primary tumor from which they originated. Furthermore, in all cases where multiple metastases in the same patient were analyzed, the mutation was detected in every single metastasis. From 1 patient as many as six metastases were available, and all of them retained the arginine mutation found in the primary tumor. Together, these results indicate that N-*ras* codon 61 mutations are stable during melanoma tumor progression, and furthermore, that the metastasizing cells are recruited from cell clones of the primary tumors carrying the N-*ras* mutations, and that in patients with multiple metastases, there is a clonal relationship between the different metastases.

In none of the patients with primary tumors that were wt for N-*ras* codon 61 did mutations arise in the metastases. This indicates that N-*ras* codon 61 mutations are not the critical event for metastasis initiation. That N-*ras* mutations do not play a major role in metastasis initiation has been suggested already in previous studies. In these studies, the mutation frequency between unrelated primary and metastatic melanomas were compared, and mutations were found to occur no more commonly in the metastases (9, 11, 16). Although *ras* mutations may be without importance for metastasis initiation, our finding that they are preserved in the metastases suggests that they may still have an important influence on tumor progression in the subgroup of melanomas in which they occur.

Our finding that in a subset of melanoma patients N-*ras* codon 61 mutations are early events that may be of importance for tumor progression and clonal expansion is in good agreement with the results of two experimental studies by Chin *et al.* (21), in which transgenic mice models have been used to determine the effect of activated *ras* on melanoma development. In mice that are deficient for Ink4a, melanocyte-specific expression of H-ras^{G12V} leads to development of nonmetastatic melanomas at high frequency (21). Furthermore, in a model where

the H-*ras*^{G12V} expression was under control of doxycycline, the same investigators showed that tumors regressed upon doxycycline withdrawal (22). These experiments demonstrate that activated *ras* has a role not only in melanoma initiation but also in melanoma tumor maintenance.

To date, only a few other studies have analyzed paired primary and metastatic melanomas for *ras* mutations. In all cases, the number of patients has been limited. Ball *et al.* (11) did not find a relationship between *ras* mutations in primary tumors and metastases, whereas in two other studies, the *ras* mutations present in the primary tumors were also detected in the corresponding metastases (8, 18). Thus, our study of paired primary and metastatic melanomas from 53 different patients constitutes the most extensive study performed and shows clearly that there is a strong relationship between the N-*ras* genotype of primary melanomas and corresponding metastatic lesions.

In SSCP analyses of DNA extracts from the majority of tumors with N-*ras* mutations, the mutant and wt bands had similar intensities. This finding demonstrates that the N-*ras* codon 61 mutations are present in the majority of melanoma cells, not only in a minor fraction of them. Moreover, it indicates that most tumor cells are heterozygous and carry one mutant and one wt allele. There was evidence of loss of the wt allele only in rare cases. This corresponds well with the documented effect of mutated N-*ras* as a dominant oncogene.

We found that the N-*ras* codon 61 mutations had no significant influence on patient overall survival. However, our series of melanoma patients were selected to consist exclusively of patients who had developed metastases and were thus enriched for patients with a poor prognosis. Therefore, this set of patients is not optimal for an analysis of the possible prognostic impact of N-*ras* codon 61 mutations. This fact may also explain the lack of significant influence of previously documented prognostic factors, such as tumor ulceration and gender on patient survival in our study. Despite this, our results are similar to those reported by Van Elsas *et al.* (16); the classical N-*ras* codon 12, 13, and 61 mutations do not have an impact on survival. In contrast, the N-*ras* codon 18 mutation, which was recently detected in a subset of primary melanomas, seems to significantly improve patient outcome, because tumors carrying this mutation seem to lack metastasizing capacity (18).

In summary, by screening a large series of paired primary and metastatic melanomas for N-*ras* codon 61 mutations, we showed both that the mutations arise early in melanoma development, because they were detected in the rgp as well as in precursor lesions, and also that they are preserved during tumor progression into the vgp and metastatic lesions. The recent finding that B-raf mutations occur at high frequency in melanomas (23) further supports the importance of the ras-raf-MEK-ERK pathway in melanoma. This makes activated N-*ras* and the ras-signaling pathway attractive targets for future therapeutic trials in melanoma patients. Induction of immune responses against mutated *ras* (24) as well as the use of chemotherapeutic agents acting on ras (25, 26) may be successful therapeutic strategies.

ACKNOWLEDGMENTS

We thank Bo Nilsson for help with statistical analyses.

REFERENCES

1. Clark, W. H., Jr., Elder, D. E., Guerry, D., IV, Epstein, M. N., Greene, M. H., and Van Horn, M. A study of tumor progression: the precursor lesions of superficial spreading and nodular melanoma. Hum. Pathol., *15:* 1147–1158, 1984.

2. Joneson, T., and Bar-Sagi, D. Ras effectors and their role in mitogenesis and oncogenesis. J. Mol. Med., *75*: 587–593, 1997.

3. Bos, J. L. *ras* oncogenes in human cancer: a review. Cancer Res., *49:* 4682–4689, 1989.

4. Der, C. J., Finkel, T., and Cooper, G. M. Biological and biochemical properties of human *ras*^{H} genes mutated at codon 61. Cell, *44:* 167–176, 1986.

5. Trahey, M., and McCormick, F. A cytoplasmic protein stimulates normal N-*ras* p21 GTPase, but does not affect oncogenic mutants. Science (Wash. DC), *238:* 542–545, 1987.

6. Polakis, P., and McCormick, F. Structural requirements for the interaction of p21^{ras} with GAP, exchange factors, and its biological effector target. J. Biol. Chem., *268:* 9157–9160, 1993.

7. Raybaud, F., Noguchi, T., Marics, I., Adelaide, J., Planche, J., Batoz, M., Aubert, C., de Lapeyriere, O., and Birnbaum, D. Detection of a low frequency of activated *ras* genes in human melanomas using a tumorigenicity assay. Cancer Res., *48:* 950–953, 1988.

8. 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.

9. 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.

10. Shukla, V. K., Hughes, D. C., Hughes, L. E., McCormick, F., and Padua, R. *ras* mutations in human melanotic lesions: K-*ras* activation is a frequent and early event in melanoma development. Oncogene Res., *5:* 121–127, 1989.

11. 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. Investig. Dermatol., *102:* 285–290, 1994.

12. Carr, J., and Mackie, R. M. Point mutations in the N-*ras* oncogene in malignant melanoma and congenital naevi. Br. J. Dermatol., *131:* 72–77, 1994.

13. Platz, A., Ringborg, U., Månsson Brahme, E., and Lagerlöf, B. Melanoma metastases from patients with hereditary cutaneous malignant melanoma contain a high frequency of N-*ras* activating mutations. Melanoma Res., *4:* 169–177, 1994.

14. Jafari, M., Papp, T., Kirchner, S., Diener, U., Henschler, D., Burg, G., and Schiffmann, D. Analysis of *ras* mutations in human melanocytic lesions: activation of the *ras* gene seems to be associated with the nodular type of human malignant melanoma. J. Cancer Res. Clin. Oncol., *121:* 23–30, 1995.

15. 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.

16. 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.

17. 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. Investig. Dermatol., *111:* 757–761, 1998.

18. 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.

19. 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. Investig Dermatol., *117:* 1483–1489, 2001.

20. Van der Lubbe, J. L. M., Rosdorff, H. J. M., Bos, J. L., and Van der Eb, A. J. Activation of N-*ras* induced by ultraviolet irradiation *in vitro*. Oncogene Res., *3:* 9–20, 1988.

21. Chin, L., Pomerantz, J., Polsky, D., Jacobson, M., Cohen, C., Cordon-Cardo, C., Horner, J. W., II, and DePinho, R. A. Cooperative effects of *INK4a* and *ras* in melanoma susceptibility *in vivo*. Genes Dev., *11:* 2822–2834, 1997.

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. 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.

24. Hunger, R. E., Brand, C. U., Streit, M., Eriksen, J. A., Gjertsen, M. K., Saeterdal, I., Braathen, L. R., and Gaudernack G. Successful induction of immune responses against mutant *ras* in melanoma patients using intradermal injection of peptides and GM-CSF as adjuvant. Exp. Dermatol., *10:* 161–167, 2001.

25. Jansen, B., Schlagbauer-Wadl, H., Kahr, H., Heere-Ress, E., Mayer, B. X., Eichler, H. G., Pehamberger, H., Gana-Weisz, M., Ben-David, E., Kloog, Y., and Wolff, K. Novel Ras antagonist blocks human melanoma growth. Proc. Natl. Acad. Sci. USA, *96:* 14019–14024, 1999.

26. Rowinsky, E. K., Windle, J. J., and Von Hoff, D. D. Ras protein farnesyltransferase: a strategic target for anticancer therapeutic development. J. Clin. Oncol., *17:* 3631–3652, 1999.

Clinical Cancer Research

Screening of N-*ras* Codon 61 Mutations in Paired Primary and Metastatic Cutaneous Melanomas: Mutations Occur Early and Persist throughout Tumor Progression

Katarina Omholt, Sofia Karsberg, Anton Platz, et al.

Clin Cancer Res 2002;8:3468-3474.