

Relevance of Ultraviolet-Induced N-ras Oncogene Point Mutations in Development of Primary Human Cutaneous Melanoma

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Intermittent or recreational exposure to sunlight is thought to contribute to development of human cutaneous melanoma. We investigated the incidence of ras oncogene mutation in human cutaneous melanoma in connection to sun-exposed body sites in the patient, using a large series of DNA samples derived from paraffin-embedded material as well as from fresh tumor samples and cell lines. We first show that, of the ras family, predominantly N-ras is activated (15%), whereas rarely H-ras or K-ras are mutated. The occurrence of N-ras mutations correlates with continuous exposure to sunlight of the primary tumor site. Of all tumors initiated on chronically sun-exposed body sites, 26% contained mutated N-ras, in contrast to 0% of sun-protected melanomas. Melanoma lesions obtained from patients from North or Central Europe contained fewer N-ras mutations (12%) as compared with patients from Australia (24%). Mutations were specifically associated with nodular melanoma and to a lesser extent with lentigo malignant melanoma. N-ras mutations did not correlate with metastasis or survival parameters. This study identifies a subset of cutaneous melanomas that contain in the primary lesion ultraviolet-induced N-ras mutations, which are maintained through further progression. (Am J Pathol 1996, 149:883–893)

The incidence of cutaneous melanoma and the resulting mortality have increased considerably during the last decade, although recent studies indicate that the mortality rate may be leveling off in cohorts born after 1960.^{1–4} For the nonmelanoma skin cancer squamous cell carcinoma, the increasing occurrence has been clearly linked to the escalating levels of ultraviolet radiation (UVR) and also to altered sun habits.⁵ Epidemiological studies have indicated that a linear correlation exists between squamous cell carcinoma incidence and UV dose.⁵ In agreement with this notion, xeroderma pigmentosum patients suffering from reduced or abrogated UV damage repair have a 1000-fold increased risk for developing any type of skin cancer.⁶

The relationship between UV and melanoma is much more complex. First, this relationship is not dose dependent, with the exception of the development of lentigo malignant melanoma, which, as such, behaves similar to nonmelanoma skin cancer. Second, the different subtypes of melanoma each show a distinctive causal relationship to sun exposure.⁷ Intermittent or recreational (peak) exposure to sunlight and repeated instances of sunburn are described to be risk factors for the development of melanoma.^{8,9} In contrast, occupational exposure to UV seems to decrease the relative risk of developing some forms of melanoma, suggesting that melanoma is not simply caused by accumulating UV dose.^{9,10} Other factors thought to be involved in determination of this relative risk are specific for the host, such as skin phototype and presence of dysplastic nevi or total nevus count.^{11,12}

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The mutational activation through UV of *ras* oncogenes and the p53 tumor suppressor gene in human skin cancer have been studied extensively. In squamous cell carcinoma, UV-induced damage to DNA results in mutational activation of *ras* and p53 genes in a high proportion of primary tumors.¹³⁻¹⁵ Particularly, the p53 mutations described mainly occurred at lesions opposite of possible pyrimidine dimers and are characterized by C→T and CC→TT transitions, pointing to UVR as the carcinogenic agent involved.¹⁶ In addition, UVR induces p53 mutations in premalignant skin lesions in man.¹⁷ Furthermore, in xeroderma pigmentosum patients, a mutation spectrum indicative for UV-induced cyclobutane damage has been clearly shown both for the p53 gene¹⁸ and the *ras* genes.¹⁹ A melanoma cell line derived from a xeroderma pigmentosum patient was shown to carry mutated N-*ras*.²⁰ *In vitro*, *ras* genes can be activated by UVR,^{21,22} and in a murine model, *ras* activation was detected in UV-induced skin tumors.²³

A number of groups have reported the presence of activated *ras* genes in human melanoma lesions.²⁴⁻³¹ The incidence of mutated *ras* varied from 6 to 36%, whereas most mutations were detected in N-*ras* and some in H-*ras*. Peculiarly, two studies also reported prominent K-*ras* activations that could not be confirmed by others.^{30,31} *ras* mutations have been clearly linked to particular stages of differentiation phenotype²⁸ and to malignant progression.³¹ In a previous study, we have shown that in a group of 37 melanoma patients 7 carried mutated N-*ras*, and most importantly, activation occurred only in primary melanomas that had been chronically exposed to sunlight.²⁹

To assess the general relevance of UVR for N-*ras* activation in melanoma, we have now analyzed more than 250 primary melanomas, metastases, and cell lines from different geographical areas in Europe and Australia. The overall incidence of N-*ras* activation was 15%, with similar frequencies detected in the groups of primary and metastatic lesions. Aberrations in the N-*ras* gene predominantly occur in primary tumors arising on chronically sun-exposed body sites. This study provides evidence for UVR being involved in the activation of the N-*ras* oncogene in a substantial group of melanomas on sun-exposed body sites.

Materials and Methods

Tumor Material, Histology, and Patient Data

Melanoma material was collected through the Melanoma Cooperative Group of the European Organiza-

tion for Research and Treatment of Cancer. Paraffin-embedded (archival) samples as well as purified DNA preparations were entered in this study. Of each series of paraffin slides (5 to 10 μm), one was stained using hematoxylin and eosin and diagnosis was confirmed by the pathologist. Tumor-rich parts of the slide were indicated. Data on the particular tumor sample (eg, Breslow thickness, Clark level of invasion, type of melanoma, and whether primary or metastasis) were provided as well as data on the patient's sun history and other related subjects, such as skin phototype, use of UVA lamps, and indication of xeroderma pigmentosum. Furthermore, the precise location of the primary tumor in each case was indicated. Finally, follow-up data (as of December 1994) were obtained on the course of the disease, occurrence of metastasis, and date of death.

DNA Isolation

Tumor tissue was collected from one or two slides. After deparaffinization with two successive washes with xylene, tissue was washed with 96% ethanol twice and dried with acetone. Protein was digested in 100 μl of PK2 buffer (10 mmol/L Tris, pH 8.3, 1 mmol/L EDTA, 0.5% Tween-20) containing 50 $\mu\text{g}/\text{ml}$ proteinase K (Boehringer, Mannheim, Germany) overnight at 56°C. The resulting mixture was heated to 95°C for 5 minutes and stored on ice for 15 minutes, after which the solution was cleared by centrifugation. This crude DNA extract was further purified using silica preparations.³² Briefly, the DNA was adsorbed to silica preparations (Janssen Chimica, Beersse, Belgium) in guanidium-thiocyanate-containing buffer, washed, and eluted in 10 mmol/L Tris, pH 8, 1 mmol/L EDTA at 56°C. This material was stored at 4°C.

Polymerase Chain Reaction (PCR)-Single-Stranded Conformation Polymorphism (SSCP) Analysis

Of the DNA extract, 0.5 to 3 μl was used for amplification of N-*ras*-specific exon 1 and 2 sequences using the following primers: for exon 1, 5'-GACTGAGTACAACTGGTGG (N1a) and 5'-GGCCTCACCTCTAGGTG (N1b) were used yielding a fragment of 118 bp; for exon 2, a 103-bp fragment was generated using 5'-GGTGAAACCTGTTTGTGGA (N2a) and 5'-ATACACAGAGGAAGCCTTCG (N2b).³³ Alternatively, 5'-CTGGTGTGAAATGACTGAGT (N1c) plus N1b and 5'-GTTATAGATGGTGAAACCTG (N2c) plus N2b were used to generate an exon 1 fragment of

130 bp and an exon 2 fragment of 112 bp. For amplification of H-ras, the following primer sequences were used: 5'-CAGGCCCTGAGGAGCATG (H1a), 5'-GTATTCGTCCACAAAATGGTTCT (H1b), 5'-TCCTG-CAGGATTCCTACCGG (H2a), and 5'-GGTTCACCTG-TACTGGTGGG (H2b), generating fragments of 113 and 194 bp for exons 1 and 2, respectively. For K-ras amplification, the following primers were employed: 5'-GGCCTGCTGAAAATGACTGA (K1a), 5'-GTCCTG-CACCAGTAATATGC (K1b), 5'-TTCCTACAGGAAG-CAAGTAG (K2a), and 5'-CACAAAGAAAGCCCTCCC-CA (K2b). The K-ras primers generated 162- and 128-bp products for exons 1 and 2, respectively. The 20- μ l amplification mixture further consisted of 250 μ mol/L dATP, dTTP, and dGTP, 50 μ mol/L dCTP (Pharmacia, Woerden, The Netherlands), 0.4 μ Ci of dCTP (Amersham, Wycombe, UK; 3000 Ci/mmol), 50 pmol/L of each primer, 0.04 U of *T. thermophilus* DNA polymerase, and 2 μ l of 10X buffer (Sphaero Q, Leiden, The Netherlands). Cycling was performed using the following program: 4 minutes at 95°C; 36 cycles of 30 seconds at 95°C, 60 seconds at 50°C, and 30 seconds at 72°C; and 5 minutes at 72°C. For SSCP analysis,³⁴ 4 μ l of the amplified mixture was mixed with gel-loading buffer without NaOH. One-half of this solution was denatured at 95°C for 4 minutes and loaded next to the nondenatured sample onto an 8% polyacrylamide gel containing either 10% glycerol/1X TBE (89 mmol/L Tris, 89 mmol/L boric acid, 2 mmol/L EDTA) or 5% glycerol/0.5X TBE. Throughout this study it was found that consecutive use of both gel conditions for each sample yielded the most accurate information. N-ras exon 1 mutations were more readily identified on 5% glycerol/0.5X TBE gels, and exon 2 aberrations were better visualized using 10% glycerol/1X TBE. Gels were run for 4 to 6 hours at 30 W at room temperature. Vacuum-dried gels were exposed to Fuji RX X-ray film for 6 to 72 hours.

Direct Sequencing

The DNA extract was reamplified for sequence analysis as described above using 250 μ mol/L of each dNTP. The PCR fragment was gel purified using the Mermaid procedure (Stratagene, La Jolla, CA), and 10 to 50 ng of the fragments was sequenced by using γ -³²P-labeled primers and the T7 system (Pharmacia) or by using the CircumVent procedure (New England Biolabs, Beverly, MA).

Statistical Analysis

Statistical analysis was carried out using the SPSS statistical package for Windows. Possible correla-

tions between *ras* mutations and several parameters, including primary site of the tumor, origin, and sun exposure, were evaluated using either the χ^2 analysis or Fisher's exact *t*-test. Multiple variables were tested for their relevance to the presence of N-ras mutations by multiple (linear) regression analysis. Survival of patients after diagnosis of primary or metastatic melanoma and interval between primary and metastatic disease were analyzed in mutation (and other) groups using Kaplan-Meier analysis and log-rank statistics.

Results

Detection of N-ras Mutations in Paraffin-Embedded Material

DNA samples were prescreened for aberrations in the genomic sequence of N-ras by using PCR-SSCP analysis. Usually, the procedure we followed was composed of two PCR experiments using different primer pairs for either exon 1 or exon 2 sequences of N-ras. Next, both exon 1 and exon 2 samples were run through SSCP gels using two different conditions, as we found in the course of this study that some mutations shifted visibly better in 10% glycerol/1X TBE gels than in 5% glycerol/0.5X TBE, and *vice versa* (see Materials and Methods). The SSCP gels were analyzed, and samples that were suspected to contain mutated *ras* alleles were used for direct sequencing analysis. Four aberrations were identified in a representative example of a SSCP analysis of 21 cell lines and fresh tumor samples originating from patients diagnosed in Australia (Figure 1). The simultaneous analysis of PCR products of exons 1 and 2 is facilitated by loading control amplifications for each exon individually (Figure 1, last two lanes).

Different N-ras Mutations in Primary Tumors

Paraffin-embedded material from samples 238 and 239 (the latter histologically subdivided into parts a and b) were analyzed for exon 1 mutations in N-ras (Figure 2). Samples 239a and 239b represent two distinct local metastases of primary tumor 238. Satellite 239b contains a 13Gly \rightarrow Val mutation (Figure 2B; sequence data not shown). In contrast, both the primary tumor 238 and satellite lesion 239a carry a 13Gly \rightarrow Arg mutation that is obvious in the top panel (Figure 2A). This demonstrates that the N13Arg mutation is present in a substantial amount of tumor cells in the primary tumor and in local metastasis

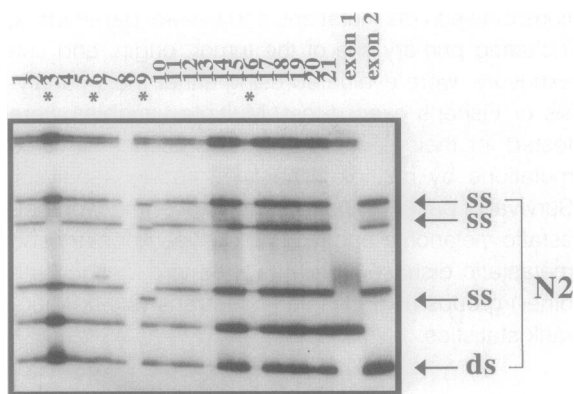


Figure 1. *N-ras* mutations in 21 Australian melanoma samples. Fragments from exons 1 and 2 of the *N-ras* gene were amplified and subjected to SSCP gel electrophoresis, using 10% glycerol and 1X TBE. Samples are loaded as follows: lane 1, 40-786; lane 2, 40-783; lane 3, 40-755; lane 4, 40-725; lane 5, 40-713; lane 6, 40-208; lane 7, 40-034; lane 8, 40-024; lane 9, 40-179; lane 10, 40-438; lane 11, 40-016; lane 12, 40-774A; lane 13, 40-774B; lane 14, 40-547; lane 15, 40-815; lane 16, 40-745; lane 17, 40-454; lane 18, 40-798; lane 19, 40-438; lane 20, 40-811; lane 21, 40-303. Lanes on the right of each gel panel were loaded with control exon 1 amplification product (generated from a mixture (1:1) of wild-type and 13-val-mutated *N-ras* genomic clones), followed by control exon 2 product (generated from the same mixture of plasmids). Samples carrying mutated *N-ras* are indicated with an asterisk. Sample 8 did not yield any detectable fragments in this particular experiment.

239a, whereas the progenitor cells for metastasis 239b containing the N13Val mutation are not detectable in tumor 238 (all three tumor areas were estimated to contain approximately 80 to 90% tumor cells). In contrast, in five other patients, different *N-ras* mutations were detected in the primary tumor (cf Table 1), indicating that this is a rather common phenomenon in melanoma.²⁹

Fifteen Percent of All Human Cutaneous Melanomas Carry Mutated *N-ras*

Using a similar technology, point mutations in the *K-ras* and *H-ras* gene were determined. PCR-SSCP analysis of 68 tumor samples revealed only one aberration in *H-ras* (a 12Gly→Val substitution; cf tumor 40-254, Table 1) and none in *K-ras* (Figure 3). We concluded from this series of samples that *H-ras* (1/68, <2%) and *K-ras* activation (0/68) do not play a significant role in the etiology of the melanomas analyzed.

In total, 272 independent tumor samples, consisting of 175 primary tumor samples, 63 metastases, and 32 cell lines (for 2 samples the origin was unknown) were analyzed for *N-ras* mutations. Of 272 samples, 42 (15%) carried one or more mutations in *N-ras*, of which all but 4 were confirmed by direct sequencing (Table 1).

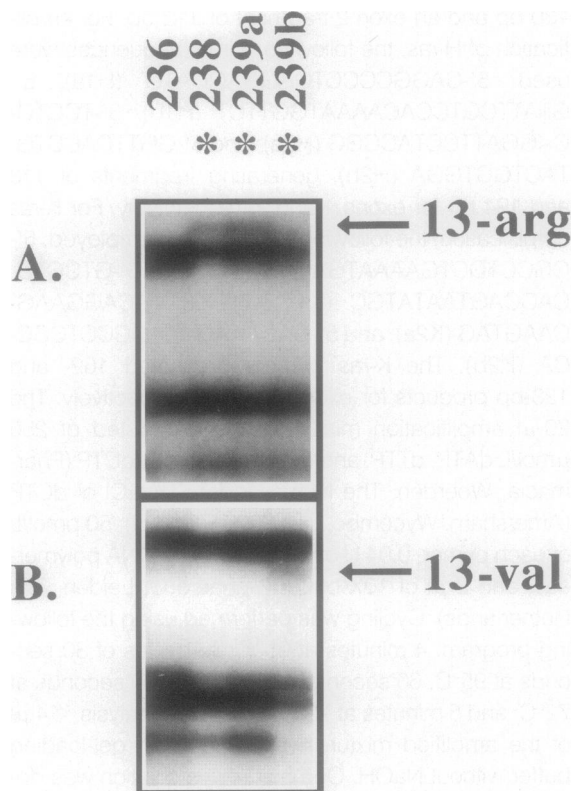


Figure 2. *N-ras* mutation analysis of paraffin-embedded melanoma samples. Samples 236, 238, and 239 (cf Table 1) were used to amplify *N-ras* exon 1 fragments. Samples 239A and 239B represent two separable local metastases from primary tumor sample 238. Unrelated sample 236 shows the gel pattern for wild-type *N-ras* exon 1 PCR products. **A:** SSCP analysis using 5% glycerol, 0.5X TBE during electrophoresis. **B:** Result obtained using 10% glycerol, 1X TBE. Samples bearing mutant *N-ras* alleles are marked with an asterisk. On the right side of both panels the position of fragments shifting in the gel is indicated by an arrow, and the resulting amino acid substitution is shown.

As is apparent from Table 1, 20 tumors were found to contain one or more mutations in exon 1 of *N-ras* (mostly in codon 13 and only four times in codon 12), whereas 22 tumors carried mutations in exon 2 (codon 61). The spectrum of amino acid substitutions resulting from these mutations is more or less restricted to 13-val, 61-lys, 61-leu, and 61-arg. The three position 61 mutations were all previously shown to be highly transforming in focus-forming assays and all resulted in an approximately 10-fold reduced GTPase activity.³⁵ Human *N-ras* genes carrying the four mutations generated by PCR mutagenesis were expressed in Rat-1 fibroblasts and found to be highly transforming (data not shown). Furthermore, a number of these mutated samples contained two different mutations at the same codon. In two patients it was found that distinct metastases contained one of the two different mutations. These data

Table 1. Summary of ras Mutations Detected in 265 Melanoma Cases

Number of cases	Subclassified diagnosis*	Affected ras gene and codon†	Mutation (amino acid substitution)
2	LMM, SSM	N12	GGT→CGT (arg)
8	LMM (2), SSM (3), NM (3)	N13	GGT→GTT (val)
3	LMM (1), SSM (2)	N13	GGT→GAT (asp)
2	SSM, MM	N13	GGT→CGT (arg)
7	NM (4), MM (2), unknown (1)	N61	CAA→AAA (lys)
6	SSM (2), NM, MM (2), unknown (2)	N61	CAA→CTA (leu)
3	SSM, NM, Uncl	N61	CAA→CGA (arg)
4	LMM, SSM, NM, unknown	N61	NR‡
1	NM	N12	GGT→GAT (asp)
		N13	GGT→GTT (val)
2	LMM, MM	N13	GGT→GTT/GAT (val/asp)§
1	NM	N13	GGT→GTT/CGT (val/arg)
1	SSM	N13	GGT→GAT/CGT (asp/arg)
1	Unknown	N61	CAA→CTA/CGA (leu/arg)
1	NM	N61	CAA→CGA (arg)
		H12	GGC→GTC (val)

*Diagnosis of primary lesions was done at the collaborating institute. MM, malignant melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; LMM, lentigo malignant melanoma; Uncl, unclassifiable melanoma; Unknown, occult primary lesion.

†Indicated are the ras gene affected and the codon that carries the mutation. N12 denotes a mutation in codon 12 of the N-ras gene.

‡NR, SSCP result only; no result from direct sequencing.

§Two different mutations are identified by direct sequencing in the same codon of the N-ras gene.

can be explained by a single event of UV adduct formation (see Discussion).

N-ras Mutation Is Correlated with Chronic Sun Exposure of the Primary Body Site

Next, N-ras mutation frequency was analyzed according to sun exposure of body sites where the primary melanomas arose (Table 2). This revealed a highly significant correlation between solar exposure and incidence of mutated N-ras ($P < 0.005$). Chronically UV-exposed primary tumors show a twofold enhanced mutation incidence when compared with tumors induced on intermittently exposed primary body sites. Strikingly, 38 tumors taken from sun-protected sites showed no N-ras mutation at all. When we compared the incidence of N-ras mutation according to primary body sites irrespective of solar exposure, a similar trend, although not significant, was observed. The highest incidence of mutant ras was found in tumors arising on body sites such as the face or head (22%) compared with the limbs (15%) or the trunk (11%). Furthermore, in a subset of 90 tumors for which sun history data of the patient were available, we found an enhanced N-ras mutation incidence associated with occupational or chronic exposure to sunlight when compared with recreational (intermittent) exposure (11 mutations in 49 chronically exposed patients (22%) and 5 mutations in 41 intermittently exposed patients (12%)). These observations suggest that UV dose accumulation, instead of UV peak exposure, results in more frequent N-ras activation. The origin of the analyzed

patients was categorized into North and Central Europe (The Netherlands, Belgium, Germany, and France), Mediterranean area (Greece, Italy, and Israel), or Australia (plus one patient originating from Angola, Africa). N-ras mutations occurred less frequently in Northern Europe ($P < 0.02$) and more frequently in Australia ($P = 0.060$, not significant). An intriguing observation made was that male patients seemed to carry more N-ras-mutated melanomas than female patients did (27 of 140 = 19.3% for males and 15 of 130 = 11.5% for females; $P = 0.087$). However, this difference could be explained by the fact that males in our study had experienced more occupational sun exposure and therefore showed a higher N-ras mutation incidence. Finally, in our series, N-ras mutation was not related to skin phototype (not shown). Altogether, the data stress the fact that melanoma incidence (related to peak or intermittent sun exposure) and N-ras activation incidence follow different rules.

Prognostic/Diagnostic Significance of Mutated N-ras in Melanoma Patients

Mutations were found predominantly in nodular melanoma (28%) and lentigo malignant melanoma (23%) as compared with superficial spreading (14%) or unclassified melanoma (4%, also including 6 cases of acral lentiginous melanoma; Table 3). The group of malignant melanomas represents a number of tumors collected in Australia without clinical subclassification, of which 15% contained mutated N-ras. Apparently, N-ras was preferentially activated in

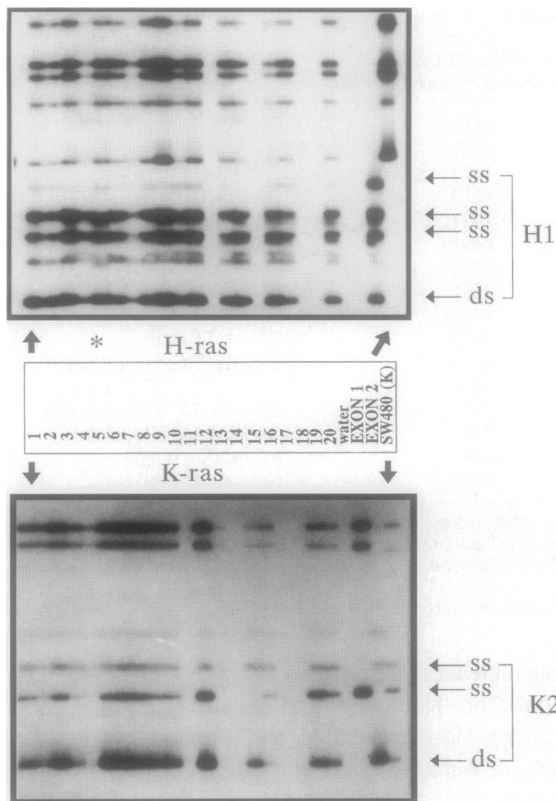


Figure 3. *H-ras* and *K-ras* mutation analysis in human melanoma tumor material. PCR-SSCP analysis for *H-ras* and *K-ras* in separate reactions was carried out for 20 melanoma samples. For both *H-ras* (top panel) and *K-ras* (bottom panel), control amplifications were performed for exons 1 or 2 separately, using as a template genomic DNA from melanoma cell line IGR39, which was previously shown to be wild type for *N-ras*, *H-ras*, and *K-ras*.²⁹ Furthermore, a control amplification was done for *K-ras* exons 1 and 2, with DNA isolated from colon carcinoma cell line SW480, that carries a K12-val mutation. The *H-ras* mutation in sample 4 is indicated by an asterisk (left side). Samples 1 to 20 are loaded in the following order: 40-851, 40-852, 40-854, 40-254, 40-485, 40-016, 40-024, 40-034, 40-179, 40-208, 40-291, 40-303, 40-352, 40-358, 40-438, 40-464, 40-496, 40-496C, 40-544A, 40-544B.

the particularly aggressive nodular melanoma as well as in lentigo malignant melanoma, a disease associated with chronic UV exposure.

N-ras activation was found to occur with similar frequency in primary or metastasized tumors as well as in cell lines (Table 4). Therefore, a subclass of 15% of melanomas seems to exist, characterized by

an early activation (ie, in the melanoma lesion *in situ*) of an *N-ras* allele, that is present throughout progression to disseminated disease (Table 4), indicating that *N-ras* mutation is not involved in metastasis. This is further strengthened by the observation that *N-ras* aberrations in primary melanoma were not correlated to Breslow thickness or Clark level of invasion in our study (not shown). These data also show that *N-ras* activation in melanoma cell lines is not a culture artifact, allowing the biological significance of *ras* activation in human melanoma to be studied using cell lines.

Biological Significance of *N-ras* Mutation in Melanoma

When survival analysis was performed, the presence of *N-ras* mutations did not seem to influence the total survival after diagnosis of the first melanoma (not shown). However, the survival period after diagnosis of metastasized disease (Figure 4A) seemed to be extended in melanoma patients with mutated *ras* (mean period is 31.3 months; SD = 28.0 months) as compared with wild-type *N-ras* (mean is 18.7 months; SD = 19.7 months). This effect is not statistically significant ($P = 0.10$), but as a trend it might suggest that, in contrast to findings in other human cancers and in contrast to its oncogene function, mutated *ras* may be a favorable prognostic factor in human melanoma.

A significantly ($P < 0.01$) extended survival was detected for patients from Northern and Central Europe (98 ± 7.8 months) as compared with Mediterranean (125 ± 34 months) or Australian patients (69 ± 9.5 months). The latter group of patients experiences a particularly shortened ($P < 0.001$) interval period between diagnosis of primary and metastatic disease (Figure 4B). This may be partly explained by assuming that the Australian melanomas are diagnosed at a more advanced stage of disease. Alternatively, 71% of the malignant melanomas in this series were derived from Australia, and this group may represent a specifically aggressive

Table 2. Correlation between Sun Exposure of the Primary Tumor Site and *N-ras* Mutation in Human Melanoma Patients

N-ras status	Sun exposure of the primary tumor site			Total samples
	Chronic	Intermittent	Rare	
Mutated N-ras	19 (25.7%)*	20 (13.8%)	0 (0%)	39
Wild-type <i>ras</i>	55	125	38	218
Total	74	145	38	257

*Indicated are number of cases and column percentages (in parentheses).
 $\chi^2 = 13.4$ (DF = 2); $P = 0.0013$.

Table 3. *N-ras Status and Diagnosis of the Primary Melanoma Lesion*

N-ras status	Diagnosis of primary tumor					Total cases
	MM	LMM	NM	SSM	Uncl/ ALM	
Mutated N-ras	6 (15.4%)	6 (23.1%)	13 (27.7%)*	12 (14.1%)	1 (3.7%)†	38
Wild-type N-ras	33	20	34	73	26	186
Total	39	26	47	85	27	224

Numbers represent number of cases per group, with the percentage of mutated samples within the column given in parentheses. MM, malignant melanoma (this group consists mainly of archival patient material from Australia and The Netherlands; no subclassification is known); LMM, lentigo malignant melanoma; NM, nodular melanoma; SSM, superficial spreading melanoma; Uncl, unclassifiable melanoma; ALM, acral lentiginous melanoma.

*Significantly more N-ras mutations in NM ($\chi^2 = 4.83$; $P < 0.05$).

†Lower mutation frequency ($\chi^2 = 3.83$; $P = 0.050$; Fisher exact two-tailed, $P = 0.056$).

group of tumors. A third possible explanation emerges from the comparison of the survival of males and females with melanoma. In our series of paraffin-embedded material, two-thirds of the Australian material was collected from male patients. Male patients experience a shortened period to metastasis (72.2 ± 7.6 months; $P = 0.039$) as compared with females (109.3 ± 8.3 months) and consequently experience a shorter survival period (see Discussion).

Discussion

Detection of N-ras Mutations in Paraffin-Embedded Melanomas by PCR-SSCP Analysis

The combination of PCR techniques with the sensitive detection of mutated PCR fragments using SSCP gel electrophoresis potentially allows the genetic characterization of archival tumor material. This routinely paraffin-embedded material can be histologically classified and subdivided, thereby generating the possibility of identifying molecular genetic events during progression of the tumor. PCR-SSCP analysis has been applied to the detection of different genetic aberrations.^{33,36,37} A major problem we encountered during this study was the lack of any detectable PCR product in highly pigmented samples. In most cases, this could be surmounted by adsorption of DNA to silica particles,³² although in a minority of preparations (4 of 321) it still proved impossible to amplify

N-ras fragments. To exclude false negative SSCP results it was necessary to use two different SSCP gel conditions, because not all base changes produced visible band-shifts under all conditions. To exclude false positives, all samples suspected of a mutation by SSCP analysis were sequenced and shown to contain a mutation. Initially, we also sequenced approximately 20 samples that did not show any shifts on SSCP, which in no case revealed an N-ras mutation, indicating that the gross majority of mutations was detected by our prescreening procedure.

N-ras, but Not H-ras or K-ras, Mutations Arise in 15% of Primary Cutaneous Melanomas

In agreement with previous reports, 15% of all tested primary cutaneous melanomas carried an N-ras mutation. H-ras or K-ras activation were not found in significant numbers, suggesting that only mutation of the N-ras gene is involved in tumor development. This corroborates the findings of Albino et al^{24,28} but contrasts the high incidence of K-ras mutations in human melanoma described by others.^{30,31} Remarkably, the H12Val mutation was detected in a melanoma cell line that also contained an N61Arg mutation (cf Table 1), allowing the conclusion that the H12Val mutation by itself has no functional significance.

All of the N-ras mutations described in this series of melanomas have been identified previously in other malignancies.³⁸ Most of the N-ras amino acid substitutions detected in this study have been shown

Table 4. *N-ras Activation Not Correlated to Late Progression of Human Melanoma*

N-ras status	Type of material analyzed			Total cases
	Primary tumor	Metastasis	Cell line	
Mutated N-ras	27 (15.4%)	10 (15.9%)	5 (15.6%)	42
Wild-type N-ras	148	53	27	228
Total	175	63	32	270

Indicated are number of cases and column percentages (in parentheses).

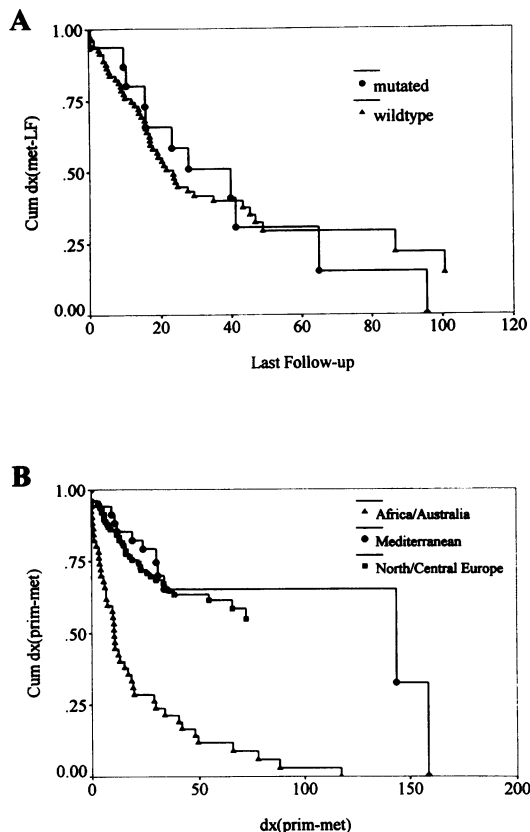


Figure 4. Survival parameters for the patient groups from three continents carrying wild-type or mutated *N-ras* alleles. **A:** Survival as measured after diagnosis of metastasis (Cum dx(met-LF)) was compared for patients carrying wild-type *N-ras* or mutated *N-ras*. Means are 31.3 ± 9.1 months for mutated patients (5 of 16 patients censored) and 18.7 ± 5.1 months (37 of 81 censored) for wild-type patients ($P = 0.10$, log-rank analysis). **B:** The interval period between diagnosis of primary melanoma and metastasis (Cum dx(prim-met)) was compared for groups of patients from Northern and Central Europe, the Mediterranean area, and Australia (1 Central African patient). Mean values are 93 ± 6.2 months (North/Central Europe), 104 ± 13 months (Mediterranean), and 49 ± 9.1 months for Australian patients (log-rank value 11.5, $P < 0.001$).

previously by others to be highly transforming.^{35,39} We confirmed the transforming capacity of the four major *N-ras* mutants (N13Val, N61Leu, N61Lys, and N61Arg) in focus-forming assays. Furthermore, such oncogenic mutations were shown to result in impaired GTP hydrolysis³⁵ and insensitivity to regulation of GTPase activity by the p120-GAP and neurofibromin.^{40,41} As an example, the 61Leu mutation was found to be highly oncogenic as a result of enhanced GTP/GDP exchange combined with decreased GTPase activity.⁴²

UVR Involved in the Mutagenesis of N-ras in Melanoma

In carcinomas of the pancreas, lung, and colon, the varying mutation spectra as measured in the *K-ras*

gene have been interpreted as an indication for the etiological involvement of different carcinogens.³⁸ Similarly, the activation spectrum found in human melanomas can be indicative for a carcinogenic agent involved. The wild-type sequence at codons 12, 13, and 61 of the *ras* genes contains sites for potential pyrimidine dimerization, a lesion that is frequently induced by UVR.¹⁶ In this study, all observed point mutations occur opposite these putative pyrimidine dimers, ie, CCA CCA for wild-type codons 12 and 13 of *N-ras* (Table 1). Moreover, the presence of two *N-ras* mutations in the same melanoma lesion in separate cells can be explained by one event of UVR-induced pyrimidine dimerization. Primary melanoma 83070 contains both N13Val and N13Asp mutations (Table 1) in roughly equal amounts of different cells.²⁹ The induction of one pyrimidine dimer lesion in codon 13 may explain the presence of two mutations in different cells if the UV adduct survives one round of replication. In that case, two repair events may arise from one UV-induced lesion leading to two different mutations.⁴³ The sequential induction of two independent codon 13 lesions in two different cells is considered less likely.

We demonstrate that *N-ras* mutations in melanoma preferentially arise on sun-exposed body sites and show a UV-dose-dependent activation pattern. In particular, *N-ras* mutations are detected to a significantly lesser degree in melanomas arising in North or Central European patients as compared with Australian and Mediterranean patients. Occupational exposure to sunlight elevates the incidence of mutated *N-ras*. Also, primary tumors localized in the face or on the head carry more mutated *N-ras* alleles than tumors arising on the trunk (chronic versus intermittent exposure). This contrasts with the concept of melanoma induction by intermittent or peak exposure to UV. On the other hand, it is not unexpected, as isolated molecular mutagenic events are anticipated to follow more simple dose-response rules. This study provides molecular evidence for the carcinogenic action of UVR in human melanoma. UV irradiation induces these *N-ras* mutations and consequently lies at the molecular basis of this subset of melanomas. Therefore, *N-ras* activation can be considered as a marker for the involvement of UVR in the etiology of these melanomas in a fashion similar to p53 activation in nonmelanoma skin cancer.^{15,17,44} Mutation of p53 has not been investigated in large groups of primary melanoma, although a conclusion from some studies is drawn that p53 is not involved in the generation of primary melanoma.⁴⁵ We are currently resolving the question of whether p53 (mu-

tated by UVR) plays a role in this series of primary melanomas.

The significance of UVR for human melanoma has been debated for many years, because no linear correlation could be found between UV dose received and relative risk of developing melanoma. Epidemiological data suggested that high levels of UV exposure may even be protective in this respect.⁹ However, a consistent deleterious effect of intermittent or recreational sun exposure has been observed in a number of studies.^{1,2} This was explained as a consequence of high doses of carcinogenic UV (peak exposure) acting on relatively unprotected skin.⁸⁻¹⁰ Furthermore, sunburn episodes⁴⁶ during or before adolescence (induction of increased numbers of nevi) and migration to sunny areas before the age of 10⁹ increase the risk of developing nonlentigo malignant melanoma. This leads to a hypothetical chain of events in which UV initiates the development of multiple nevi in childhood, after which subsequent peak exposure to UV in young adult life promotes the formation of malignant melanoma at later ages. In that sense UV may prove to be a complete carcinogen,¹⁶ likely also involved in the induction of *ras* mutations.

Biological Significance of Mutated N-ras in Melanoma Patients

In our melanoma series, no obvious correlation could be found between N-ras activation and progression, as defined by Clark level of invasion, Breslow thickness, or tendency to metastasize (Figure 4). Because the frequency of mutations in primary tumors was identical to that in metastases, N-ras activation is probably involved in the biology of the primary melanoma, although it may also contribute to progression.

In colon cancer as well as in lung cancer and childhood leukemia a direct detrimental prognostic value has been assigned to the detection of a mutated *ras* gene.⁴⁷⁻⁴⁹ In our series of melanoma patients overall survival does not seem to differ between the group carrying mutated *ras* and the wild-type group. We screened a distinct group of patients with metastatic melanoma and found that *ras* mutation was associated with favorable response to immunotherapy, using α -interferon plus interleukin-2 (U. Keilholz and A. van Elsas, unpublished). This was interpreted as an indication that melanoma cells carrying mutated N-ras are more susceptible to eradication by natural killer cells⁵⁰ or by cytotoxic T lymphocytes.⁵¹ The possible notion that mutated *ras*

oncogenes may be associated with improved clinical course of the disease would be remarkable. However, the group of melanoma patients studied in this contribution was not specifically selected for immunotherapy treatment, and only a nonsignificant slightly improved survival after metastatic onset was detected in patients with N-ras mutations (Figure 4A).

Altogether, we suggest that the activation of the N-ras oncogene occurs in a subset of melanomas that received a substantial amount of UVR. This activation invariably is induced in the primary lesion and therefore plays a role in the early stages of melanoma development or progression. Its significance for metastasis and survival remains unclear.

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