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

Andrea van Elsas,* Shuraila F. Zerp,* Silvia van der Flier,* K. Margreet Krüse,* Corlien Aarnoudse,* Nicholas K. Hayward,† Dirk J. Ruiter,‡ and Peter I. Schrier* From the Department of Clinical Oncology,* University Hospital Leiden, Leiden, The Netherlands; the Department of Human Genetics,† the Queensland Institute of Medical Research, Brisbane, Australia; and the Department of Pathology,‡ University Hospital Nijmegen, Nijmegen, The Netherlands

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 mutational activation through UV of ras onco-
genesis and the p53 tumor suppressor gene in human
skin cancer have been studied extensively. In squa-
mous 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 Particu-
larly, the p53 mutations described mainly occurred
at lesions opposite of possible pyrimidine dimers
and are characterized by C-\rightarrow T and CC-\rightarrow 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. Particularly, 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 activa-
tion was 15%, with similar frequencies detected in
the groups of primary and metastatic lesions.
Aber-
rations 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 onco-
genese 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 Organi-
tation 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 \u00bcm), 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 pre-
cise 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,
ocurrence 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 µg/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 min-
utes, after which the solution was cleared by centrifu-
gation. This crude DNA extract was further purified
using silicon preparations.32 Briefly, the DNA was ad-
sorbed to silica preparations (Janssen Chimica,
Beersae, Belgium) in guanoidium-thiocyanate-con-
taining 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 ampli-
fication of N-ras-specific exon 1 and 2 sequences
using the following primers: for exon 1, 5'-GACT-
GAGTACAAACTGGTTGG (N1a) and 5'-GGGCCTCCT-
CACCTAGGTG (N1b) were used yielding a frag-
ment of 118 bp; for exon 2, a 103-bp fragment was
generated using 5'-GGTGAAACCTGTGTGTGGA
(N2a) and 5'-ATACACAGAGGACCTC (N2b).33
Alternatively, 5'-CTGGTGATGAATGACTGAGT (N1c)
plus N1b and 5'-GTTATAGATGTTGAAACCTG (N2c)
plus N2b were used to generate an exon 1 fragment
of
104 mmol/L boric acid, 2 mmol/L EDTA) or 5% glycerol/0.5X TBE gels 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/0.5X TBE gels. Throughout this study it was found that consecutive use of both gel conditions for each sample containing either 10% glycerol/1 X TBE (89 mmol/L Tris, 0.5X buffer without NaOH). One-half of this solution was dried gels were exposed to Fuji RX X-ray film for 6 to 72 hours.

**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 amplification for each exon individually (Figure 1, last two lanes).

**Direct Sequencing**

The DNA extract was reamplified for sequence analysis as described above using 250 μmol/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-
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).
N-ras Activation by Sun Exposure in Human Melanoma

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

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

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

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

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

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
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 melanomas 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.

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

<table>
<thead>
<tr>
<th>N-ras status</th>
<th>Sun exposure of the primary tumor site</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chronic (25.7%)</td>
</tr>
<tr>
<td>Mutated N-ras</td>
<td>19 (25.7%)</td>
</tr>
<tr>
<td>Wild-type ras</td>
<td>55</td>
</tr>
<tr>
<td>Total</td>
<td>74</td>
</tr>
</tbody>
</table>

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

\[ \chi^2 = 13.4 \text{ (DF = 2); } P = 0.0013. \]
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. 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, 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. but contrasts the high incidence of K-ras mutations in human melanoma described by others. 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. Most of the N-ras amino acid substitutions detected in this study have been shown

---

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

<table>
<thead>
<tr>
<th>N-ras status</th>
<th>MM (15.4%)</th>
<th>LMM (23.1%)</th>
<th>NM (27.7%)</th>
<th>SSM (14.1%)</th>
<th>Uncl/ALM (3.7%)</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated N-ras</td>
<td>27</td>
<td>10</td>
<td>15</td>
<td>5</td>
<td>1</td>
<td>42</td>
</tr>
<tr>
<td>Wild-type N-ras</td>
<td>146</td>
<td>53</td>
<td>27</td>
<td>32</td>
<td>228</td>
<td>270</td>
</tr>
</tbody>
</table>

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 lentigious 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, \( P = 0.056 \)).

---

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

<table>
<thead>
<tr>
<th>N-ras status</th>
<th>Primary tumor</th>
<th>Metastasis</th>
<th>Cell line</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutated N-ras</td>
<td>27 (15.4%)</td>
<td>10 (15.9%)</td>
<td>5 (15.6%)</td>
<td>42</td>
</tr>
<tr>
<td>Wild-type N-ras</td>
<td>146</td>
<td>53</td>
<td>27</td>
<td>228</td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>63</td>
<td>32</td>
<td>270</td>
</tr>
</tbody>
</table>

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

\[ \text{Survival parameters for the patient groups from three continents carrying wild-type or mutated N-ras alleles. A: Survival as measured after diagnosis of metastasis (Cum. dist (met-LF)) was compared for patients carrying wild-type N-ras or mutated N-ras. Means are 51.3 ± 9.1 months for mutated patients (5 of 16 patients censored) and 18.7 ± 5.1 months (7 of 81 patients censored) for wild-type patients (a = 0.10, log-rank analysis). B: The interval period between diagnosis of primary melanoma and metastasis (Cum. dist (prim-met)) was compared for groups of patients from Northern and Central Europe, the Mediterranean area, and Australian (Central African patients). Mean values are 9.1 ± 6.2 months (North/Central Europe), 104 ± 13 months (Mediterranean), and 49 ± 9.1 months for Australian patients (log-rank value 11.5, p < 0.001).} \]

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 hydrolysis and insensitivity to regulation of GTPase activity by the p120-GAP and neurofibromin. 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.

**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. 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. 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. 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 different mutations. The sequential induction of two independent codon 13 lesions in two different cells is considered less likely.

We demonstrate that N-ras mutations in melanomas 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.
ated 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. However, a consistent deleterious effect of intermittent or recreational sun exposure has been observed in a number of studies. This was explained as a consequence of high doses of carcinogenic UV (peak exposure) acting on relatively unprotected skin. Furthermore, sunburn episodes during or before adolescence (induction of increased numbers of nevi) and migration to sunny areas before the age of 10 increase the risk of developing nonmelanocytic 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, 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. 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 between the group carrying mutated ras and the wild-type group, 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 cells or by cytotoxic T lymphocytes. 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.

**Acknowledgments**

We thank the following collaborators, who provided us with the tumor material and accompanying patient data: Drs. Bröcker (Wurzburg, Germany), Pologeorgis (Athens, Greece), Vacca (Bari, Italy), Cesarini (Villejuif, France), Avril and Duvillard (Paris, France), Kleeberg (Hamburg, Germany), Schadendorf (Berlin, Germany), Lienard (Brussels, Belgium), Azizi (Tel Hashomer, Israel), Hardmeier (Münsterlingen, Switzerland), and Osanto (Leiden). Nelleke Gruis, Anne-Marie Cleton, and Edwin Abein are gratefully acknowledged for valuable discussions. We thank J. Hermans for advice on the statistical analyses and Susanne Osanto and Wilma Bergman for critically reading this manuscript.

**References**

33. Suzuki Y, Orita M, Shiraishi M, Hayashi K, Sekiya T: ...


42. Feig LA, Cooper GM: Relationship among nucleotide exchange, GTP hydrolysis, and transforming potential of mutated ras proteins. Mol Cell Biol 1988, 8:2472–2478


