N-ras Mutations are Common in Melanomas from Sun-Exposed Skin of Humans but Rare in Mucosal Membranes or Unexposed Skin

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Ras mutations, preferentially in codon 61 of the N-ras oncogene, are common in human cutaneous melanomas. In this study, we questioned the association between ras mutations in primary melanomas and sun exposure. DNA was extracted from formalin-fixed primary melanomas: 28 at chronically sun-exposed head and neck areas, 18 at sites subject to intermittent sun exposure, and 28 from unexposed mucosal membranes (vulva/vagina, anus/rectum, palate). Mutations of both exons of H-, K-, and N-ras genes were examined by polymerase chain reaction/single-strand conformation polymorphism and by direct nucleotide sequencing of the polymerase chain reaction amplified exons. Thirty-two per cent of the head and neck melanomas and 11% of the melanomas from intermittently sun-exposed skin had N-ras codon 61 mutations; comparatively only 7% of the tumors from the unexposed areas had such mutations. One vulvar melanoma had an N-ras codon 12 mutation. No H-ras or K-ras mutations were detected in any sample. The frequency of N-ras exon 2 mutations in melanomas of typically sunbathed skin was compared for the first time with that in melanomas of areas completely protected from sun exposure. The significantly higher frequency ($p = 0.04$, chi square exact test) of these N-ras mutations on the head and neck demonstrates their UV-light induction in a subset of melanomas explaining one of the molecular effects of UV light in human skin. ***Key words: human melanoma/mutation analysis/ras genes/UV exposure. J Invest Dermatol 111:757–761, 1998***

The incidence of human cutaneous melanoma is increasing worldwide, with the highest incidence reported in Queensland, Australia (MacLennan *et al*, 1992). Whereas the number of cutaneous melanomas has increased annually by 6% in Sweden over the last decades (Thörn *et al*, 1989), melanoma in the mucosal membranes and adjacent unexposed glabrous skin from the vulva/vagina has decreased by 3.2% annually (Ragnarsson-Olding *et al*, 1993). This comparison and epidemiologic data suggest that sunlight, presumably UV radiation, is the main etiologic agent (Longstreth *et al*, 1992). Experiments with animal models, including fish and marsupials, also demonstrate the *in vivo* induction of melanoma by UV irradiation (Ley *et al*, 1989; Setlow *et al*, 1989; Anders, 1991; Ley, 1997). The most direct effect of UV light on skin is its interaction with the genetic material (DNA) of cells resulting in point mutations.

Alterations in the ras proto-oncogenes are commonly found in solid tumors of humans, including sporadic cutaneous melanomas (Bos, 1989). The three types of human ras genes, H-ras, K-ras, and N-ras, encode GTP-binding proteins involved in key functions of signal transduction and, thereby, in the control of cellular proliferation. Ras activation occurs by mutations preferentially in codons 12, 13, and 61 (Barbacid, 1978). Despite great variation in early reports on ras mutation frequencies in human cutaneous melanomas (Raybaud *et al*, 1988; Albino *et al*, 1989; Shukla *et al*, 1989; van't Veer *et al*, 1989), subsequent studies including large numbers of patients and refined methodologies, agree convincingly that the N-ras proto-oncogene, activated by mutation of codon 61, is the predominant ras alteration in cutaneous melanomas (Ball *et al*, 1994; Carr and Mackie, 1994; Platz *et al*, 1994, 1996; van Elsas *et al*, 1996). Of these tumors, nodular melanoma (NM) is the subgroup with the highest incidence of N-ras mutations (Jafari *et al*, 1995; van Elsas *et al*, 1996). Additionally, the expression of N-ras p21 is increased in a large percentage of primary melanomas and metastases (Platz *et al*, 1995a). A correlation between N-ras mutation and expression and glutathione transferase Pi expression levels in melanomas may have implications for drug resistance (Platz *et al*, 1995b).

Irradiation experiments *in vitro* on cloned human N-ras sequences (van der Lubbe *et al*, 1988) as well as *in vivo* experiments on mice (Pierceall *et al*, 1992), indicated a link between UV exposure and N-ras activation. Later, the occurrence of ras mutations in primary human cutaneous melanoma was found dependent on anatomical localization, geographic distribution, and the extent of UV-light exposure (van Elsas *et al*, 1996). When tumors from chronically sun-exposed body sites were compared with tumors from intermittently and rarely exposed sites, the incidence of ras mutation was highest in tumors on the face and on the head. Additionally, the mutation frequency was higher in Australian patients than in patients from northern and central Europe (van Elsas *et al*, 1996).

To explore one of the mechanisms of UV-induced carcinogenesis in greater detail, we analyzed mutations of all three ras genes in primary human melanomas from three anatomical areas: the head and neck area (chronic sun exposure), other skin sites (intermittent sun exposure), and mucosal membranes with adjacent skin (complete absence of sun exposure).

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Abbreviations: NM, nodular melanoma; SSM, superficial spreading melanomas.

The questions asked were these: Which ras genes are mutated and what type of mutation can be found in melanomas from the three anatomical areas? What is the frequency of ras mutations in these three anatomical areas? Are the mutations identified consistent with UV-light induction?

MATERIALS AND METHODS

Preparation of tumors Seventy-four formalin-fixed and paraffin-embedded primary melanomas were used for this study. Of these tumors, 28 originated from chronically sun-exposed head and neck areas (excluding the scalp), 18 from less exposed areas (i.e., the abdomen, back, and legs), and 28 from completely unexposed areas: the vulva (covered by glabrous skin and mucosal membrane), vagina (mucosal membrane only), anus and rectum (glabrous skin and mucosal membrane), and palate (mucosal membrane only). The histogenetic subtypes of these melanomas are listed in **Table I**.

From each paraffin bloc 4 μm and 20–40 μm thick sections were cut. The 4 μm thick sections were then stained with hematoxylin and eosin and used to guide the dissection of tumor tissue from surrounding tissue in the 20–40 μm thick sections. Tumor sections were deparaffinized with xylene twice, washed with 70% ethanol twice followed by two acetone washes, and then dried. Proteins were digested in 500 μl PK2 buffer (10 mM Tris, pH 8.3, 1 mM ethylenediamine tetraacetic acid, 0.5% Tween-20) containing 50 μg proteinase K per ml (Boehringer Mannheim GmbH, Mannheim, Germany) overnight at 56°C. DNA was extracted and purified with the Wizard DNA clean-up system (Promega, Madison, WI), and dissolved in 50 μl H_2O.

Polymerase chain reaction (PCR) and single-strand conformation analysis (SSCP) Two consecutive PCR were used to amplify exon 1 and 2 of the H-, K-, and N-ras genes, with nested or seminested primers applied in the second amplification. For the first PCR, 0.05 μg of genomic DNA was used in a 10 μl PCR mixture. The PCR product was diluted 1/20 with H_2O and 1 μl was incubated for the second PCR. Amplification was performed using a DNA thermal cycler (Perkin Elmer, System 9600). Samples were denatured for 4 min at 94°C, incubated for 30 cycles [15–20 cycles in the second PCR (one cycle = 30 s at 94°C, 30 s at 56–65°C, 30 s at 72°C)], and finally extended for 7 min at 72°C. All primers (**Table II**) were synthesized by Scandinavian Gene Synthesis AB (Köping, Sweden). The use of a PCR workstation (UV cabinet) and of aerosol-resistant pipette tips and multiple negative controls minimized the risk of cross-contamination.

The final PCR products, labeled by incorporation of α-^{32}P dCTP, were denatured in denaturing buffer for 10 min at 92°C, and SSCP was carried out on nondenaturing 7.5% acrylamide gels in the presence of 10% glycerol at 18°C, 40 W and without glycerol at 5°C, 30 W. The gel-temperature conditions were strictly controlled by using a thermostatic circulation chamber attached to the glass plate of the gel. Dried gels were exposed to X-ray film.

Nucleotide sequencing Sequence analysis was performed by direct sequencing of the PCR products using the fmol DNA Sequencing System (Promega). The nested primers (**Table II**) were used as sequencing primers. Fragment resolution was carried out on 10% acrylamide-urea gels at 50°C, 30 W. The gels were soaked in a solution containing 10% methanol and 10% acetic acid, then dried and exposed to X-ray film.

Statistical analyses The software StatXact (Cytel Software, Cambridge, MA) was used for chi-square exact test and chi-square exact test for trend (Mehta *et al*, 1984).

Table I. Histogenetic types of the primary melanomas in relation to sun exposure of the anatomical site

Anatomical site	Histogenetic type[a]	Male, n = 37	Female, n = 37	All, n = 74
Sun-exposed areas, n = 28	SSM	9	6	15
	LMM	2	0	2
	NM	8	2	10
	unclassifiable	1	0	1
Intermittently sun-exposed areas, n = 18	SSM	9	1	10
	LMM	0	0	0
	NM	4	2	6
	unclassifiable	0	2	2
Unexposed areas, n = 28	SSM	0	5	5
	MLM	1	10	11
	NM	0	6	6
	unclassifiable	3	3	6

[a]SSM, superficial spreading melanoma; LMM, lentigo maligna melanoma; NM, nodular melanoma; MLM, mucosal lentiginous melanoma.

RESULTS

Genomic DNA from human melanomas was obtained by extraction from dissected formalin-fixed 20–40 μm thick sections of the tumors. PCR amplification of H-ras, K-ras, and N-ras exons 1 and 2 resulted in fragments of the expected size, as verified by agarose gel electrophoresis. The PCR products were analyzed by SSCP. Fourteen of the 74 samples showed bandshifts. One sample had a bandshift for N-ras exon 1, and 13 samples had bandshifts for N-ras exon 2. None of the samples had H-ras or K-ras bandshifts. The relative SSCP band intensities were equal in normal and shifted bands of N-ras exon 2 in six samples (e.g., **Fig 1*a***, *lane 3*, ***b***), but reduced in the shifted bands in seven samples (e.g., **Fig 1*a*,** *lane 4*). The latter seven samples contained an abundance of infiltrating lymphocytes, as visible in **Fig 1(*c*)**. In contrast, one sample with an N-ras exon 1 bandshift had a marked reduction of the wild-type SSCP band.

When samples with SSCP bandshifts were analyzed by direct nucleotide sequencing, point mutations were clear in the N-ras oncogene. All mutations but one were in codon 61 of exon 2. The actual base changes are summarized in **Table III** and **Fig 2** shows

Table II. Primers used in PCR for exons 1 and 2 of N-ras, H-ras, and K-ras

		Sequence (5′→3′)	product length (bp)	annealing temp (°C)	number of cycles
N-ras exon 1	forward	ATGACTGAGTACAAACTGGT	111	60	30
	reverse	CTCTATGGTGGGATCATATT			
	nested reverse	ATCTACAAAGTGGTTCTGGA	90	60	20
N-ras exon 2	forward	GATTCTTACAGAAAACAAGTG	157	56	30
	reverse	ATGACTTGCTATTATTGATGG			
	nested forward	CAAGTGGTTATAGATGGTGA	118	60	15
	nested reverse	ATACACAGAGGAAGCCTTCG			
H-ras exon 1	forward	ATGACGGAATATAAGCTGGT	123	60	30
	reverse	CGCCAGGCTCACCTCTATA			
	nested reverse	TGGTTCTGGATCAGCTGGAT	80	62	15
H-ras exon 2	forward	AGGTGGTCATTGATGGGGAG	108	65	30
	reverse	AGGAAGCCCTCCCCGGTGCG			
	nested reverse	CTCCCCGGTGCGCATGTACT	100	60	15
K-ras exon 1	forward	ATGACTGAATATAAACTTGT	111	60	30
	reverse	CTCTATTGTTGGATCATATT			
	nested reverse	TCCACAAAATGATTCTGAAT	89	60	20
K-ras exon 2	forward	AAGTAGTAATTGATGGAGAA	109	60	30
	reverse	AGAAAGCCCTCCCCAGTCCT			
	nested forward	GAAACCTGTCTCTTGGATAT	92	60	20

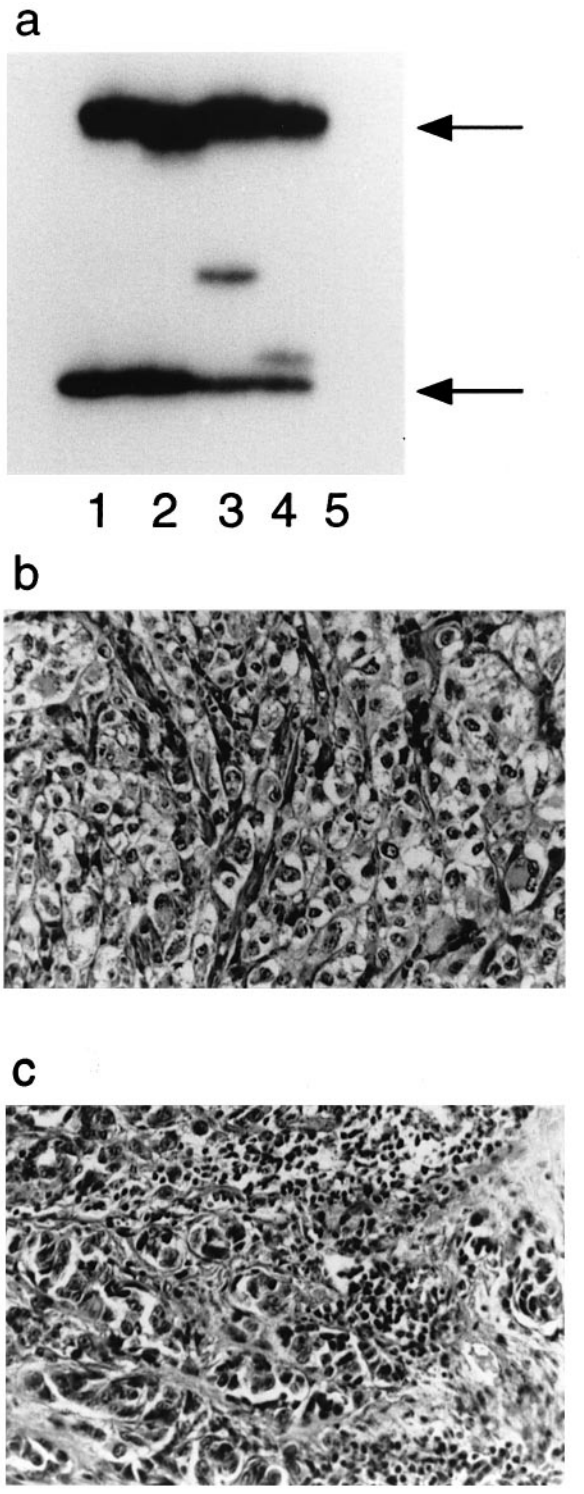

Figure 1. SSCP and histology examples. (*a*) Representative examples of SSCP results. *Lane 1*, sample C5; *lane 2*, sample C11; *lane 3*, sample C13; *lane 4*, sample C14; *lane 5*, neg control. *Arrows* indicate wild-type band. (*b*) Hematoxylin-stained section of sample C13, homogeneous tumor-cell population. (*c*) Hematoxylin-stained section of sample C14, lymphocyte infiltration among tumor cells.

representative examples of sequencing results. Of the exon 2 mutations, nine were in melanomas from chronically sun-exposed head and neck area, two were in skin subjected to intermittent sun exposure, and two were from areas not exposed to sunlight ($p = 0.021$, chi-square exact test for trend). The difference in N-ras codon 61 mutations was also significant between tumors from sun-exposed and unexposed

Table III. Summary of ras mutations, anatomical site, and histogenetic type

Patient no.	Anatomical site	Histogenetic type	Mutations N-ras codon 61
C10[a]	Ear	NM	CAA(Gln)→AAA(Lys)
C11	Neck	SSM	CAA(Gln)→CTA(Leu)
C13	Neck	NM	CAA(Gln)→AAA(Lys)
C14	Temple	NM	CAA(Gln)→CGA(Arg)
C15	Neck	NM	CAA(Gln)→CGA(Arg)
C32	Ear	SSM	CAA(Gln)→CGA(Arg)
C38	Cheek	SSM	CAA(Gln)→CGA(Arg)
C43	Cheek	NM	CAA(Gln)→CGA(Arg)
C48	Ear	NM	CAA(Gln)→CTA(Leu)
I4	Abdomen	NM	CAA(Gln)→AAA(Lys)
I14	Back	SSM	CAA(Gln)→AAA(Lys)
U18	Vulva	Unclassifiable	CAA(Gln)→CGA(Arg)
U19	Vulva	NM	CAA(Gln)→CGA(Arg) Mutation N-ras codon 12
U5	Vulva	MLM	GGT(Gly)→CGT(Arg)

[a]C, I, and U stand for chronically sun-exposed, intermittently sun-exposed, and unexposed anatomical site.

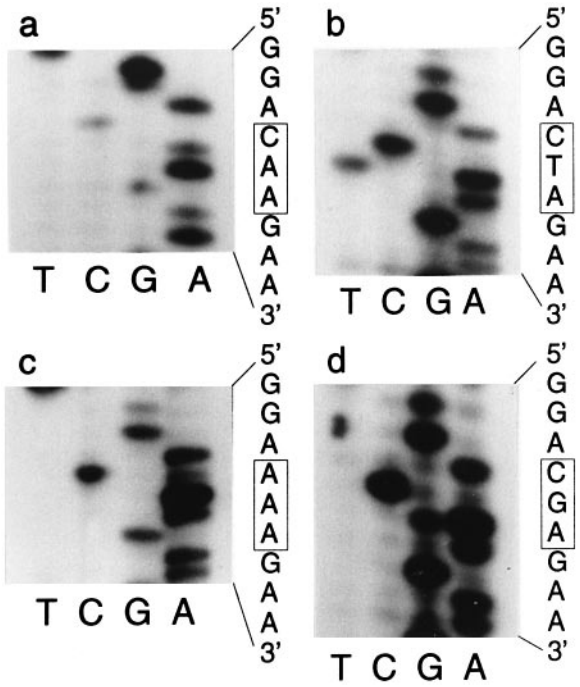

Figure 2. Representative examples of sequencing results N-ras codon 61. (*a*) Wild-type CAA Gln, patient C5; (*b*) point mutation C**T**A Leu, patient C11; (*c*) point mutation **A**AA Lys, patient C13; (*d*) point mutation C**G**A Arg, patient C14.

areas, exclusively ($p = 0.04$, chi-square exact test). A single exon 1 mutation, located in codon 12, was observed in a vulvar melanoma. Fifty-seven of the samples that migrated normally in SSCP were also analyzed for their nucleotide sequence, and all showed the wild-type sequence. With respect to melanoma subtypes, eight mutations of N-ras codon 61 were in NM, four in superficial spreading melanomas (SSM), and one in an unclassifiable melanoma. **Table IV** contains data on the distribution of N-ras mutations in relation to histogenetic subtype and sun exposure.

Melanomas with ras mutations had a mean Breslow thickness of 3.6 mm (range, 1.2–11.9 mm), compared with melanomas without ras mutation, which had a mean of 4.3 mm (range, 0.6–20.0 mm), not a significant difference (independent t test, $p = 0.52$). Even when melanomas from completely unexposed areas were excluded from the comparison, the difference was not significant between melanomas

Table IV. N-ras exon 2 mutations in relation to histogenetic subtype and sun exposure

	SSM			NM			Other[b]		
	C[a]	I	U	C	I	U	C	I	U
Melanomas	15	10	5	10	6	6	3	2	17
Mutated N-ras	3	1	0	6	1	1	0	0	1
N-ras mutation frequency (%)	20	10	0	60	17	17	0	0	6

[a]C, I, and U stand for chronically sun-exposed, intermittently sun-exposed, and unexposed anatomical site.
[b]LMM, MLM, and unclassifiable.

with ras mutations (mean thickness of 2.9 mm; range, 0.6–11.0 mm) and without ras mutations (mean thickness 2.6 mm; range, 1.2–6.6 mm) (independent t test, $p = 0.73$).

DISCUSSION

Here we have combined PCR, SSCP, and nucleotide sequence analysis to compare for the first time ras mutations in human melanomas in UV exposed *versus* completely protected sites. We have found that this combination of methods is sensitive and reproducible. An essential step in the procedure is the dissection of tumor cells from formalin-fixed tissues to avoid contamination by normal cells, which can dilute mutant alleles with the wild-type and, thereby, obscure the results. The SSCP runs were carried out using several different temperatures and glycerol concentrations. As a result, 14 samples showed bandshifts in SSCP. Subsequent nucleotide sequence analysis confirmed the presence of point mutations in all 14 samples. In addition, 57 of the samples lacking SSCP bandshifts but also analyzed by nucleotide sequencing, all contained only the wild-type sequences. The dissection procedure, the strictly controlled SSCP conditions, and the extensive use of nucleotide sequence analysis minimized any possibility that mutations might have escaped detection. The frequency of 19% N-ras mutations we found in a total of 74 primary melanomas from 74 patients with sporadic melanoma is in agreement with the 15% figure published in a recent report on mutation screening of 272 melanomas tested similarly (van Elsas *et al*, 1996). Earlier reports that relied largely on oligonucleotide hybridization experiments resulted in variations of mutational frequencies that may be partially explained by technical difficulties with the assay procedures (Raybaud *et al*, 1988; Albino *et al*, 1989; Shukla *et al*, 1989; van't Veer *et al*, 1989). Furthermore, those studies were small and difficult to compare due to the lack of specific information like tumor thickness or Clark's levels or data on sun exposure.

All of the 14 ras mutations registered in the present investigation were in the N-ras gene. Six N-ras codon 61 mutant samples had balanced allelic band intensities, and originated from homogeneous tumor-cell populations (**Fig 1*b***). The seven samples with N-ras codon 61 mutations that showed reduced intensities of the shifted SSCP bands were all from tissue sections containing large numbers of infiltrating lymphocytes, resulting in an excess of wild-type alleles (**Fig 1*c***). All these 13 samples consisted of heterozygous tumor-cell populations. In contrast the N-ras codon 12 mutant sample with a marked intensity reduction of the wild-type SSCP band most likely was hemizygous or homozygous for the mutant allele. The preferential mutation of N-ras codon 61 in cutaneous melanomas has previously been documented in several reports (Ball *et al*, 1994; Carr and Mackie, 1994; Platz *et al*, 1994; Jafari *et al*, 1995; van Elsas *et al*, 1996). Lentigo malignant melanoma is the subtype of melanoma associated with long-term constant sun exposure. NM and SSM, however, are more frequent than lentigo malignant melanoma in the head and neck and other areas. In fact only two of the 46 cutaneous tumors in this study belong to the lentigo malignant melanoma subclass, and neither of them had ras mutations. Eight of 22 nodular melanoma (36%) had N-ras exon 2 mutations whereas only four of 30 SSM (13%) had such mutations, which is in accordance with previous notified results by Jafari *et al* (1995) and van Elsas *et al* (1996). Furthermore, we found that 60% of NM from chronically sun-exposed areas and only 17% of NM from intermittently or unexposed areas had such mutations (**Table IV**).

The N-ras wild-type codon 61 CAA (Gln) contains a TT pyrimidine doublet in the noncoding strand and is a site for formation of potential mutagenic cyclobutane dimeres and (6–4) pyrimidine photoproducts, as demonstrated by UV irradiation experiments *in vitro* (Törmänen and Pfeifer, 1992). Furthermore the 3′ nucleotide in the noncoding triplet may be converted to a 8-oxo-deoxyguanosine by UV-light induction (Yamamoto *et al*, 1992), leading to mispairing with adenosine. The cloned N-ras gene has been mutationally activated in all three positions of codon 61 after *in vitro* UV irradiation followed by transfection experiments, resulting in CGA Arg, AAA Lys, and CTA Leu codon (van der Lubbe *et al*, 1988). The N-ras codons 61 mutant triplets we observed in the tumors from chronically and intermittently sun-exposed localizations were five CGA Arg, four AAA Lys, and two CTA Leu, all consistent with possible UV induction via the above-mentioned primary photoproducts. The two mutation-containing tumors from intermittently sun-exposed localizations were from patients with recorded severe sunburn and pronounced sun-bathing behavior. Two additional CGA Arg mutations in tumors from the vulva were obviously induced via a mechanism other than UV induction.

No significant correlation existed between ras mutation and Breslow thickness in this series of melanomas. Similarly, a European study of 175 primary melanomas revealed a highly significant correlation between sun exposure and N-ras mutation, but no obvious correlation between N-ras mutation and tumor progression as defined by Clark level of invasion, Breslow thickness, or tendency to metastasize (van Elsas *et al*, 1996). In contrast, a previous report (Ball *et al*, 1994) demonstrated a significant correlation between Breslow thickness and N-ras mutation. Analyses of larger randomized samples are needed in order to resolve these contradictions.

Our results provide clear answers to the three questions initially asked. First, mutations appeared exclusively in the N-ras oncogene and consisted, with a single exception, of point mutations in codon 61. The single exception was a codon 12 mutation found in a primary melanoma of the vulva. Second, the frequencies of N-ras codon 61 mutations were: 32% in primary melanomas from head and neck areas with chronic sun exposure, 11% from sites with intermittent sun exposure, and 7% from unexposed body localizations (vulva, vagina, anus and rectum, palate). Third, the N-ras codon 61 mutations in the melanoma examined here are consistent with known mechanisms for UV induction.

These results strongly support the likelihood that UV light induces N-ras codon 61 mutations in a subset of primary cutaneous melanomas and that these mutations play a role in the early stages of melanoma development.

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