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N-*ras* Mutations in Human Cutaneous Melanoma from Sun-Exposed Body Sites

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In 7 of 37 patients with cutaneous melanoma, mutations in the N-*ras* gene were found. The primary tumors of these seven patients were exclusively localized on body sites continuously exposed to sunlight. Moreover, the *ras* mutations were all at or near dipyrimidine sites known to be targets of UV damage. Two primary tumors were biclonal with respect to *ras* mutation. An active role for UV irradiation in induction of the mutations is suggested.

In animals, a wide range of xenobiotic agents are capable of inducing mutations in the *ras* oncogene family (2). Little is known, however, about the involvement of mutagenic agents in the induction of *ras* mutations in humans. For human cutaneous melanoma, there is epidemiological evidence suggesting a role of UV irradiation in occurrence of the tumor (18).

N-*ras* activated in melanoma. To investigate the role of UV irradiation in the activation of *ras* genes, we studied 63 tumor samples from 37 cutaneous melanoma patients (Table 1) for mutations in H-, K-, or N-*ras*. These samples included 10 primary tumors, 40 metastases, and 13 cell lines, all of the non-lentigo maligna type. To analyze mutational activation of *ras* genes, polymerase chain reaction-amplified DNA samples were prepared as described previously (17) either from DNA obtained from fresh tumor specimens and cell lines or from Formalin-fixed and paraffin-embedded material (16). All samples contained at least 50% tumor cells. In certain cases (e.g., primary tumors of patients 4 and 10), parts of paraffin sections that contained more than 90% tumor cells were chosen. The amplified DNA was spotted onto nylon membranes and hybridized to probes 20 nucleotides long as described elsewhere (21) (Fig. 1).

Mutations were found in 7 of 37 patients (19%). These mutations were exclusively present in the N-*ras* gene in codon 12, 13, or 61. In all cases, the normal N-*ras* allele was also detected.

Biclonality with respect to N-*ras*. We found two different mutations in the primary tumors of patients 4 and 10 (Fig. 1 and Table 2). In patient 4, the two mutations were present in the same codon, N-*ras* codon 13 (N-*ras*13); in patient 10, they were in adjacent codons, N-*ras*12 and N-*ras*13. In patient 4, two different metastases of the tumor could be analyzed; one harbored the mutation N-*ras* valine, and the other harbored N-*ras* asparagine. In patient 10, only cells with one particular mutation (N-*ras*12 Asp) had metastasized, since the two local metastases as well as the inguinal lymph node metastasis harbored the mutation. This result implies that both primary tumors consisted of at least two cell populations with different *ras* mutations. Apparently, they both were able to metastasize, although there may have been some preference (N-*ras*12 in patient 10).

Primary tumors. From four of the seven patients with N-*ras* mutations (patients 4, 10, 20, and 31; Table 2), primary tumor samples as well as metastases were available. In all cases, the mutations were already present in the primary tumor. For one other patient (patient 16), the N-*ras* mutations may also have been present in the primary tumor, since all 14 metastases harbored the same mutation (N-*ras*61 Lys). These data imply that mutational activation of the N-*ras* gene must have occurred during development of the primary tumor. Mutated *ras* genes, usually N-*ras*, have been detected previously in melanoma metastases and cell lines but not in primary tumors (1, 11, 12, 15). In one study, an N-*ras* mutation was found to be present in one of five cell lines established from different metastases of one patient (1). This latter result might be an argument for the involvement of *ras* mutations in some of the metastatic conversions of melanomas. Our results suggest that the mutational activation is an earlier event.

N-*ras* mutations induced by UV. Epidemiological studies have revealed a correlation between exposure to sunlight and the development of cutaneous melanoma, especially of the lentigo maligna type but also of the more common types studied here (5–8, 19). This finding suggests that UV irradiation damages the DNA of melanocytes that might contribute to the development of neoplasia. Other evidence for such an etiology of melanoma comes from the finding that in xeroderma pigmentosum patients, deficient in repairing UV-damaged DNA, the incidence of cutaneous melanoma is higher than in normal individuals (5, 6, 18). Since UV exposure can induce mutations in N-*ras* in vitro (20), we asked whether UV irradiation might be the xenobiotic agent that induces the N-*ras* mutations in these human cutaneous melanomas. We therefore investigated the precise sites of the tumors on the bodies of melanoma patients with respect to sun exposure. Exposure was categorized as continuous, intermittent, or rare (5–8, 18; see footnote *c*, Table 1). For 31 of the patients, localization of the primary tumor was indicated in the clinical record: in 10 patients on continuously exposed sites, in 16 on intermittently exposed sites, and in 5 on rarely exposed sites (Table 1). It was found that primary tumors with activated N-*ras* were localized exclusively on continuously sun-exposed body sites; for the two patients from whom only metastasized material could be analyzed, the primary tumors were also localized on sun-exposed body

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TABLE 1. Melanoma tumor material analyzed for *ras* gene mutations

Patient[a]	Sex[b]	Site of primary tumor	Exposure to sunlight[c]	Material tested[d]	Mutated *ras* gene[e]
1	F	First digit of foot	Intermittent	c	
2	M	Lower leg	Intermittent	c	
3	F	Vulva	Rare	m	
4	M	Finger (dorsal)	Continuous	p, m (2)[f], c	*N*-ras
5	M	Occult[g]	Unknown	m	
6	F	Lower leg	Continuous	m, c	
7	M	Shoulder	Intermittent	m	
8	M	Temple	Continuous	m	
9	F	Upper leg-knee	Intermittent	m	
10	F	Lower leg	Continuous	p, m(3)[h]	N-*ras*
11	F	Lower leg	Continuous	c	N-*ras*
12	M	Lower abdomen	Rare	m	
13	F	Ankle joint-foot	Intermittent	m	
14	U	Unknown	Unknown	m	
15	M	Shoulder	Intermittent	p	
16	M	Neck	Continuous	m (14)[i]	N-*ras*
17	M	Back	Intermittent	m	
18	F	Upper leg	Intermittent	m, c	
19	F	Hairy head	Rare	m	
20	F	Lower leg	Continuous	p, m, c	N-*ras*
21	M	Back	Intermittent	m, c	
22	M	Nipple	Intermittent	c	
23	M	Back	Intermittent	p, c	
24	M	Cheek	Continuous	c	
25	M	Hairy head	Rare	c	
26	M	Back	Intermittent	c	
27	M	Upper leg	Intermittent	m	
28	M	Occult	Unknown	m	
29	M	Occult	Unknown	m	
30	M	Occult	Unknown	m	
31	F	Nose	Continuous	p, m	N-*ras*
32	F	Cheek	Continuous	p	N-*ras*
33	F	Occult	Unknown	m	
34	F	First digit of foot	Intermittent	p	
35	M	Shoulder	Intermittent	p	
36	F	Hairy head	Rare	m	
37	F	Upper leg	Intermittent	p	

[a] All patients were Caucasians and lived in The Netherlands.
[b] F, female; M, male; U, unknown.
[c] Continuous, Body site commonly exposed to sunlight; intermittent, body site exposed to sunlight when patient was wearing bathing suit, etc.; rare, body site rarely or never exposed sunlight (7, 8); unknown, patients with occult primary melanoma ($n = 5$) or unknown clinical history (patient 14).
[d] p, Primary tumor; m, metastasis; c, established cell line of primary tumor (patient 23) or of metastasis (all other cases).
[e] Mutations of codons 12 and 61 of H-*ras* and codons 12, 13, and 61 of K- and N-*ras* were analyzed.
[f] The two metastases were diagnosed and surgically removed 2 and 9 months after the primary tumor.
[g] Occult, Not manifest or detectable by clinical methods alone.
[h] Two metastases were removed 16 months after the primary tumor; one was removed 29 months after the primary tumor.
[i] The 14 metastases were removed 8 years (1 metastasis), 8.5 years (1 metastasis), 9 years (1 metastasis), and 9 years, 4 months (11 metastases) after the primary tumor.

sites. As discussed above, in at least one of these cases the primary tumor most likely contained the mutated N-*ras* allele as well. This result showed a significant correlation ($P < 0.001$) between continuous sun exposure and *ras* mutation, suggesting that UV irradiation was the mutagenic agent. The fact that we found no N-*ras* mutations at intermittently exposed sites does not exclude a role for UV irradiation in the development of these tumors (7); it is quite

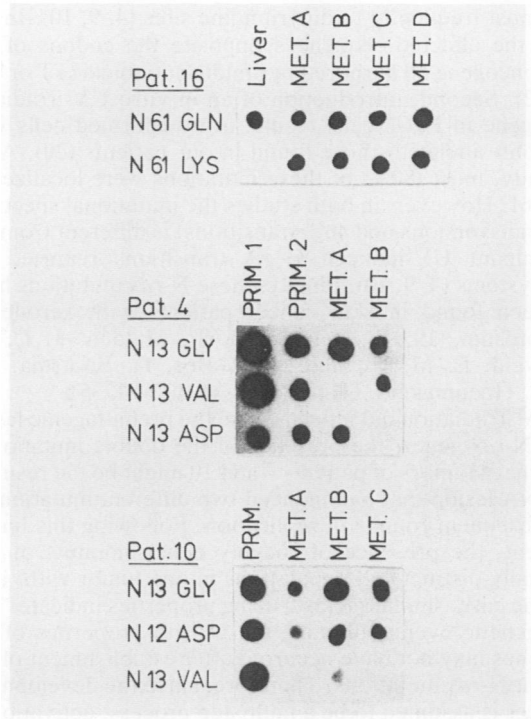

FIG. 1. *ras* gene mutations in melanomas of three representative patients. Primary tumors (PRIM.) and metastases (MET.) of patients 16, 4, and 10 (see Tables 1 and 2) were analyzed. The upper rows represent hybridizations with probes specific for the normal sequence in and around codon 61 (N61 Gln) or codon 12-13 (N13 Gly) of the N-*ras* gene. The other rows represent hybridizations with probes specific for sequences mutated in codon 12, 13, or 61. For instance, the N61 Lys probe is specific for a mutation in codon 61 of the N-*ras* gene that alters the normal codon for Gln (CAA) in a codon for Lys (AAA). For patient 4, two sites (PRIM. 1 and PRIM. 2) of one primary tumor are shown.

conceivable that UV-induced genetic alterations other than in N-*ras* were involved.

Mechanism. Our finding of an in vivo correlation between UV exposure and *ras* gene activation is supported by several other findings. First, UV-induced lesions appear to be tar-

TABLE 2. Presence of N-*ras* gene mutations[a] during tumorigenesis

Patient	Primary tumor[b]	Metastasis[c]	Cell line[d]	Sequence change in N-*ras*
4	N13 Val, N13 Asp	N13 Val (Met. B) N13 Asp (Met. A)	N13 Val[e]	GGT→GTT GGT→GAT
10	N12 Asp, N13 Val	N12 Asp (Met. A–C)	NE	GGT→GAT GGT→GTT
11	NA[f]	NA	N61 Arg	CAA→CGA
16	NA	N61 Lys (Met. A–N)	NE	CAA→AAA
20	N61 Lys	N61 Lys	N61 Lys	CAA→AAA
31	N61 Lys	N61 Lys	NE	CAA→AAA
32	N61 Lys	NA	NE	CAA→AAA

[a] All specimens contained a normal N-*ras* allele (N12 Gly, N13 Gly, or N61 Gln).
[b] Mutations are indicated as N13 Val, etc., representing a mutation coding for (in this example) valine at N-*ras* codon 13.
[c] Met. A through Met. N designate individual metastases.
[d] Established from metastases. NE, Not established.
[e] Cell line derived from Met. B.
[f] NA, Not available.

geted most frequently to dipyrimidine sites (4, 9, 10). In our study, the affected sequences opposite the codons of the N-*ras* oncogene all harbored pyrimidine doublets (TT or CC; Table 2). Second, introduction of an in vitro UV-irradiated N-*ras* gene in Rat-2 cells results in transformed cells with mutations similar to those found in our patients (20). As in our study, most (80%) of these mutations were localized at codon 61. However, in both studies the mutational spectrum (60% transversions and 40% transitions) is different from the predominant UV-induced G→A transitions reported for other systems (4, 9, 10). Finally, these N-*ras* mutations have also been found in skin cancer patients with xeroderma pigmentosum (19; W Keijzer, M. P. Mulden, J. C. M. Langeveld, E. M. E. Smit, J. L. Bos, D. Bootsma, and J. H. J. Hoeijmakers, Cancer Res. **49**:1229–1235).

If UV irradiation did indeed cause the premutagenic lesion in the N-*ras* genes, the presence of the double mutation in the primary tumors of patients 4 and 10 might be the result of one DNA lesion that had induced two different mutations in two subsequent rounds of replication. Following this line of argument, the presence of roughly equal amounts of two genetically distinct cell populations in one tumor with, in at least one case, similar metastasizing properties indicates that other genetic events affecting the growth properties of the cell clones may not have occurred after establishment of the different N-*ras* mutations. Therefore, since the development of cancer is assumed to be a multistep process, activation of N-*ras* might be the last event in the development of the two biclonal primary tumors. Whether the N-*ras* mutation occurred at a similar stage of tumor development in the other cases is unknown.

Conclusions. Although an increasing number of human tumors have been screened for the occurrence of *ras* gene mutations (3), only a few cases are known in which a xenobiotic agent may have been involved in the actual induction of the mutation. For instance, a correlation has been found between the inducing agents in cigarette smoke and activation of *ras* genes in human adenocarcinoma of the lung (13, 14). Furthermore, the differences found between the K-*ras* mutational spectra of adenocarcinoma of the colon (22) and adenocarcinoma of the pancreas (17) or lung (13, 14) suggest the involvement of different carcinogens (17). Our finding of a correlation between exposure to sunlight and N-*ras* gene activation in human cutaneous melanoma supports the idea that the mutations can be induced by external factors. Altogether, a coherent picture emerges which indicates that mutation-inducing agents can activate *ras* oncogenes, which in turn may contribute to tumor development.

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