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Genetics

A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma

(UV light/tumor suppressor genes)

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ABSTRACT **Sunlight is a carcinogen to which everyone is exposed. Its UV component is the major epidemiologic risk factor for squamous cell carcinoma of the skin. Of the multiple steps in tumor progression, those that are sunlight-related would be revealed if they contained mutations specific to UV. In a series of New England and Swedish patients, we find that 14/24 (58%) of invasive squamous cell carcinomas of the skin contain mutations in the p53 tumor suppressor gene, each altering the amino acid sequence. Involvement of UV light in these p53 mutations is indicated by the presence in three of the tumors of a CC → TT double-base change, which is only known to be induced by UV. UV is also implicated by a UV-like occurrence of mutations exclusively at dipyrimidine sites, including a high frequency of C → T substitutions. p53 mutations in internal malignancies do not show these UV-specific mutations. The dipyrimidine specificity also implicates dipyrimidine photoproducts containing cytosine as oncogenic photoproducts. We believe these results identify a carcinogen-related step in a gene involved in the subsequent human cancer.**

The frequency of skin cancers induced by sunlight in the United States approaches that of all other cancers combined and is doubling each decade (1–3). Ninety-five percent of these are non-melanoma skin cancers, resulting in one-third as many deaths as melanoma (4).

Epidemiology has identified causal agents for many human cancers, including skin cancer (5), and reagents such as retroviruses have revealed genes that become oncogenic when mutated (6). Yet, the events between a human carcinogen and the human tumor mutations are unknown. Several questions central to oncology converge on these missing events. In the case of squamous cell carcinoma of the skin, they include the wavelength of light, the gene that absorbs the photon, the DNA photoproduct, the contribution of mutagenesis versus systemic effects of sunlight such as immunosuppression, the type of mutation, possible hotspot sequences, and confidence that the genetic alterations observed in a tumor participated in tumor formation.

Squamous cell carcinoma of the skin is an ideal cancer for determining which of the multiple steps in tumorigenesis are carcinogen-related for the following reasons.

(*i*) The carcinogen is known; in lightly pigmented individuals of all races, the majority of skin cancers are due to sunlight. Of these, squamous cell carcinoma is more sunlight-dependent than basal cell carcinoma or melanoma (1, 7). This carcinogen is physically well-defined, whereas agents such as tobacco smoke are complex mixtures.

(*ii*) UV light produces distinctive mutations, leaving a "signature" in the DNA. Mutations due to direct absorption of UV light by DNA are predominantly C → T transitions at dipyrimidine sites, including CC → TT double-base mutations, in organisms from viral to human (refs. 8–12 and references therein). Because CC → TT base changes are only known to be caused by UV, their presence identifies UV as the mutagen. The appearance of C → T transitions exclusively at dipyrimidines is also unique to UV; chance would dictate that one in four occurs at monopyrimidines. In contrast, UV-induced C → A and nonadjacent double mutations can be caused by other agents and are therefore noninformative. Some target systems show additional types of mutations after UV (refs. 13, 14, and references therein), but these do not affect the use of CC → TT and dipyrimidine C → T mutations as analytic tools.

The specificity of UV mutagenesis results from the fact that the most frequent UV photoproducts involve bonds between adjacent pyrimidines on the same strand (9, 11, 15), even though UV also produces other photoproducts (reviewed in ref. 16).

(*iii*) These distinctive UV-induced mutations do not appear to depend greatly on genetic background. In the extreme case of excision-repair defective xeroderma pigmentosum patients, mutation frequency is increased and the incidence of skin tumors is elevated ≈2000-fold, but the UV mutation spectrum still consists almost exclusively of dipyrimidine C → T substitutions, including CC → TT (12, 17, 18). This situation contrasts with chemical carcinogens requiring metabolic activation by the heterogenous family of cytochrome P-450 enzymes.

For these reasons, we would predict that a gene involved in a sunlight-related step of human squamous cell carcinoma of the skin would carry a CC → TT double-base mutation or a mutation at a dipyrimidine site, usually C → T. We report here that the majority of squamous cell carcinomas of the skin contain mutations in the p53 tumor suppressor gene and that these are of the type caused by UV radiation.

MATERIALS AND METHODS

Tissue. Archived paraffin blocks of neutral-formalin-fixed squamous cell carcinomas were chosen for which the tumor originated in sun-exposed skin and the epidermis consisted of at least 50% contiguous invasive carcinoma. Patients were from the greater New York City or Uppsala, Sweden, areas. In some cases, multiple samples from the same tumor were coded anonymously. The squamous cell carcinoma line SCC 13 was the gift of J. Rheinwald (Dana–Farber Cancer Insti-

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Abbreviations: UVA, UVB, and UVC, UVA region (320–400 nm), UVB region (290–320 nm), and UVC region (100–290 nm) of the UV light spectrum.

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tute, Boston). The patient had received x-ray therapy (5600 R; 1 R = 0.258 mC/kg) and methotrexate.

DNA Amplification. Unstained 5-μm paraffin sections were extracted and the DNA was amplified by the PCR (19). In some cases, normal tissue flanking the carcinoma was isolated for separate amplification. Negative controls were included in each set of reactions, reactions were segregated from post-PCR analyses, and positive displacement pipettes and a UV-irradiated biohazard hood were used. Formaldehyde, in which the tumor samples were fixed, leads to T → G transversions and transversions at C (20). Amplification buffers and primers used were as follows: exon 2, buffer D, 5'-ACTGCCTTCCGGGTCACTGC-3' and TGGATCCACTCACAGTTTCC; exon 4, buffer J, AATGGATGATTTGATGCTGTCCC and CTCAGGGCAACTGACCGTGC; exon 5, buffer D, TTCCTCTTCCTGCAGTACTC and GCCCCAGCTGCTCACCATCG; exon 6, buffer D, CTGATTGCTCTTAGGTCTGG and AGTTGCAAACCAGACCTCAG; exon 7, buffer J, GTGTTGTCTCCTAGGTTGGC and AAGTGGCTCCTGACCTGGAG; exons 8 to 9, buffer J, AGTGGTAATCTACTGGGACG and ATTCTCCATCCAGTGGTTTC. Amplification was for 50 cycles of 94°C, 30 sec/55°C, 1 min/72°C, 30 sec after initial 94°C, 5 min. Buffer compositions were as follows: buffer D, 84 mM Tris, pH 9.0/43 mM KCl/1.3 mM $MgCl_2$/0.01% gelatin; buffer J, 50 mM Tris, pH 9.0/50 mM KCl/1.5 mM $MgCl_2$/0.01% gelatin. Exon 4 used 100 μM tetramethyl ammonium chloride with 62°C annealing and 1-min extension for 75 cycles.

PCR Sequencing. Primers and primer-dimers were removed by purifying on 4% NuSieve gels (FMC); this was followed by phenol extraction of melted gel slices. Direct DNA sequencing of amplified PCR products was performed by annealing an unlabeled primer to the denatured PCR product for 2 min at 25°C, extending the primer one base with the corresponding radiolabeled deoxynucleotide triphosphate and Sequenase for 2 min at 25°C, adding Sequenase termination mixes (United States Biochemical), and sequencing for 5 min at 37°C (S. Nassim and D.E.B., unpublished).

RESULTS

Squamous Cell Carcinoma of the Skin. We chose p53 as a candidate gene because point mutations at many sites can lead to cell transformation and because human papilloma virus (the E6 protein of which binds p53) can transform human keratinocytes (21). In addition, mutated p53 causes premalignant lesions in mouse skin (22, 23). Like other tumor suppressor genes, p53 leads to tumors when inactivated by deletion; in addition, dominant negative mutations code for a mutant protein that inactivates the wild-type protein. Mutated and deleted p53 genes are frequently observed in human internal malignancies, and inherited mutation of p53 underlies the Li–Fraumeni cancer-family syndrome (24, 25).

Paraffin sections of invasive squamous cell carcinomas from sun-exposed skin were surveyed by direct DNA sequencing of PCR amplification products of the p53 gene. The majority of exons 2 and 4–9 were examined; these regions contained 95% of the codons in the five evolutionarily conserved domains (25) as well as substantial flanking regions.

Of 24 samples, 8/11 (72%) from New England patients and 6/13 (46%) from Swedish patients showed point mutations in the p53 gene (Fig. 1 and Table 1). These were confirmed by sequencing the opposite strand of the amplified product and by performing a second amplification reaction. The possibility that the mutation was inherited could be excluded for the majority of samples (Table 1). One sample carried two mutations. In three of the mutated tumors (21%), the normal allele was absent. The true deletion frequency may be higher, since normal tissue in the tumor will obscure a homozygous mutant, and loss of a normal allele will not be observed by sequencing if the other allele is also normal.

Most mutations occurred within conserved regions III–V, and at conserved amino acids nearby, leading to a predicted amino acid change (Table 1). Mutations in these regions often result in dominant negative inactivation of wild-type p53 (24). The mutations observed in nonconserved regions led to a predicted acid-to-base replacement in the acidic amino terminus, to stop codons in the 5' third of the gene, and to a stop codon in the nuclear targeting sequence (26). These mutations could abrogate the suppressor function of one of the alleles, just as would the allelic losses observed. Ten of the 15 point mutations occurred at codons reported in other tumor types, and 8 of these resulted in amino acid substitutions previously reported at these sites (refs. 25, 27–31, and references therein). But the nucleotide changes were different.

Three of the tumors (23% of base substitutions) contained CC → TT double-base changes, which evidence indicates are specific to UV (Table 1). These make it possible to infer the carcinogen from the mutation in an individual tumor. In addition, 100% of the mutations occurred at a dipyrimidine

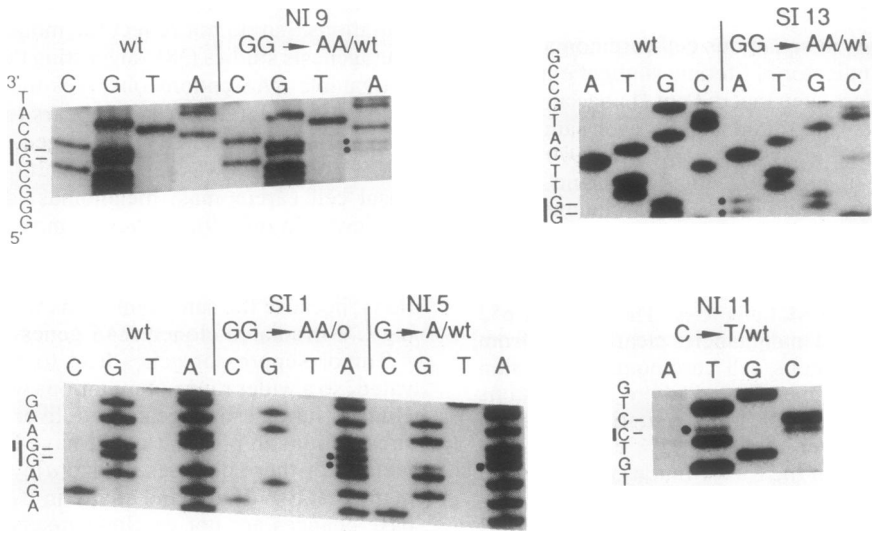

FIG. 1. Mutations in the p53 tumor suppressor gene in invasive squamous cell carcinomas of the skin. The DNA sequence is shown to the left, with the mutated bases indicated by a line. The mutant bands are indicated by filled circles. CC → TT mutations are shown as the GG → AA mutations on the other strand and are specific to UV light. wt, Wild type.

Table 1. Mutations in the p53 gene in invasive squamous cell carcinoma of the skin

Tumor	Age, yr	Sex	Site	Codon	Sequence	Base change	Amino acid change
NI 6	86	♀	Preauricular	7	tCt	C → G	Asp → His
NI 9	77	♀	Chest	56	tcttCa	C → A	Glu → stop
SI 2	82	♂	Preauricular	104/105	gcct	ΔC	Gly → Ala . . . stop
SI 20	82	♂	Temple	104/105	gcct	ΔC	Gly → Ala . . . stop
SI 16	69	♀	Scalp	151	cCccc	C → A	Pro → His
SI 15	69	♀	Hand	152	cccCc	C → T	Pro → Ser
NI 4	76	♂	Front scalp	179	acCa	C → A	His → Asn
NI 3	68	♂	Cheek	245	gcCg	C → A	Gly → Cys
NI 9	77	♀	Chest	245	gCCg	CC → TT	Gly → Asn
SI 13	80	♀	Nose	247–248	aCC*g	CC → TT	Asn-Arg → Asn-Trp
SCC 13	56	♀	Side of face	258	ttCc	C → T	Glu → Lys
NI 11	76	♂	Cheek	278	tCct	C → T	Pro → Ser
SI 1	85	♂	Face	285–286	tCCt	CC → TT	Glu-Glu → Glu-Lys
NI 5	89	♂	Forehead	286	tCct	C → T	Glu → Lys
NI 8	75	♂	Postauricular	317	cccCa	C → T	Gln → stop

The sequence is written 5′ → 3′ for the strand containing the pyrimidine. A wild-type allele was observed in all cases except SI 1, SI 15, and SCC 13. Sample NI 9 contained two point mutations. For SI 2, 13, 15, 16, and 20 and NI 4 and 11, an inherited mutation at the site could be excluded based on the presence of a normal sequence in a section of normal tissue or in a second tumor. For SI 13 and 16 and NI 3, 6, and 9, the mutant band was present at less than a 1:1 ratio to the wild-type band; these samples were also those that contained <70% neoplastic cells. SI, Sweden; NI, New York; uppercase letter of sequence, base mutated; ΔC, deletion of a C; C*, cytosine known to be methylated at this site.

sequence, with C → T transitions predominating (62%). These characteristics are expected only if the mutagenic lesion was a dipyrimidine photoproduct induced by UV light.

The mutations observed also included transversions such as C → A, which are noninformative because they can be caused by many different mutagens. Yet, the overall spectrum agrees numerically with that expected for mutations induced by UVC (100–290 nm) (refs. 8–10, 12, 32, and references therein) and UVB (290–320 nm) (11, 33) (UVA, UVB, and UVC indicate UVA, UVB, and UVC regions of the UV light spectrum). [In shuttle vectors, the UVB dipyrimidine mutations are accompanied by multiple separated mutations in one molecule, caused by single-strand breaks (34)]. In published UVC and UVB mutagenesis studies, the frequency of C → T transitions is typically 60–75% of base substitutions, the frequency of CC → TT is 5–15%, and the frequency of C → A is 5–15%. We found 62%, 23%, and 31%, respectively. Other mutation types, such as the C → G substitution and two one-base deletions we observed, are reported by others at low frequencies. T → C substitutions are reported at a frequency of 1–26%, depending on the gene studied.

The UV-like mutations in squamous cell carcinoma of the skin are not simply an indicator of lifetime sunlight exposure because no mutations were seen that did not change an amino acid, although 950 bases were sequenced for each sample. In addition, only two mutations were seen in the 970 bases flanking codons 12 and 61 of the *HRAS*, *KRAS*, and *NRAS* genes in 20 of our squamous and basal cell carcinomas (data not shown). These two mutations were C → T substitutions at codon 12 of *KRAS* and *NRAS*, capable of activating *RAS* to an oncogene.

Comparison with Internal Malignancies. The types of p53 mutations seen in 97 internal malignancies clearly differ from those found here for squamous cell carcinoma of the skin (Fig. 2). In the internal malignancies, no CC → TT mutations were seen (histogram b). Only 62% of the mutations occurred at dipyrimidines, even fewer than the 75% expected randomly; furthermore, many of the C → T mutations seen were not located at dipyrimidines (histograms a and c, respectively). Instead, histogram d shows that one-third of the p53 mutations in the internal malignancies were C → T mutations at CG sequences, indicative of mutations due to spontaneously deaminated 5-methylcytosine (35). C → T mutations at CG sites never occurred in squamous cell carcinomas of the skin. Since the deamination mechanism requires a CG sequence, it cannot generate double-base CC mutations such as CC → TT. Importantly, the tumors surveyed for these comparisons included squamous cell carcinomas of sunlight-shielded organs, lung and esophagus (36, 37).

For each of mutation types a–d, Fisher's exact test was used to compare pairwise the proportion of such mutations in the internal malignancies versus the proportion in either the squamous cell carcinomas or the UV studies. The two-tailed P values are significant at a level of $P < 0.02$ (except $P = 0.2$ for the comparison of p53 mutations in histogram c). In contrast, the distribution of mutations among types a–d is qualitatively similar between squamous cell carcinoma of the skin and UV mutagenesis studied in marker genes.

DISCUSSION

Point Mutations in Tumors. The *RAS* genes have previously been used to link chemical mutagens to mutations in animal tumors (38). Yet, many carcinogens produce *RAS* mutations that do not reflect the mutation specificity seen in mutagenesis studies (38), suggesting that the small number of activatable *RAS* codons obscures the relation between carcinogen and mutation. This appears to be the case in human skin. Mutated, deleted, rearranged, or amplified *HRAS*, *KRAS*, or *NRAS* was reported in 10–40% of squamous and basal cell carcinomas, melanomas, and keratoacanthomas (reviewed in ref. 39). However, most mutations were transversions rather than C → T transitions. These probably reflect phenotypic selection acting on UV-induced mutations, because the same mutations were seen after transfecting UV-irradiated cloned *RAS* genes.

Tumor suppressor genes lead to cancer by being inactivated, so a wider range of mutations will alter the phenotype. Human lung, esophageal, and liver tumors carried base substitutions in p53 that could have been generated by risk factors for these diseases, benzo[*a*]pyrene and nitrosamines in the first two tissues and aflatoxin B_1 in liver (25). But these base changes are not carcinogen-specific; for example, the G → T base substitution caused by benzo[*a*]pyrene is also the major mutation caused by aflatoxin B_1 (25). This mutation is also caused spontaneously (40).

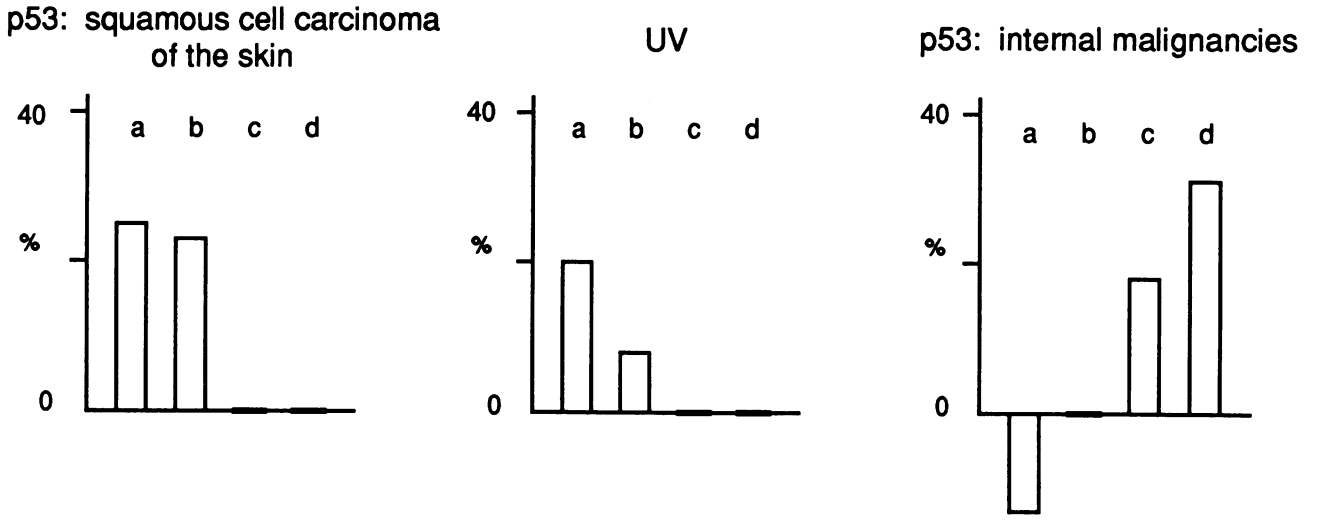

FIG. 2. Diagnostic types of base substitutions in p53 in 13 squamous cell carcinomas of the skin compared with p53 mutations reported in 97 internal malignancies (refs. 25, 27–31, and references therein) and compared with 66 UV mutations studied in endogenous genes in mammalian cells (10, 12). The comparisons are limited to endogenous genes because CG frequencies are underrepresented in mammalian DNA. The vertical axis indicates percentage of total skin squamous cell carcinoma p53 mutations, UV mutations, or internal malignancy p53 mutations that are of mutation type a, b, c, or d. Histograms: a, mutation located at a dipyrimidine site (in excess of the 75% expected randomly); b, CC → TT double-base substitution; c, C → T substitution not at a dipyrimidine site; d, C → T substitution at a CG dinucleotide.

In contrast, the CC → TT double-base substitutions are specific for UV (41, 42). Double-base mutations are rarely produced by other mutagens; hydrogen peroxide and cisplatin cause occasional double events but not CC → TT (43, 44). Spontaneous mutagenesis *in vivo* can lead to single, but not double, C → T substitutions, and these do not predominate (40).

Sunlight-Related Events in Human Skin Cancer. The widespread presence of uniquely UV-like point mutations in the p53 gene enables us to answer the questions raised in the Introduction, reconstructing the sunlight-related events leading to squamous cell carcinoma of the skin.

(*i*) The wavelengths contributing most to the p53 point mutations were evidently in the UVB region, the same wavelengths that most efficiently lead to tumors in animals (reviewed in ref. 39). This is because dipyrimidine photoproducts are produced in the skin's basal layer in greater amounts by UVB than by sunlight doses of UVA (320–400 nm) (45). Although UVC produces these photoproducts even more efficiently than UVB in isolated DNA, it is screened out at least 10^5-fold by the ozone layer (46).

(*ii*) A gene in which sunlight absorption led to skin cancer was p53, a tumor suppressor gene.

(*iii*) The oncogenic DNA photoproducts in p53 were cytosine-containing cyclobutane pyrimidine dimers, pyrimidine-pyrimidone (6-4) photoproducts, or the Dewar isomer of the (6-4) photoproduct (reviewed in refs. 16, 47). The cyclobutane dimer has been implicated in cutaneous tumors in fish and opossum (48, 49) and in mutagenesis in human cells (9). The p53 mutation in tumor SI 13 evidently occurred at a cyclobutane dimer, because the 3′ cytosine at that site has been observed to be methylated (35), and it is known that cytosine methylation will block (6-4) photoproduct production (15).

(*iv*) The predominant mutations were C → T and CC → TT transitions. Because the sunlight-induced mutations appeared in tumors of the skin but not in internal malignancies, they were responsible for the tissue specificity of the carcinogen. In contrast, deamination of 5-methylcytosine at CG sequences (35) will occur in all tissues. The absence of mutations at T, despite the high frequency of T photoproducts (9), is expected from the "A rule" (reviewed in ref. 50) that DNA polymerase preferentially inserts adenine opposite noninstructive lesions. This process generates the correct sequence opposite thymines but at cytosines leads to C → T substitutions.

(*v*) The UV-like p53 mutations in squamous cell carcinoma of the skin identify mutagenesis of p53 as a sunlight-related step in tumor progression and therefore directly connect a human carcinogen with a gene involved in the subsequent tumor. Since most individuals' sunlight exposure occurs primarily in childhood (51), the p53 mutations may have occurred in our patients >50 years previously. This event would have been preceded or followed by other events that are part of the development (52) of dysplasia, actinic keratosis, or carcinoma *in situ* toward invasive squamous cell carcinoma.

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