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Genetics

Mutation hotspots due to sunlight in the p53 gene of nonmelanoma skin cancers

(UV light/DNA photoproducts/multistage carcinogenesis)

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ABSTRACT **To identify the sites in the p53 tumor suppressor gene most susceptible to carcinogenic mutation by sunlight, the entire coding region of 27 basal cell carcinomas (BCCs) of the skin was sequenced. Fifty-six percent of tumors contained mutations, and these were UV-like: primarily CC → TT or C → T changes at dipyrimidine sites. Such mutations can alter more than half of the 393 amino acids in p53, but two-thirds occurred at nine sites at which mutations were seen more than once in BCC or in 27 previously studied squamous cell carcinomas of the skin. Seven of these mutation hotspots were specific to skin cancers. Internal-cancer hotspots not located at dipyrimidine sites were not mutated in skin cancers; moreover, UV photoproducts were absent at these nucleotides. The existence of hotspots altered the process of inactivating p53 in BCC compared to other cancers: allelic loss was rare, but 45% of the point mutations were accompanied by a second point mutation on the other allele. At least one of each pair was located at a hotspot. Sunlight, acting at mutation hotspots, appears to cause mutations so frequently that it is often responsible for two genetic events in BCC development.**

Basal cell carcinomas (BCCs) are the most frequent cancer in the United States. Where light-skinned populations are heavily exposed to sunlight, the lifetime incidence of BCC exceeds 50% (1). BCCs are tumors of the skin keratinocyte and resemble the basal layer of the epidermis; they spread almost exclusively by local invasion and tend to remain diploid (2). The less frequent squamous cell carcinomas (SCCs) show greater cornification, have a greater tendency to metastasize, and usually become aneuploid. These processes are initiated by sunlight, because the incidence of both tumors correlates with outdoor exposure, low latitude, and fair skin. One role of sunlight is to induce genetic damage, since individuals with xeroderma pigmentosum, who are unable to repair UV-induced DNA photoproducts, have a 2000-fold increased frequency of these tumors (3). Mutations due to such damage have been found in the p53 tumor suppressor gene in SCC (4). Neither the sites in p53 most susceptible to sunlight nor the number of genetic alterations caused by sunlight are known.

Early events in skin carcinogenesis cannot be observed directly. But the distinctive mutations produced by UV light allow early events to be inferred from mutations observed in a tumor (4). Tumor suppressor genes offer a clearer view of the original mutagenic event than do oncogenes: an oncogene's requirement for a particular gain of function constrains the ability of a base substitution to lead to a tumor, so that some mutations made by the original carcinogen will never be found in tumors. In contrast, a tumor suppressor gene needs only to be inactivated, so that many different base changes will be effective.

The p53 tumor suppressor gene, apparently coding for a transcription factor regulating a cell cycle checkpoint (5), can be inactivated by allelic loss, by small deletions, and by point mutations that cause aberrant splicing, stop codons, or other null phenotypes (6). In addition, some p53 mutations lead to a dominant-negative phenotype, characterized by inactivation of proteins produced by the normal p53 allele. Identification of mutation hotspots in the p53 gene in nonmelanoma skin cancers reveals that they are linked to UV light and that, in BCC, they may have enabled sunlight to mutate both alleles of the p53 gene in the cell of origin.

MATERIALS AND METHODS

Tissue. DNA was isolated from surgically removed tumors (YB and HB; from Yale and Harvard clinics, respectively) or from archived paraffin blocks of neutral-buffered formalin-fixed BCCs for which the section consisted of at least 50% contiguous carcinoma (NB; from a New York City clinic) (4, 7). YB tumors were removed by Mohs' surgery, yielding the central tumor mass relatively free of surrounding normal stroma (7). The percentage of tumor cells in a sample was estimated by two dermatopathologists, using hematoxylin/eosin-stained formalin-fixed biopsy sections or, for archived blocks, a section adjacent to those used for DNA isolation. Histological types included nodular, infiltrating, and superficial BCCs.

DNA Amplification, PCR Sequencing, and Allelic Loss. Exons of the p53 gene were amplified in at least two independent PCR reactions. For YB samples, normal DNA from blood was also amplified. Amplification buffers, primers, cycling conditions, and negative controls were as described (4). In some cases, additional intronic primers were used (8). Amplification conditions were as follows: exons 2–4, buffer JI with a final formamide concentration of 5%, primers 5′-ACTGCCTTCCGGGTCACTGC-3′ and 330, annealing at 58°C, and extension for 1.5 min; exons 5 and 6, buffer J with 5% formamide and 0.1 mM tetramethylammonium chloride, primers 312 and 256, annealing at 50°C, and extension for 1 min; exon 7, buffer D, primers E7Li and 238; exons 8 and 9, buffer D, primers 316 and 317, extension for 1 min; exon 10, buffer D, primers 670 and 671; exon 11, buffer D, primers 564 and E11Ri. Buffer JI was 50 mM Tris, pH 9.0/3.0 mM $MgCl_2$/0.01% gelatin; other buffers were as described in ref. 4. Direct DNA sequencing of both strands of purified PCR products was performed as described (9). To determine allelic loss, regions in p53 flanking the polymorphic codon 72 (10)

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Abbreviations: BCC, basal cell carcinoma; SCC, squamous cell carcinoma.
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were amplified from DNA isolated from the tumor and from normal blood.

DNA Photoproducts at the DNA Sequence Level. Internal-malignancy mutation hotspot codon 175 was on a 261-bp *Hin*dIII–*Eco*RI fragment from a TA cloning vector (Invitrogen) containing exon 5 of a PCR-amplified p53 gene, and hotspot codon 273 was on a 132-bp *Dde* I fragment from a PCR amplification of exons 8 and 9 cloned into pBluescript II SK$^-$ (Stratagene). Skin cancer hotspot codons 177 and 278 lie on these same fragments. Skin cancer hotspot codon 196 was contained on a 105-bp *Stu* I–*Taq* I fragment of amplified exon 6. Since the cytosine bases in CG dinucleotides of the p53 gene are methylated in some tissues *in vivo*, the cloned fragments were also methylated *in vitro* with CpG methylase (New England Biolabs). Identification of the location and frequency of UV-induced cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6–4) photoproducts at individual bases in the p53 gene, as bands on DNA sequencing gels, was as described (11).

Immunohistochemistry. Unbaked paraffin sections were deparaffinized and hydrated, endogenous peroxidase activity was quenched by a 20-min incubation in 0.1% hydrogen peroxide, and sections were permeabilized by a 30-min incubation in 0.05% saponin. Sections were then incubated in normal goat serum for 30 min to prevent nonspecific interactions. p53 immunocomplex was generated by overnight 4°C incubation with a 1:4000 dilution of CM1 rabbit polyclonal antibody (NovoCastra, Newcastle, U.K.; the manufacturer's recommended dilution is 1:100). This antibody detects both wild-type and mutant p53 protein. The immunocomplex was detected by a 45-min incubation with biotinylated anti-rabbit antibody followed by a 30-min incubation with avidin–biotin-peroxidase complex (Elite Vectastain ABC; Vector Laboratories). Positive staining was visualized with diaminobenzidine. Staining was restricted to tumor cells and was absent when CM1 antibody was omitted.

RESULTS

Sunlight-Induced p53 Mutations in BCC. Sequencing the entire p53 coding region (exons 2–11) identified point mutations in 56% of BCCs (15/27) (Table 1). Most p53 mutations in tumors have been sought in the evolutionarily conserved regions of exons 5–8 (6); one-quarter of the BCC mutations were outside these exons. Germ-line DNA from the same patients was wild type. One hundred percent of the base substitutions occurred at sites of adjacent pyrimidines, and 80% were CC → TT or C → T (Table 1). This is the result expected if UV light were the mutagen (4). There was no strand preference for the pyrimidine at which the mutation occurred. In contrast to p53, only one mutation was seen in the *HRAS*, *KRAS*, and *NRAS* genes in 10 BCCs, representing a total of 20 kb of sequence on the two alleles (data not shown). That mutation, in codon 12 of *NRAS* in tumor HB 3, was a C → T substitution at a dipyrimidine site, changing glycine to aspartic acid and capable of activating *RAS* to an oncogene.

Table 1. p53 mutations in human BCCs

Tumor	Age	Sex	Site	p53 LOH	% Ab area	Codon	Sequence	Base change	Amino acid change
YB 4	73	M	Nose	N	100	196	tcC*g	C → T	Arg → stop
YB 11	70	M	Cheek	NI	100	342	tcC*g	C → T	Arg → stop
YB 12	73	M	Temple	NI	100	100	tccCa	C → T	Gln → stop
						177	ccCcc	C → T	Pro → Leu
YB 13	87	M	Chin	N	100	248	tCC*g	CC → TT	Arg → Gln
YB 17	79	M	Shoulder	N	100	46–47	cCCc	CC deleted	Pro → Gly . . . stop
YB 21	48	M	Shoulder	NI	100	248	tCC*g	CC → TT	Arg → Gln
NB 1	79	F	Forehead		100				
NB 2	86	M	Nose		100				
NB 3	83	F	Nose		100				
HB 2	73	M	Neck		100				
YB 10	82	M	Cheek	N	90	196	tcC*g	C → T	Arg → stop
						278	tCct	C → T	Pro → Ser
YB 15	90	F	Forehead	NI	90	178	cCac	C → A	His → Asn
NB 5	88	F	Nose	Y	90	257–258	ttCCa	CC → TT	Leu-Glu → Leu-Lys
YB 8	56	M	Shoulder	N	90				
YB 18	44	F	Nose	N	90				
YB 5	70	M	Nose	NI	80	196	tcC*g	C → T	Arg → stop
						280	ctCt	C → T	Arg → Lys
						282	acC*g	C → T	Arg → Trp
NB 6	73	M	Cheek		80	278	tCct	C → T	Pro → Ser
						281	tCtct	C → T	Asp → Asn
YB 7	65	M	Nose	NI	80				
YB 9	83	M	Nose	N	80				
YB 20	68	F	Cheek	N	80				
YB 6	70	M	Nose	N	10				
YB 19	81	F	Nose	N	5	342	tCC*g	CC → TT	Phe-Arg → Phe-stop
YB 1	65	M	Chest	NI	1				
YB 2	61	M	Leg	NI	0	249	ccTcc	T → A	Arg → Trp
YB 14	60	M	Scalp	NI	0	51	ttCa	C → A	Glu → stop
HB 1			Face						
HB 3	75	F	Nose			294	ctCccc	C → A	Glu → stop

LOH, presence (Y) or absence (N) of loss of heterozygosity; NI, locus noninformative. Blank spaces indicate that no mutations were found or that antibody-staining or loss-of-heterozygosity data were not available. Tumors are arranged in order of decreasing percentage of antibody (Ab) area, which represents the percentage of tumor cells staining positively with CM1 antibody. Mutations in boldface type were accompanied by another p53 point mutation in the same tumor. DNA sequence flanking the mutated site is shown for the strand containing the pyrimidine. *Cytosine is at a CG sequence and thus is potentially methylated.

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p53 Mutation Hotspots in Nonmelanoma Skin Cancers. Though over half of the 393 amino acids in p53 can be altered by a C → T base substitution at a dipyrimidine site, 67% of the mutations in BCC occurred at sites also found in other skin tumors. To identify sites in p53 most susceptible to carcinogenic mutation by sunlight, the present BCC data were combined with previous SCC data (4, 12), as well as our subsequent SCC results (unpublished). Data for which the body site of tumors was not reported were omitted (13). In a collection of 42 mutations, each site observed more than once represents >3% of the total; these nine sites are shown in Fig. 1. These sites are analogous to the six mutation hotspots in internal cancers, which are each the site of 3–10% of the observed mutations (6).

This hotspot spectrum contains seven sites that were hotspots only in skin cancers. Two of the six internal-cancer hotspots, codons 245 and 248, were also hotspots in skin tumors, and two others, codons 249 and 282, were represented once. However, mutations in skin tumors were never observed at the internal-cancer hotspots at codon 175 or codon 273 (Fig. 1). These are the only internal-cancer mutation hotspots at which the mutating base is not flanked by another pyrimidine and so should be incapable of forming UV photoproducts.

UV Photoproducts at p53 Mutation Hotspots. To determine the role of UV photoproducts in causing mutation hotspots, we measured the frequency of cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6–4) photoproducts at internal-cancer hotspot codons 175 and 273 and in skin-cancer hotspot codons 177, 196, and 278. In a highly repeated DNA sequence, frequencies in cloned DNA differed from those *in vivo* only by a constant shielding factor (14). Fig. 2 shows an autoradiogram of UV photoproducts in the vicinity of codon 175, on the nontranscribed strand. Neither UV photoproduct was present at the bases that mutate in internal cancers (brackets). Photoproducts were also absent from the transcribed strand (data not shown). Since the internal-cancer hotspots are located at sites of cytosine methylation, which blocks formation of (6–4) photoproducts (15), measurements were made both with and without *in vitro* methylation, with similar results (not shown). Fig. 2 also shows UV photoproducts at a nearby hotspot specific for skin cancers, codon 177. UV photoproducts were frequent at this site (dots). These photoproduct frequencies were not notably higher than at surrounding sites at which a C → T substitution would change the amino acid. Similar results were found at internal-cancer hotspot codon 273 and skin-cancer hotspot codons 196 and 278 (data not shown). Thus, UV photoproducts are a prerequisite for a skin-cancer hotspot, but factors in addition to initial photoproduct frequency are influential.

Point Mutations in Two Alleles of BCCs. The typical pattern of p53 mutation in internal cancers is a point mutation in one allele and loss of the other allele. In informative BCCs, loss of heterozygosity within the p53 gene was observed in only 8% (1/12) of the cases (Table 1). The tumor samples were pure enough to show loss of heterozygosity, since allelic loss on other chromosomes was observed in 57% of the same tumors (7). In contrast, 45% of the p53 point mutations were accompanied by a second point mutation in the same tumor (Table 1). In each case, at least one of the mutations was at a mutation hotspot.

To determine whether the mutations were on separate alleles, PCR amplification products from tumors YB 5 and YB 10 were cloned. DNA sequencing of multiple clones showed that the two mutations never appeared in the same clone (Fig. 3). For these tumors and NB 6, the mutations could also be separately amplified by PCR. The independence of the mutations in tumor YB 12 was not determined, because the mutations were on different PCR products separated by a large intron. For YB 5, one allele contained the codon 282 mutation and the other contained mutations at codons 196 and 280.

Null Phenotypes in BCC. All p53 mutations in BCC altered the predicted amino acid (Table 1). For 13 of the 20 point mutations, information about the biological effect of the amino acid substitution was available. All were putative null mutations: a stop codon (codons 51, 100, 196, 294, and 342), a site where mutations cooperate poorly with *ras* for transformation (codon 281) (16), or mutations identical to those found in the germ line in Li-Fraumeni cancer families or in families with a history of osteosarcoma (codons 248, 258, and 282) (17–19). These germ-line mutations render p53 unable to suppress the growth of cells *in vitro* (20). Moreover, biological effects were known for six of the skin cancer mutation hotspots. These were all null mutations; in addition to those above, codon 245 mutations are found in Li-Fraumeni families. In tumors with more than one mutation, at least one mutation led to a predicted null phenotype.

Homogeneously Aberrant p53 Protein in BCC. Cells with p53 mutations can usually be identified by immunohistochemical visualization of over-stabilized protein (21). In BCC, 92% (23/25) of the tumors stained positively for elevated levels of p53 protein (Table 1). The fraction of tumor cells staining positively for p53 was usually 80–100%, and

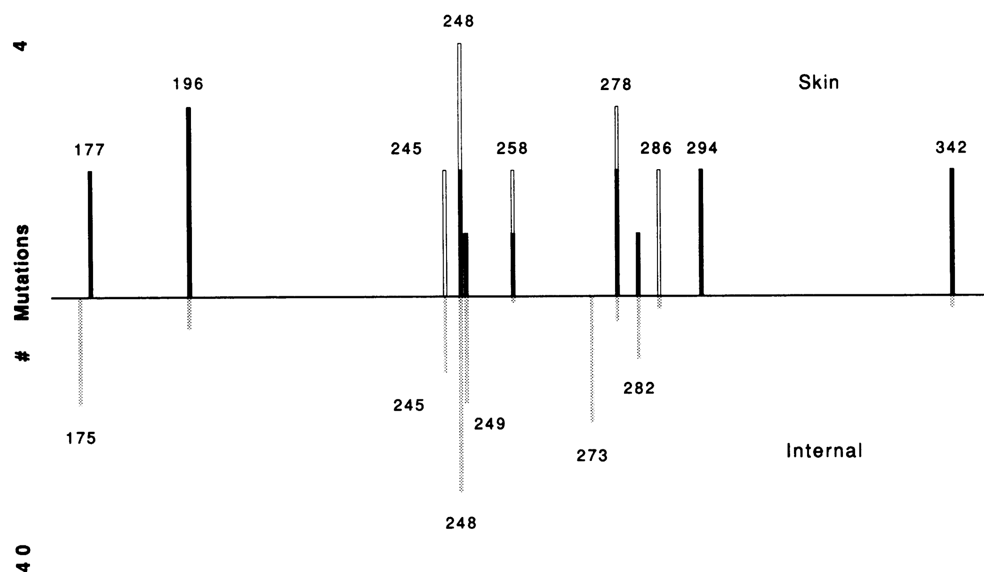

FIG. 1. Mutation hotspots in the p53 tumor suppressor gene in nonmelanoma skin cancers (upper histogram: filled bars, BCCs; open bars, SCCs) compared to internal malignancies (lower histogram: stippled bars). Hotspots were mutated in ≥3% of 42 skin tumors (codons 177, 196, 245, 248, 258, 278, 286, 294, and 342) or in ≥3% of 313 internal tumors reported in the literature (codons 175, 245, 248, 249, 273, and 282); scales for upper and lower histograms differ. Also shown are the number of internal-malignancy mutations reported at the skin cancer hotspots, and vice versa.

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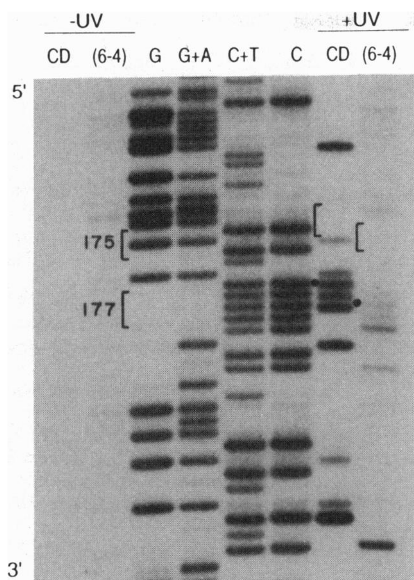

FIG. 2. UV photoproducts at the codon 175 internal-cancer mutation hotspot and flanking bases. UV-irradiated cloned DNA fragments were analyzed for the location and frequency of the two major UV photoproducts, the cyclobutane pyrimidine dimer and the pyrimidine-pyrimidone (6–4) photoproduct. Gel bands indicate the location of the photoproducts; band intensity is proportional to photoproduct frequency. Brackets span the possible gel locations of UV photoproducts at the bases mutated at codon 175 in internal cancers; they differ in position because of the different migration rates of the two photoproducts. Codon 177 is a mutation hotspot only in skin cancers. Dots indicate the gel location of UV photoproducts corresponding to the bases mutated in skin cancers. The top strand, 5'-GGAGGTTGTGAGGCGCTGCCCCCACCATGAGCGCTGC-TCA-3' is shown. Codons of interest are underlined. −UV, unirradiated; +UV, UV irradiation at 100 J/m^2; CD, cyclobutane dimers; (6–4), pyrimidine-pyrimidone (6–4) photoproducts; G, A, T, and C, Maxam–Gilbert DNA sequencing reactions.

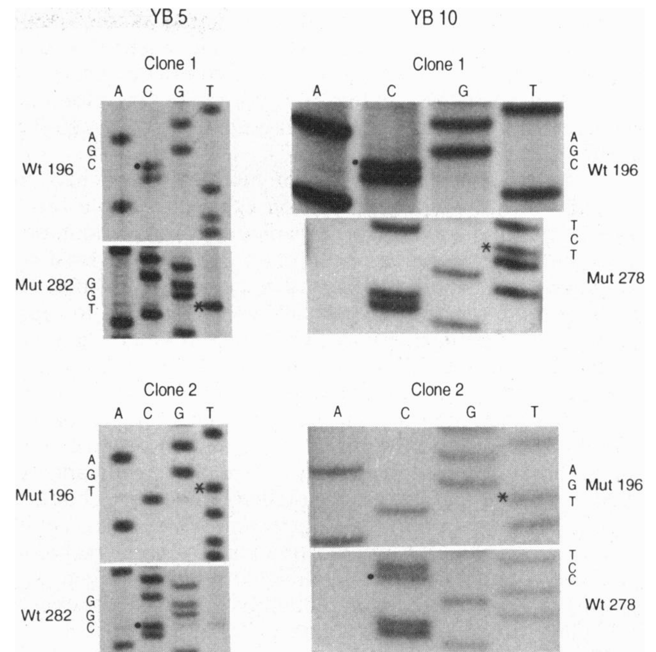

FIG. 3. p53 mutations on separate alleles. The two alleles of tumors YB 5 and YB 10, which carried multiple p53 mutations, were separated by cloning PCR amplification products. Ten clones from each tumor were then sequenced. If mutations were on separate alleles, each clone would carry one mutation but not the other. Shown are one example of each type of clone. For YB 5, codons 196 and 282 are shown; for YB 10, codons 196 and 278 are shown. The wild-type allele is indicated by Wt and a closed circle. The mutated allele is indicated by Mut and an asterisk.

staining was similar in each nodule of a tumor. Staining was present not only in advanced deeply invasive areas of the tumors but also in early superficially invasive areas (data not shown). Adjacent normal cells did not stain. The absence of coding sequence mutations in half the immunopositive tumors was probably not due to contaminating normal cells, since four of the six mutation-negative tumors tested for allelic loss on other chromosomes were pure enough to show such loss (7). Positive p53 immunostaining in BCC has also been reported by others (22).

DISCUSSION

Carcinogenic Mutations. Three criteria for implicating mutations as causal were met in the BCCs containing mutated p53: (*i*) mutations changed the predicted amino acid, (*ii*) mutations were specific to the original carcinogen, and (*iii*) mutations were present in the majority of tumor cells. The first two criteria were also examined and met in SCC. The absence of p53 coding sequence mutations in 40% of BCCs, despite aberrant protein stability, implies that there are other mechanisms for inactivating p53.

Molecular Origin of Sunlight Mutation Hotspots. Direct absorption of UVC (100–290 nm) or UVB (290–320 nm) by DNA leads primarily to photoproducts joining adjacent pyrimidines. The resulting mutations are primarily CC → TT double-base substitutions and C → T substitutions at dipyrimidine sites and are, as far as is known, unique to UV light (4). The CC → TT mutations eliminate a large class of chemical and physical agents, leaving UV, oxygen radicals (23), and aflatoxin (24). The predominance of C → T mutations at dipyrimidine sites eliminates the latter two agents: Oxygen radicals cause all possible base substitutions and mutate isolated pyrimidines. Aflatoxin causes primarily C → A and C → G substitutions, including CC → AA, CC → AG, and CC → AT. Mutations in skin tumors were found only where UV-like mutations could alter the amino acid.

Conversely, p53 mutations in skin tumors were never observed at the two internal-malignancy hotspots at which the mutating base is a monopyrimidine, codons 175 and 273, and photoproducts were not found there. The difference in mutation frequency between internal and skin cancers at these codons (37/313 versus 0/42) is statistically significant ($P = 0.01$ by Fisher's exact test, two-tailed).

Mutation hotspots can originate from a phenotypic preference for a particular amino acid substitution or from enhanced effects of DNA damage at a site. The skin-cancer-specific hotspots cannot all be explained by phenotypic selection, since (*i*) skin-cancer-specific hotspots 196, 294, and 342 mutate to stop codons and (*ii*) internal-cancer-specific hotspot 273 has a mouse homolog that is part of a dipyrimidine sequence (25) and underwent a C → T mutation in a UV-induced fibrosarcoma (26). In contrast, UV mutation hotspots can originate from base-to-base differences in excision repair rate (27). Similarly, the location of three of the six BCC mutation hotspots at CG dinucleotides suggests that they may be due to the 10^6-fold acceleration of the cytosine deamination rate by cyclobutane dimers (28, 29), occurring at 5-methylcytosine. Preferred sites of sunlight-induced mutation may thus be determined by the combination of photoproduct frequency and the presence of an endogenous, tissue-specific DNA modification.

Cellular Effect of Mutation Hotspots: Inactivating Both p53 Alleles by Sunlight in BCC. Hotspots altered the process of inactivating p53 in BCC compared to other cancers. The typical pattern in internal malignancies is point mutation of one p53 allele and loss of heterozygosity in the other. The

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frequency of allelic loss in 116 carcinomas of the breast, esophagus, bladder, colon, and ovary, and in neurofibromas, ranged from 60% to 90% (30–35). In contrast, only 8% of BCCs had allelic loss within the p53 gene. This difference from internal cancers is statistically significant ($P = 10^{-4}$ by Fisher's exact test, two-tailed).

Instead of allelic loss, 26% of mutated BCCs had one sunlight-related point mutation on each allele; as a result, 45% of mutations were accompanied by another mutation. Two mutations were also seen in an SCC (4). At least one mutation of each pair was located at a mutation hotspot. The pairs of mutations on separate alleles are unlikely to represent tumor heterogeneity in BCC, since no more than two mutated alleles were found in a tumor and the tumors stained homogeneously. In internal cancers, the frequency of two p53 mutations reported in the same primary tumor was 10-fold lower, 3% (5/180) (refs. 4, 6, and 30–35, and references therein). This difference is statistically significant ($P = 0.005$ by Fisher's exact test, two-tailed), although it should be noted that these reports did not examine the entire coding sequence. In skin cancers, all five pairs of point mutations included a mutation with a predicted null phenotype, suggesting that loss-of-function mutations fulfill the inactivating role played by allelic loss in internal cancers.

The presence of two sunlight-induced mutations in one cell is remarkable, even when mutation hotspots exist. The frequency of mutations induced in the *HPRT* gene of cultured human cells by a single sublethal dose of UV light is $<10^{-5}$ per cell generation (36). The frequency of two events in the same cell would then be $<10^{-10}$, and more than two events appear to be required for BCC development (7, 37). In comparison, the number of skin epithelial cell generations at risk can be estimated at only 10^{14} (38). Therefore, either (*i*) some events are unexpectedly frequent or (*ii*) inducing a mutation in a previously mutated cell is made numerically possible by clonal expansion after the first mutation (39). In either case, sunlight appears to cause mutations frequently enough to generate both hits in the p53 tumor suppressor gene.

Since epidemiology indicates that the sunlight exposure most significant for BCC occurs before age 15 (40), it is likely that many of these UV-related p53 mutations occurred in the cell of origin 50–80 years ago. A striking example of the effectiveness of sunlight is Gorlin syndrome, in which an inherited tumor suppressor gene mutation leads to BCCs 40–50 years earlier than in the general population (7, 41). These tumors still arise primarily on sun-exposed skin. Evidently, the sun-exposed skin of normal children contains cells with p53 mutations, but these cells have not yet acquired other genetic alterations required for BCC.

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