p53 Mutation in Nonmelanoma Skin Cancers Occurring in Psoralen Ultraviolet A-Treated Patients: Evidence for Heterogeneity and Field Cancerization

Robert S. Stern, Svetlana Bolshakov*, Arun J. Nataraj,† and Honnavara N. Ananthaswamy*

Department of Dermatology, Beth Israel Deaconess Medical Center, Boston, Massachusetts, U.S.A.; *Department of Immunology, The University of Texas, MD Anderson Cancer Center, Houston, Texas, U.S.A.; †Johnson Graduate School of Management, Cornell University, Ithaca, New York, U.S.A.

A combination of psoralens and ultraviolet A radiation is widely used to treat psoriasis. Long-term, high-dose exposure to psoralen + ultraviolet A is associated with an increased risk of nonmelanoma skin cancer, particularly squamous cell carcinoma. In this study, we used *p53* mutations as a molecular marker to determine the separate contributions of psoralen + ultraviolet A and other ultraviolet exposures, such as ultraviolet B for skin cancer development in psoralen + ultraviolet A-treated psoriasis patients. The results indicated that of 69 tumors analyzed, 37 (54%) tumors had one or more *p53* mutations. Of 37 tumors with mutations, 17 (46%) tumors had only ultraviolet-type mutations, two (5%) tumors had only psoralen + ultraviolet A-type mutations, and 18 (49%) tumors had both types of mutations. Interestingly, psoralen + ultraviolet A-type *p53* mutations were more frequent than ultraviolet type in tumors arising in patients with high-dose exposure to psoralen + ultraviolet A. Field cancerization and tumor heterogeneity appeared to occur frequently in the same patient and even in the same tumor. This study's data suggest that psoralen + ultraviolet A-induced *p53* mutations may play an important part in the development of nonmelanoma skin cancer in psoralen + ultraviolet A-treated patients, but these mutations are likely to act in concert with the effects of other carcinogenic exposures, particularly ultraviolet B, in the development of skin cancer. ***Key words: psoriasis/skin neoplasms/tumor suppressor gene. J Invest Dermatol 119:522–526, 2002***

In 1974, photochemotherapy for psoriasis utilizing oral 8-methoxypsoralen and ultraviolet A (PUVA) was introduced for the treatment of psoriasis (Parrish *et al*, 1974; Haber, 1974). PUVA is highly effective and widely used in treating psoriasis, vitiligo, cutaneous T cell lymphoma, and other skin disorders. In PUVA-treated patients, a concentration-dependent increase in both the risk of squamous cell carcinoma (SCC) and, with very long-term treatment melanoma has been demonstrated (Stern *et al*, 1984, 1997, 1998). The risk of SCC is most elevated in patients with high-dose exposure to PUVA, particularly those with exposure to at least 200 PUVA treatments (Stern *et al*, 1998). The increased risk of SCC may be a consequence of a multitude of effects following PUVA treatment, direct as well as indirect. In addition to its mutagenic and carcinogenic effects, PUVA is immunosuppressive (Kripke *et al*, 1983; Morison and Kripke, 1984). Long-term immunosuppressive therapy is a substantial risk for SCC (Lindelöf *et al*, 2000). In addition, PUVA influences cellular functions and release of cytokines, which might have an impact on the progression of skin cancer (Tokura *et al*, 1999).

Psoriasis patients exposed to high doses of PUVA therapy are also likely to have had substantial exposure to natural sunlight, UVB phototherapy, topical corticosteroids, and a variety of systemic therapies, including oral retinoids and methotrexate. These exposures might act independently or as cocarcinogens with PUVA (Stern and Laird, 1994; Stern *et al*, 1998). More recently, immunosuppressive therapies for psoriasis, which are likely to substantially impact on the risk of SCC, have been introduced (Stern, 1989); however, these therapies were not in widespread use within our study cohort.

PUVA is a potent mutagen and carcinogen (Pathak *et al*, 1959; Ananthaswamy, 1985; Sage and Bredberg, 1991; Sage *et al*, 1993; Nataraj *et al*, 1996; Gasparro *et al*, 1998). In experimental systems, it induces characteristic mutations in *p53* and other genes that are quite different from those induced by UVB (Sage and Bredberg, 1991; Sage *et al*, 1993; Nataraj *et al*, 1996, 1997; Peritz and Gasparro, 1999). This could be attributed to the fact that PUVA induces different types of DNA damage than UVB. Whereas PUVA induces monofunctional adducts and DNA cross-links (Ben-Hur and Elkind, 1973; Song and Tapley, 1979; Sage, 1993; Gunther *et al*, 1995; Gasparro *et al*, 1998), UVB induces mainly cyclobutane-type pyrimidine dimers and (6–4) photoproducts (Rosenstein and Mitchell, 1987). In addition to these photoproducts induced by the direct action of PUVA and UVB on cellular DNA, other oxidative lesions such as 8-oxo-2′-deoxyguanosine (8-oxodG) are also induced indirectly by UVB and PUVA

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Reprint requests to: Dr. Honnavara N. Ananthaswamy, Department of Immunology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, U.S.A. Email: hanantha@mdanderson.org

Abbreviations: BCC, basal cell carcinoma; NMSC, nonmelanoma skin cancer; PUVA, psoralen and UVA; SSCP, single strand conformation polymorphism

via reactive intermediates (Rosen *et al*, 1996; Zhang *et al*, 1997; Liu *et al*, 1999; Cooke *et al*, 2001). It is interesting to note that both pyrimidine dimers and 8-oxodG are induced and excreted in the urine of human volunteers exposed to suberythemal doses of UVA lamps used in PUVA therapy (Cooke *et al*, 2001); this implies that 8-oxodG may play a part in the development of SCC in PUVA-treated patients. In fact, 8-oxodG has been shown to be mutagenic (Kuchino *et al*, 1987). Although multiple mechanisms may contribute to the occurrence of nonmelanoma skin cancer (NMSC) in PUVA-treated patients, an in-depth analysis of *p53* mutations in tumors arising in PUVA-treated patients could help clarify the relative contribution of the mutagenic and carcinogenic effects of PUVA and UVB.

The *p53* suppressor gene is a major target for carcinogen-specific mutations. Human skin cancers arising in the general population (non-PUVA-treated patients) as well as UV-induced mouse skin cancers exhibit a high frequency of UV signature (C to T and CC to TT) mutations (reviewed in Nataraj *et al*, 1995; Brash *et al*, 1996). In contrast, PUVA-induced mouse skin cancers contain *p53* mutations at 5′-TpA/ApT or 5′-TpT sequences that are quite distinct from those found in UV-induced skin cancers (Nataraj *et al*, 1996). In addition, previous studies have shown that SCC arising in PUVA-treated psoriasis patients from Austria, contained both UVB and PUVA signature mutations (Nataraj *et al*, 1997); however, Wang *et al* (1997) found that tumors arising in PUVA-treated patients from the United States had only the UVB type of mutations. Similarly, Seidl *et al* (2001) recently demonstrated that basal cell carcinomas (BCC) arising in PUVA-treated psoriasis patients contained mostly UV-type mutations in the *p53* gene. The reason for this discrepancy could be attributed to the fact that we may have overestimated the frequency of PUVA-type mutations in our study because we considered some C to T mutations at nondipyrimidine sites as PUVA type. As C to T mutations at nondipyrimidine sites are also found in skin cancers arising in the general population, they cannot be considered as PUVA type. Therefore, additional studies using a large sample size and patient population are needed to determine if PUVA contributes to the increased incidence of SCC by exerting its mutagenic and carcinogenic effects directly on target genes and cells. We therefore analyzed a total of 113 specimens from 69 tumors collected from 29 PUVA-treated patients for the presence of PUVA and UVB signature mutations in the *p53* gene. In addition, we determined the extent of heterogeneity in *p53* mutations both within the same tumor and in separate tumors that arose in the same patient. If specific mutations in the *p53* gene are present in tumors arising in PUVA-treated patients, we can hypothesize that the mutagenic effects of PUVA and/or UVB played a part in the etiology of those tumors (Nataraj *et al*, 1997; Gasparro *et al*, 1998).

MATERIALS AND METHODS

The PUVA Follow-up Study was organized in 1975 and includes many of the first patients with psoriasis to be treated with oral photochemotherapy (Stern *et al*, 1979). The study has prospectively followed for nearly 25 years in 1380 patients first treated with PUVA in 1975 or 1976. As of 1998, there were approximately 900 surviving patients. From 1975 to July 1998, we had documented 2693 BCC or SCC among 384 of the 1380 originally enrolled patients. The observed increase in risk of SCC among cohort members was dose related. Compared with the increase in risk associated with PUVA use, other exposures, including UVB phototherapy, methotrexate, and topical tar were, at most, modest risk factors for the development of SCC and BCC in this cohort (Stern and Laird, 1994). The PUVA Follow-up Study submitted 113 separate specimens from SCC and BCC for mutational analysis to the laboratory of Dr Ananthaswamy. All specimens were submitted without any information concerning patient or tumor characteristics to prevent any bias before the final analysis. The patients incurred no additional risks as a result of this study, which was conducted with the approval of The University of Texas M.D. Anderson Cancer Center Institutional Review Board.

DNA extraction Two methods were used to extract DNA from paraffin-embedded sections. The first was the sonication method (Heller *et al*, 1991), with minor modifications. Five to 10 μM sections were deparaffinized with xylene, suspended in 100–200 μl of lysis buffer (40 nM KCl mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 0.01% gelatin, 0.5% Tween 20, and 0.5 mg proteinase K per ml), and sonicated with sterile glass beads (Sigma, St Louis, MO) at 55°C for 30 min using a Branson Model 2200 (Branson Ultrasonic, Danbury, CT). The samples were then boiled for 15 min and spun for 1 min. The resulting supernatant was stored at –20°C until used. In the second method, after deparaffinizing, samples were washed two times with 100% and 70% ethanol, dried in a heating block, and suspended in Release-IT reagent (CPG, Inc., Lincoln Park, NJ). The tissues were grounded using disposable pestles and lyzed directly in amplification tubes in a thermal cycler, according to the manufacturer's instructions. Lysed samples were stored at –20°C. Each sample was screened for *p53* mutations by both methods.

Polymerase chain reaction–single strand conformation polymorphism (PCR–SSCP) analysis Exons 4–9 of the *p53* gene were amplified separately using specific upstream and downstream primers encompassing intron–exon junctions. All primers were custom synthesized by Genosys Technologies, Inc. (The Woodlands, TX). Five microliters of the lysates were used as templates in a 50 μl solution containing 10 mM Tris–HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 150 μl each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxycytidine triphosphate, and deoxythymidine triphosphate; 2.5 μCi of [α-^{32}P]deoxycytidine triphosphate; upstream and downstream primers (10 μM each); and 5 U AmpliTaq (Perkin Elmer, Foster City, CA). The reaction mixture was overlaid with mineral oil (Sigma, St Louis, MO) and PCR was carried out in a DNA thermal cycler (Perkin Elmer) as follows. The DNA was denatured at 94°C for 4 min followed by two cycles at 94°C (1 min), 65°C (1 min), and 72°C (1 min); five cycles at 94°C (1 min), 60°C for 15 min to complete the extension. An aliquot (3 μl) of the PCR product was subjected to SSCP analysis on 5% Mutation Detection Enhancement (FMC Bioproducts, Rockland, ME) acrylamide gels with 10% glycerol, as described previously (Kanjilal *et al*, 1993). The gel was run at 6 W for 14–18 h, dried and exposed to autoradiographic film. Extreme precaution was taken to avoid contamination, and blank PCR controls without DNA templates were included in every PCR reaction. In addition, human placental DNA was included in every PCR reaction as a normal control. PCR–SSCP analysis was performed three times for each sample.

Nucleotide sequencing Wild-type and shifted (mutant) bands were cut out of the dried SSCP gels, and the DNA was eluted by incubating at 80°C for 30 min in 100 μl of Tris-EDT buffer. The eluted DNA was reamplified by PCR using the same primers and conditions described above and the PCR products were analyzed on 2% agarose gel along with a Low Mass DNA ladder (Life Technologies, Gaithersburg, MD) to verify successful amplification and quantify the products for further sequencing analysis. The samples were treated with shrimp alkaline phosphatase and exonuclease I (USB, Cleveland, OH) for 15 min at 37°C and 15 min at 80°C before direct sequencing using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (USB). In some cases genomic DNA from SSCP-positive tumors was amplified by cold PCR and the products sequenced directly to confirm the mutation. In addition, to rule out PCR-generated mutations, human placental DNA was simultaneously amplified and sequenced in every experiment.

RESULTS

In this study, we examined NMSC arising in psoriasis patients who had long-term exposure to PUVA before the removal of the first tumor. All *p53* mutation analyses were performed with the laboratory blinded as to the type of tumor and whether the specimen was the only one or one of multiple specimens from a tumor. Tumor tissues were first screened by SSCP analysis for *p53* mutations in exons 4–9, because over 95% of all mutations are known to occur at these exons in human cancers (Greenblatt *et al*, 1994). Tumors harboring *p53* mutations were identified by the presence of aberrant (shifted) bands by SSCP. DNA from these bands was then eluted, reamplified, and sequenced to determine the nature of mutations.

The 69 tumors analyzed as 113 separate specimens came from 21 patients, of whom 15 (71%) had at least one tumor with a *p53* mutation. The characteristics of the 21 patients studied are summarized in **Table I**. The anatomic distribution of tumors used in this study is shown in **Table II**. Of the 69 NMSC analyzed

Table I. Characteristics of 21 patients included in this study[a]

Patient history/characteristics	Mean	Range	SD
Male 10; female 11			
Age at first tumor analyzed	60	(42–77)	12
PUVA treatment to first tumor analyzed	329	(93–613)	178
Number of tumors analyzed	3.3	(1–4)	2.8
Years of methotrexate use to last tumor (17 of 21 had used methotrexate)	5.5 years	(0–20)	4.8
Number of SCC (up to last tumor)	17	(1–93)	25
Number of BCC (up to last tumor)	6	(0–28)	9
Years from first PUVA to first tumor analyzed	14.0	(3–18)	4

[a]Skin type (one patient was not skin typed): 1 = 1 patient, 2 = 9 patients, 3 = 9 patients, 4 = 1 patient; history of exposure to ionizing radiation for psoriasis = 2 patients (10%); high exposure to UVB/tar = 4 patients (33%).

Table II. Anatomic location by tumor and tumor type

	Tumor type		
Site	SCC	BCC	All
Face/neck	3	11	14
Upper extremities	8		8
Trunk[a]	7	4	11
Lower extremities	33	3	36
Total	51	18	69

[a]Includes anogenital tumor.

for *p53* mutations, 51 were SCC (74%), including one initially diagnosed as a SCC and subsequently diagnosed as hypertrophic actinic keratosis, which is included as a SCC in our analysis. The remaining 18 tumors were BCC. A higher percentage (61%) of SCC harbored *p53* mutations compared with BCC (33%) ($p<0.05$, chi square test). Overall, 86% of SCC with two or more separate slides or blocks analyzed had a *p53* mutation and 67% of such tumors had a PUVA-type mutation. BCC were significantly more likely to come from the head and neck than SCC (**Table II**). Some tumors had multiple independent specimens (blocks or histologic slides) analyzed. Therefore, a given tumor might have all PUVA-type mutations, all UVB-type mutations, mutations of both types, or no mutations.

We detected a total of 83 *p53* mutations in 55 (49%) of 113 specimens. Of the 15 patients with PUVA-type or UVB-type mutations detected in one or more specimens, one patient (6.7%) had tumors with only PUVA mutations, six patients (40%) had tumors with only a UVB-type mutation, seven patients (46.6%) had both UVB and PUVA-type mutations in their tumors, and one patient (6.7%) had a tumor with mutations classified as ''other'' type (**Fig 1*A***). All but one patient (91%) with more than one tumor analyzed had at least one mutation. Of the 83 mutations, C to T and CC to TT transitions represented 27% and 16%, respectively, and T to A and TT to AA transversions represented 11 and 8.0%, respectively (**Fig 1*B***). Interestingly, T to C transitions occurred at a relatively high percentage (31%). Mutations occurring at 5′-TpA/ApT and 5′-TpT sites, potential sites for PUVA-induced mono-adducts and DNA cross-links, were considered to have been induced by PUVA, and C to T, CC to TT and T to C transitions at CC, CT, and TC sites were considered to have been induced by UV; however, it is quite possible that C to T transitions may also arise from indirect effects of PUVA. It is well known PUVA and UVA radiation causes DNA damage indirectly and produce reactive oxygen intermediates and oxidative lesions such as 8-oxodG (Rosen *et al*, 1996; Zhang *et al*, 1997; Liu *et al*, 1999; Cooke *et al*, 2001), which is reported to be mutagenic (Kuchino *et al*, 1987). In addition, Reid and Loeb (1993) demonstrated that reactive oxygen species such as hydroxyl radicals can induce C to T and CC to TT transitions, and Wang *et al*, 1988) showed that stable oxidation products of cytosine and thymine can induce C to T transitions. Thus, it is reasonable to speculate that some of the mutations found in skin cancers from PUVA-treated psoriasis patients could have been induced by the indirect action of PUVA or UVA (Peritz and Gasparro, 1999); however, our understanding of PUVA-induced photochemistry *in vivo* human cells is far from complete. Nonetheless the presence of mutations at these sites suggest that these sequences are potential hot spots for PUVA- or UV-induced mutations in psoriasis patients who have undergone PUVA treatment. CC to TT and C to T transitions at dipyrimidine sites occurred at codons 157–158, 247–248, and 250, which are also hotspots in NMSC from non-PUVA-treated patients (Brash *et al*, 1996). The *p53* mutation data summarized in **Table III** reveal that 34 of 83 mutations were characteristic of PUVA-induced mutations (41%), 36 mutations (44%) had the UV signature, and 13 were categorized as ''other'' type (15%), meaning they could have been induced by UV or PUVA, or they arose spontaneously during tumor progression.

Twenty-four (44%) of 55 specimens had multiple UVB and/or PUVA-type *p53* mutations. Even though some tumors from the same patient had both PUVA-type and UV-type mutations, one type occurred more frequently than the other. For example, tumor 4 from patient 1 had a total of six mutations in four specimens, of which five were PUVA type and one was UV type. Similarly, tumor 3 from patient 2 had a total of 13 mutations in six specimens, of which 10 were PUVA type, two were UV type and one other type. Conversely, tumor 2 from patient 9 had a total of six mutations in four specimens, of which five were UV type and one was PUVA type. These results suggest that more likely PUVA was the primary carcinogen in tumors 4 and 3 from patients 1 and 2, respectively, and UV was the primary carcinogen in tumor 2 from patient 9. When the characteristics of tumors that had a mutation were compared with those without mutation there were two major differences. The likelihood a tumor would have a mutation increased with the number of separate specimens analyzed from that tumor. For tumors with at least two separate specimens analyzed, 82% had mutation(s) compared with only 46% of tumors with only a single specimen analyzed ($p < 0.01$, chi square test). In fact, there was a significant association between the number of separate specimens examined and the likelihood of having a PUVA and/or UVB *p53*-type mutation (ANOVA, $p = 0.05$).

The age of patients whose tumors displayed at least one tumor with only PUVA-type mutations tended toward being younger than those whose tumors had only a UVB-type mutation, mutations of both types, or no mutation, but this did not reach statistical significance. Tumors that had only UVB mutations occurred after fewer PUVA treatments than tumors that displayed PUVA-type mutations, but this did not reach statistical significance.

DISCUSSION

We used *p53* mutations as a molecular marker to determine whether PUVA or UVB is associated with the induction of skin tumors in PUVA-treated psoriasis patients. This study demonstrates that in a U.S. population of patients treated with PUVA, 41% of all *p53* mutations detected were characteristic of those associated with PUVA exposure. Nearly three-fourths of SCC that developed after exposure to at least 300 PUVA treatments exhibited mutations of the type associated with PUVA. In addition to PUVA-type mutations, UV-type mutations were also present at a high frequency (44%) in NMSC arising in PUVA-treated patients. There was a trend for patients with only UVB-type mutations to more often be fair-skinned and burn easily and be older, but not to have more exposure to UVB for psoriasis therapy. These findings suggest that exposure to natural sunlight rather than therapeutic UVB play a greater part in inducing UVB-type mutations. The

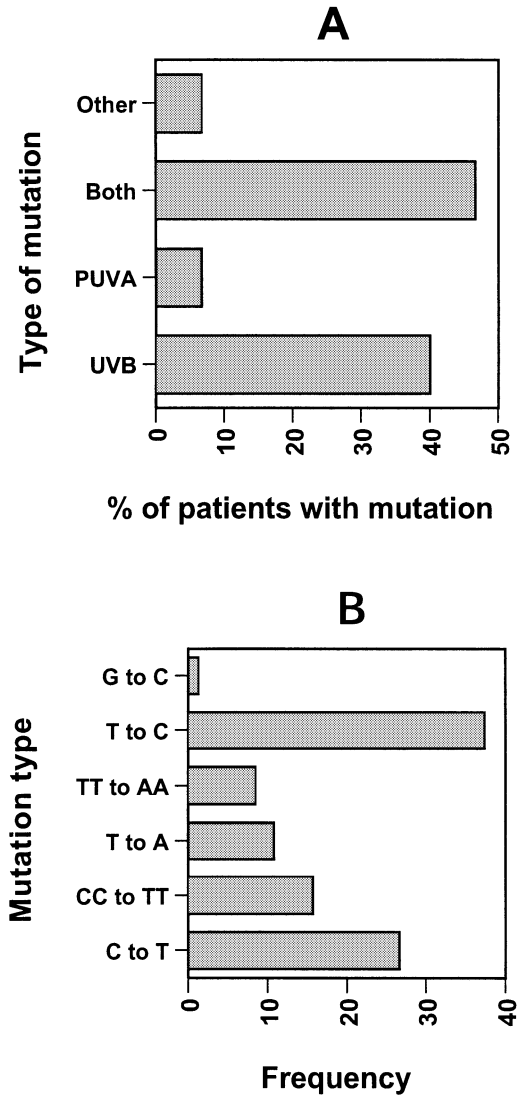

Figure 1. Type and frequency of *p53* mutations detected in skin cancers from PUVA-treated patients. *Panel A* shows percent of patients with UVB only, PUVA only, both, or other types of *p53* mutation. *Panel B* shows the frequency and type of base substitutions in skin tumors from PUVA-treated patients. Mutations arising at 5′-TpA and 5′-TpT sites are considered as PUVA type, whereas C to T, T to C, and CC to TT mutations occurring at dipyrimidine sites are considered as UV type; however, it is possible that some of these mutations could be induced indirectly via oxidative damage caused by PUVA or UVA alone.

presence of UVB signature *p53* mutations is expected because the UVA lamp source used in PUVA therapy invariably contains some UVB radiation. In addition, some of the patients may also be exposed to prior UVB phototherapy or natural sunlight post-PUVA therapy. Nonetheless, these results are somewhat different from a previous finding that about 64% of *p53* mutations detected in NMSC from PUVA-treated psoriasis patients from Austria were of PUVA type and about 32% were of UVB type (Nataraj *et al*, 1997). This discrepancy is attributed to the fact that we considered some mutations occurring at dipyrimidine sites such as 5′-CpT sites as PUVA type. In fact such mutations are also present in UV-induced skin tumors and hence cannot be considered as PUVA type. Nonetheless, Wang *et al* (1997) reported that the frequency of UVB-type mutations in SCC developing in PUVA-treated patients was comparable with that of tumors arising in non-PUVA-treated patients; however, they did not detect PUVA-type mutations in any of the tumors from PUVA-treated patients. Similarly, a recent study by Seidl *et al* (2001) has shown that BCC from PUVA-treated Austrian patients contained mostly UV-type *p53* mutations. The reason for the discrepancy between these studies and our study is unknown but could be attributed to differences in dose and extent of PUVA treatment, genotypic and phenotypic characteristics of patients, or other unknown factors. Alternatively, the differences in patient population could also account for this apparent discrepancy.

Table III. Frequency of PUVA and UV type *p53* mutations in skin cancers from PUVA-treated patients

Mutation type[a]	Number of specific mutations/ total number of mutations	Mutation frequency
PUVA	34/83	41%
UV	36/83	44%
Other	13/83	15%

[a]Mutations arising at 5′-TpA and 5′-TpT sites are considered as PUVA type, whereas C to T, T to C, and CC to TT mutations occurring at dipyrimidine sites are considered as UV type.

In about half of the specimens analyzed we failed to detect a *p53* mutation characteristic of those associated with PUVA or UVB. The reasons for this might include the following:

1 We analyzed only exons 4–9 of *p53* gene because over 95% of all mutations are known to occur at these exons in human cancers (Greenblatt *et al*, 1994). It is possible that mutations could also occur in exons 1–3 and 10 and 11 in skin cancers from PUVA-treated patients; however, we did not look for mutations at these exons.

2 Some specimens contained silent mutations. We did not count them as mutation-positive because those mutations did not change the corresponding amino acid.

3 Some of the samples may contain large amounts of normal cell contamination, which can mask detection of mutations by the direct sequencing technique used, particularly if only one *p53* allele is mutated in tumor cells.

4 A higher proportion of tumors without mutations detected were BCC, which are less strongly associated with sunlight than SCC.

Nonetheless, when multiple specimens from the same tumor were analyzed, a *p53* mutation was more likely to be detected and multiple mutations were often present. Even within single specimens, multiple mutations characteristic of both UVB and PUVA exposure, were detected in about one-seventh of specimens. Interestingly, however, a given tumor from a patient with multiple tumors, even though it had both PUVA- and UV-type mutations, one type (either PUVA or UV) of mutation often dominated over the other, which suggests that the dominating component presumably came first, creating the founder mutant cell, and one of the progeny cells acquired mutations later by the second carcinogen. Thus in tumors 4 and 3 from patients 1 and 2, respectively, PUVA could be the major carcinogen and UV the minor component. Conversely, in tumor 2 from patient 9, UV could be the major component and PUVA the minor component. As neither type of mutation was present in every sample of a tumor, *p53* mutation may not have been the first event in tumorigenesis and may not have conferred a growth advantage.

The presence of multiple *p53* mutations has also been reported in human and UV-induced mouse skin cancers and head and neck cancers (Kanjilal *et al*, 1993; Lydiatt *et al*, 1998). The presence of multiple mutations in the same tumor is consistent with our earlier observation that suggest field cancerization (Kanjilal *et al*, 1995). Field cancerization is postulated to result from prolonged exposure to carcinogens. Numerous mutations accrue throughout the field or area sharing the carcinogen exposure(s), and some of the initiated cells progress to multiple primary tumors. The presence of multiple *p53* mutations observed in PUVA-induced mouse skin cancers suggests (i) that clones harboring an initial mutation on one

allele were targets for a second mutational event on the other allele, or (ii) that these mutations may have arisen independently, perhaps in different clonal subpopulations during tumor development (Kanjilal *et al*, 1993). It is possible that mutant *p53* alleles with single base changes were targets of secondary and tertiary mutational events, perhaps because of repeated exposure to PUVA therapy or UVB exposure. Alternatively, secondary or tertiary mutations could also occur in the same *p53* allele or different alleles. A third possibility is that multiple mutations may arise independently, perhaps in different clonal subpopulations during continued PUVA therapy or during tumor development. This possibility is supported by the finding that primary tumor masses often consist of clones of cells that have different biologic properties such as karyotype, surface receptors, growth rate, and metastatic ability.

In summary, molecular studies using the *p53* gene as a marker for carcinogen-specific mutations performed in this study revealed the presence of PUVA-type mutations. UVB-type mutations, however, were observed at an equal frequency or even at a slightly higher frequency in skin cancers arising in PUVA-treated psoriasis patients. These findings are consistent with the mutagenic effects of PUVA being an important mechanism for the induction of SCC in PUVA-treated patients; however, as in the general population, where exposure to UVB radiation in sunlight is associated with the development of NMSC, mutations from UVB, both from UVB phototherapy and natural sunlight, are likely to be more important in the development of skin cancer in PUVA-treated patients. Although PUVA and UVB leaves classic mutational footprints in many tumors that develop in PUVA-treated patients and certainly play a part in the development of most SCC in patients exposed to high doses of PUVA, it is difficult to rule out the involvement of other potentially carcinogenic exposures in the development of NMSC in PUVA-treated patients.

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