Ultraviolet Radiation Induced Signature Mutations in Photocarcinogenesis

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The photons of sunlight begin a series of genetic events in skin leading to cancer. UV signature mutations provide an alternative to inherited mutations as a way of identifying genes that are involved in cancer development. They augment epidemiologic and clinical data by serving as molecular evidence for the role of UV radiation in skin carcinogenesis. Signature mutations are present in *TP53* and *PTCH*, two tumor suppressor genes responsible for non-melanoma skin cancer. We review evidence that clones of TP53-mutated cells are present in normal human and murine epidermis exposed to UVB and conclude that, in addition to being a tumorigenic mutagen, sunlight acts as a tumor promoter by favoring the clonal expansion of *TP53* mutated cells. These combined actions of sunlight result in normal individuals' carrying a substantial burden of keratinocytes predisposed to cancer. Thus cancer involves both a single-cell problem and a multi-cell problem; in skin cancer, sunlight appears to drive both. *Key words: basal cell cancer/p53/PTCH/squamous cell cancer. Journal of Investigative Dermatology Symposium Proceedings 4:6–10, 1999*

Mutations are pivotal in cancer development. In addition to their causal role, they constitute a permanent record of the events that created the cancer. In familial cancers, mutations are inherited. A genetic analysis of the patient and family members may reveal direct links between genes and tumors. Inactivated genes such as Rb in retinoblastoma (reviewed in Hooper, 1998; Gallie *et al*, 1999), APC in inherited familial adenomatous polyposis syndrome (reviewed in de la Chapelle and Peltomaki, 1998; Fearon and Dang, 1999), or BRCA1 and BRCA2 in hereditary breast cancer (reviewed in Blackwood and Weber, 1998; Duncan *et al*, 1998) have been shown to contribute to tumors. Most tumors, however, are sporadic; the mutations underlying behind these tumors occurred anew during DNA replication in each somatic progenitor cell. Yet, in order to understand sporadic cancers, the genes responsible for these tumors must be identified. This can be best achieved by using a retrospective approach in which DNA from tumors is analyzed in search of mutations. Several genes have been identified using this approach, both oncogenes and tumor suppressor genes. The role of mutations in cancer is currently unquestioned, yet important steps of the process of cancer development fall short of being explained. For example, the cause of these mutations is largely unknown. This is primarily because mutagenic agents are nearly countless whilst mutation types are limited to a few. Also, important links are yet to be discovered concerning how a single mutant cell slips past the body's surveillance system to start becoming a tumor. Steps that we find most intriguing are the earliest ones: the acquisition of the first mutation and the rise of a clone from a single mutated cell.

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Abbreviations: AK, actinic keratosis; BCC, basal cell cancer; SCC, squamous cell cancer.

UV SIGNATURE MUTATIONS

By definition, a mutation is a change in the sequence of the DNA. This change is introduced upon incorporating a non-complementary base into the strand being synthesized by DNA polymerase during replication. Because mammalian polymerases have high fidelity, mistakes most often occur when the template is altered and the enzyme misinterprets it. Numerous factors cause base alterations (**Table I**), but the resulting variety of mutations is much smaller. Mutations, therefore, do not reflect a unique base alteration and in most cases the mutagen remains untraceable. UV-induced mutations are exceptions. Certain sequence patterns, such as two pyrimidine bases (cytosine or thymine) adjacent to one another, are more susceptible to being damaged by UV than other base combinations (**Fig 1**). Thus, UV generates a rather distinct and homogeneous mutation pattern. It is also the pattern found in skin tumors most frequently. In skin tumors cytosine (C) is commonly replaced by thymine (T), but – because the photoproducts occur only where two pyrimidines are adjacent – this mutation occurs only where the C was next to a T or another C. Sometimes, two adjacent cytosines (CC) are changed to TT. Such changes occur almost exclusively as a result of UV. The C→T substitution may be instructed by the DNA photoproduct (Lawrence, 1981; Miller, 1985; Drobetsky *et al*, 1987). The "A rule" (Schaaper *et al*, 1983; Strauss, 1991) might also play a role in the formation of UV signature mutations: DNA polymerases treat some altered bases as if they were an adenine and incorporate a thymine into the daughter strand.

Coexistence of two features thus makes skin cancer an ideal candidate to study: they develop right in front of our eyes and they bear a traceable mutation. The presence of UV signature mutations helps identify the perpetrator. These signatures augment epidemiologic and clinical data by serving as molecular evidence for the role of UV radiation in skin carcinogenesis. Detecting these signatures from early clinical lesions helps identify steps of skin carcinogenesis and genes of the greatest importance.

TP53 is such a gene. Our knowledge regarding TP53 makes it one of the best-understood pathways for revealing how a cell acquires a mutation and how that mutation leads to cancer.

Table I. The most common factors that cause alterations in the chemical structure of the DNA, thus leading to mutations

Internal events	External damage to DNA
1. Spontaneous base alterations	1. UV radiation
2. Tautomeric shifts	2. Chemical agents
3. Deamination of bases	Alkylating agents
4. Depurination and depyrimidination	Cross-linking agents
5. Oxidative damage to DNA	Psoralens
	Chemicals that are metabolized into electrophilic reactants
	Base analogs
	3. Ionizing radiation

TP53 IN SQUAMOUS CELL CANCER

The TP53 tumor suppressor gene encodes a 53 kDa molecular weight protein that functions as a transcription factor. That protein regulates a number of genes that eventually lead to two major end-points: cell cycle regulation and apoptosis (Lane, 1992). Induction of TP53 protein can be seen as early as 30 min after exposure to UV (Maltzman and Czyzyk, 1984). TP53 protein induction happens at the level of reduced degradation, not transcription, because an increase in mRNA is not detected. The half-life of the wild-type TP53 protein is short and the signal usually disappears a few hours after induction (Ljungman and Zhang, 1996). Mutated TP53, on the other hand, is relatively resistant to degradation; this phenomenon can be used in immunohistochemical staining to detect cells with potentially mutated TP53 (Hall and Lane, 1994). In the immunohistochemical reaction, a mono- or polyclonal antibody is directed toward certain epitopes of the TP53 protein and the antigen-antibody binding is visualized as a nuclear staining. Because the basal level of TP53 protein expression is low in skin, a positive immunohistochemistry signal can be seen if (i) TP53 is being expressed in response to a recent physiologic stimulus such as UV (TP53 is wild-type), or (ii) the protein has failed to undergo degradation (TP53 is mutant). Some antibodies target mutated forms of TP53 by recognizing epitopes that are accessible only when an amino acid change occurs in the protein, thus allowing selective detection of mutated TP53. Yet, the definitive determination requires DNA sequencing.

Sections from squamous cell cancer (SCC) contain a large number of cells that are positive upon staining with an antibody against TP53 (Brash *et al*, 1991; Ziegler *et al*, 1993; Wikonkal *et al*, 1997). Sequencing data from a large number of skin tumors showed that TP53 is mutated in over 90% of SCC (Brash *et al*, 1991; Campbell *et al*, 1993; Ziegler *et al*, 1993; Jonason *et al*, 1996; Ren *et al*, 1996). The most common mutation was C→T at dipyrimidine sites, in about 70% of the cases. Tandem CC→TT mutations were also found at a frequency of 10%; these mutations have been shown to be exclusive to UV in different systems. The remaining 20% of mutations in SCC have also been seen in UV irradiated cells in culture but are not UV signature mutations because they can be caused by other agents. Hence, two important pieces of the cancer-formation puzzle become apparent: (i) UV is the most prominent mutagenic agent in skin, and (ii) TP53 is one gene that undergoes mutations. The high frequency of TP53 mutations, and the fact that they all change an amino acid, indicates that a mutation in this gene is a significant contributor to skin tumors. In internal cancers, TP53 mutations are more diverse and virtually no mutations resembling the characteristic UV signature were found (Greenblatt *et al*, 1994).

Given that TP53 mutations caused by UV lead to SCC, one might be able to use similar logic to find early forms of these tumors that contain cells with UV signature mutations in the TP53 gene. A condition related to sun exposure, actinic keratosis (AK), is considered by dermatologists to be a forerunner to early SCC. The transitions from severe sun damage to AK and from AK to *in situ* SCC have clear histologic features: increasing number of mitoses, more evident cellular atypia, and the appearance of the horn cysts characteristic to SCC. These stages are clearly distinguishable in the histology and appear as sequential steps (Lever, 1997). The similarity of AK to SCC is also present at the molecular level. Work in our laboratory and by others showed that AK, like SCC, contain cells that stain positively with anti-TP53 antibodies. Moreover, these cells contain mutations and the mutation pattern is similar to that of SCC (Ziegler *et al*, 1994). This finding further supports the notion that TP53 mutations appear early and play a critical role in the development of skin cancer.

To further address this question, we examined clinically normal skin. Samples of skin from healthy individuals were collected from sun-exposed and sun-protected areas. The epidermis was separated from the dermis and the presence of TP53 was detected by immunohistochemistry using anti-TP53 antibody. The epidermal sheets were then analyzed for TP53 immunopositive nuclei under a light microscope. We find clones of 60–1000 positive cells throughout sun-exposed epidermis (Jonason *et al*, 1996). The anti-TP53 staining reveals two distinct distributions of reactive keratinocytes. Sun-shielded skin harbors only few TP53 positive cells and these cells appear either isolated or in very small groups. Chronically sun-exposed skin, on the other hand, contains more keratinocytes that stain positively with the anti-TP53 antibody than does sun-protected skin and the size of these "patches" is larger.

After microdissecting cells that stained positively with the antibody, the DNA was isolated and exons of the TP53 gene were amplified by polymerase chain reaction and sequenced. The sequencing reveals that DNA of keratinocytes obtained from sun-exposed normal skin contains mutations reminiscent of those seen in SCC and AK (**Table II**). Normal skin from sun-protected skin areas does not contain mutations (Ren *et al*, 1996). Two important features of the patches were also analyzed: patch number and patch size. Patch frequency, expressed as number of patches per cm^2 skin area, shows a 10-fold difference between sun-exposed and relatively sun-shielded skin such as abdomen (**Fig. 2**). Patch size data are less straightforward to interpret because of the large variation from patch to patch; however, a clear trend toward larger patches in sun-exposed skin is apparent. Taken together, these clones in skin chronically exposed to sunlight mean that chronically sun-exposed skin itself can be considered one of the earliest steps in skin carcinogenesis.

In chronically sun-exposed skin, these clones are present at a frequency of 35 clones per cm^2 on average. The surprisingly high number of TP53 immunoreactive patches directed our attention to another fact. Comparing our numbers of TP53 patches to SCC incidence, it is clear that only a small percentage of these TP53 positive cells progress to tumors. Epidemiologic data show that less than 20% of Caucasians develop SCC during their entire life. Therefore, most of these clones either disappear or else remain but do not progress, e.g., do not acquire additional mutations. Clones of mutated cells disappear probably either by squaming off through regular keratinocyte differentiation or by going through apoptosis. A clinical observation is that human AK often regress if further sun-exposure is prevented (Marks *et al*, 1986). In addition, apoptosis frequency in AK is higher than in normal skin (Makino *et al*, 1998; Tsuji *et al*, 1998), which speaks in favor of p53 independent apoptosis being the effector mechanism.

Similar TP53 positive clones have been detected using CM-5 anti-TP53 antibody in mouse skin after chronic UVB irradiation. This set of experiments showed that TP53 positive clones arose in the skin of hairless mice after 17 d of daily UVB exposures (Berg *et al*, 1996). After 30 d of chronic UV exposure, the number of these positive cell clusters increased. TP53 positive cells have also been observed in acute experiments 12–24 h after a single UV exposure, largely because the antibody recognizes both overexpressed wild-type and mutant p53. The difference between the chronic irradiation staining and the acute experiment is that p53 immunostaining observed after a single UV exposure disappears 48–72 h after the UV treatment. Conversely, TP53 positive cells that were generated by repeated UV exposures remain reactive with the antibody as long as 56 d after the irradiation ceased. The length of time after which these cells still react with the TP53 antibody suggests that keratinocytes in the chronic irradiation experiment acquired a mutation in the p53 gene. To address this question, PAb240, an antibody that recognizes only mutated forms of p53, was used in an immunohistochemistry reaction. Epidermis from a chronic experiment contained several patches stained positive with

Table II. Patches of TP53 mutated keratinocytes in human epidermis[a]

	Age	Location	Patch	TP53 status
Sun shielded				
YC19A	24	Inner arm	1	wt
YC17A	28	Abdomen	4	wt
YC7A	29	Breast	1	wt
YC8A	32	Breast	4	wt
YC4A	40	Lower abdomen	3	wt
YC13A	41	Breast	7	wt
YC18A	44	Inner arm	2	wt
YC14A	49	Lower abdomen	0	wt
YC6A	50	Breast	8	wt
Sun exposed				
YC12A	9	Lip	46	wt
YC24A	43	Pre-auricular	23	
YC5B.1	47	Pre-auricular (left)	29	T→Stop
YC5B.2				wt
YC5C	47	Pre-auricular (right)	45	
YC2C.1	48	Lower eyelid	32	wt
YC2D.1	48	Pre-auricular	23	wt
YC2D.2				wt
YC2D.3				wt
YC2E	48	Pre-auricular	33	
YC23A	51	Upper eyelid (right)	24	
YC23B	51	Upper eyelid (left)	25	
YC15A	53	Lower eyelid	44	
YC15B.1	53	Lower eyelid	41	C→T
YC15B.2				C→T
YC1A	65	Upper eyelid (right)	36	
YC1B.1	65	Upper eyelid (left)	37	CC→TT
YC1B.2				wt
YC1B.3				wt
YC1B.4				wt
YC22A	66	Upper eyelid (right)	33	
YC22B	66	Upper eyelid (left)	25	
YC11A	74	Pre-auricular	39	
YC3A	79	Upper eyelid	27	wt

[a]YC numbers denote different individuals, letters denote different samples, and decimal numbers denote sequenced patches. Patch frequency is expressed as number of patches, regardless of their pattern, per cm^2. Base changes are indicated, otherwise wt stands for wild-type TP53. (from P.N.A.S. 24: 14025–14029, 1996)

the mutant specific antibody, whereas skin from mice after a single UV exposure never did, suggesting that the p53 gene acquired mutations after 17 d of irradiation (Berg *et al*, 1996). Given that chronic UV irradiation results in a squamous cell cancer after 30–80 wk in mice, the authors used sections of such UVB generated tumors as positive controls. Twenty of 24 samples contain positive reacting cells and nine of 10 PAb240 positive tumors contained mutations in the p53; eight of them showed a C→T transition at dipyrimidine sites and one had a CC→TT double transition. Immunoreactivity detected by the PAb240 antibody shows excellent conformity with sequencing data.

These experiments revealed another aspect of chronic UVB exposure: the TP53 positive clones gradually disappear after the UV irradiation ceases. The longer the exposure persists, the slower the disappearance. This result emphasizes the reversibility of these early precancerous lesions. The flexibility of animal experiments, especially the ability to control UV exposure, further supports the notion that TP53 clones arise as daughter cells of a single TP53-mutated cell that acquired its mutation by UV exposure.

TP53 IN BASAL CELL CANCER

Basal cell cancer (BCC) is quite different from SCC. For historic reasons, textbooks still categorize BCC together with SCC under the category "non-melanoma skin cancer". This distinction serves didactic purposes, but has little relevance to the actual biology of these tumors. The similarity between BCC and SCC is principally confined to having the originating cell in common: the keratinocyte. The keratinocyte origin explains why BCC and SCC lack pigmentation, as opposed to the dark pigmentation seen in malignant melanoma. Among other differences in the clinical picture, BCC have no clear precursor lesions (Miller, 1991). The most important difference between BCC and SCC is that BCC lack a key feature of cancers: metastasis. Some even reason that BCC do not qualify as a "cancer" because they do not metastasize. Differences between the clinical picture of BCC and SCC do not appear to stem from differences at the TP53 level. Mutation analysis of TP53 in BCC showed that 60%–100% of the tumors contain mutations in the TP53 gene (Ziegler *et al*, 1993; Pontén *et al*, 1997). Investigators needed alternative approaches to BCC pathogenesis.

PTCH IN BCC

The possibility of other genes being involved in the pathogenesis of BCC has been recently confirmed by identifying another gene that plays a significant role in BCC formation: the human homolog of the Drosophila "patched", *PTCH* (Hahn *et al*, 1996; Johnson *et al*, 1996; Gailani and Bale, 1997). The gene was first cloned from Gorlin syndrome patients. This autosomal dominant disorder, also called "nevoid basal cell carcinoma syndrome", is characterized by multiple BCC. The syndrome includes striking congenital malformations such as pits of the palms and soles, keratocysts of the jaw, midline brain malformations, spine and rib abnormalities, ectopic calcification, macrocephaly with coarse facies, and generalized overgrowth (Springate, 1986; Gorlin, 1995).

Numerous *PTCH* mutations have been identified in unrelated Gorlin patients, distributed across the gene (Chidambaram *et al*, 1996; Hahn *et al*, 1996; Johnson *et al*, 1996; Unden *et al*, 1996; Wicking *et al*, 1997). One-third of the mutations have been base substitutions, nearly all leading to premature stop codons or splice-site mutations. One-third were 1- or 2-bp deletions or insertions, resulting in frameshifts, and another third were 4- to 76-base deletions or insertions. Each kind of mutation would inactivate the protein, as expected for a

tumor suppressor gene. The unusually high frequency of deletions and insertions may be a feature of the *PTCH* gene or may simply reflect the fact that mutations were obtained by first screening for single-strand conformation polymorphisms. This assay misses many base substitutions, but will be efficient at detecting larger mutations such as deletions, insertions, or tandem substitutions. The larger mutations will be effective anywhere in the gene, so they do not reveal whether the gene has mutation hotspots or functionally important protein domains.

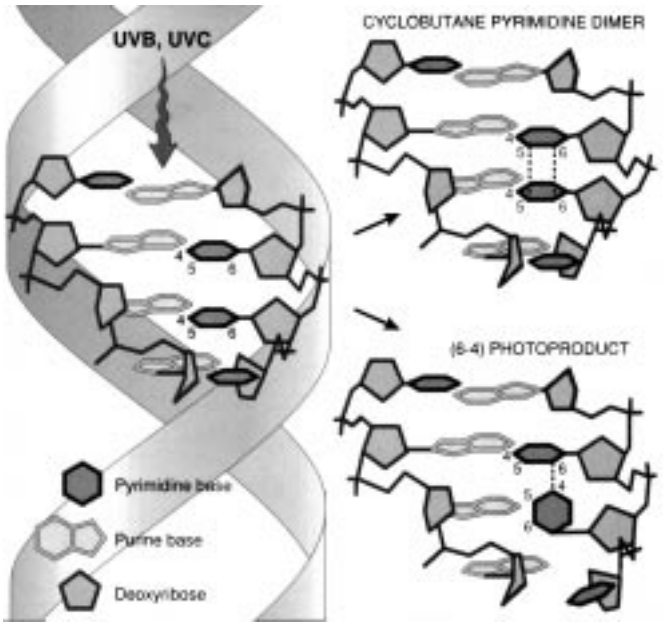

Figure 1. Two major photoproducts after UV absorption, the cyclobutane pyrimidine dimer and the (6-4) photoproduct. The close proximity of two pyrimidine rings is the foundation on which two major kinds of chemical structure rearrangement can occur. The altered bases that are formed after these rearrangements are referred to as photoproducts. The most prevalent is the cyclobutane pyrimidine dimer (*top right*). It is generated when the UV photon's energy opens the double bonds between C atoms 5 and 6 of two neighboring pyrimidine rings, whereupon two new bonds are established. These covalent bonds are formed between the participating rings and link the opposing C atoms of the two original rings. This structure is a four-member ring itself, called the cyclobutyl ring. The second major photoproduct is the pyrimidine-pyrimidone (6-4) photoproduct (*bottom right*). The foundation is again two pyrimidine bases lying next to one another. If struck by UV, similarly to the cyclobutane dimer formation, first the C-5 – C-6 double bond opens. The subsequent events are, however, different. One of the pyrimidine rings rotates and its C-4 atom forms a new bond with C-6 atom of the other ring. In this case, only one new bond is formed: between atom C-6 in one of the neighboring pyrimidone/pyrimidine rings and C-4 in the other.

Of 23 sporadic BCC, one-third had *PTCH* alterations detectable by screening for single-strand conformation polymorphisms (Gailani *et al*, 1996; Hahn *et al*, 1996; Johnson *et al*, 1996; Unden *et al*, 1996). Direct sequencing of two BCC without allelic loss or conformation polymorphisms showed point mutations in both, suggesting that nearly all BCC contain *PTCH* mutations (Gailani *et al*, 1996). The mutations in sporadic BCC were somewhat different from those of Gorlin syndrome patients. Over half were base substitutions and these often led to amino acid changes. Only a third were small or large deletions or insertions. One missense mutation near the 3′ end of the coding sequence was recently identified in a family with basal cell carcinomas but no other stigmata of the Gorlin syndrome. Thus, it is possible that some patients with multiple basal cell carcinomas, but no family history, are actually new cases of a milder form of Gorlin syndrome.

The function of PTCH is still less clear than that of TP53. PTCH belongs to the hedgehog signal transduction pathway that transmits extracellular growth and differentiation signals to the nucleus. The most common vertebrate homolog of hedgehog is sonic hedgehog (SHH). The *SHH* gene is required for correct patterning of the neural tube and somites and for anterior/posterior positioning of the limb bud (Riddle *et al*, 1993; Roelink *et al*, 1995). This list of target tissues corresponds well with the clinical features of Gorlin syndrome, which include abnormalities of the brain, ribs, vertebrae and limbs. In addition, *PTCH* is expressed in all the target tissues of sonic hedgehog. Transgenic mice overexpressing the *SHH* gene develop many features of Gorlin syndrome, and a *SHH* mutation has been identified in a BCC (Fan *et al*, 1997; Oro *et al*, 1997).

A MODEL FOR CANCER DEVELOPMENT

The multiple steps that take TP53-mutated clones into a cancer appear to have a genetic origin, with successive oncogenes or tumor suppressor genes sustaining a mutation. These intervening steps – such as a precancer – are unstable intermediates. Recent molecular and cellular discoveries tell us what clinicians have long known – that cancer is not an inevitable process. The early steps are each reversible. On a sunny day at the beach, DNA repair enzymes remove and replace most nucleotides struck and chemically rearranged by UV. Of the molecular lesions overlooked, few cause mutations; most are correctly bypassed during DNA replication and repaired later. Some cells with unrepaired DNA damage vanish by TP53-directed apoptosis. Even mutated cells do not progress inexorably. Work on DNA viruses has revealed that cells with an aberrant cell cycle undergo apoptosis (Morgenbesser *et al*, 1994; Symonds *et al*, 1994). Mutant cells thus are in equilibrium between clonal expansion and regression. This balance may explain the observation in the clinic that actinic keratoses regress in the absence of sunlight exposure (Marks *et al*, 1986). Similarly, the TP53-mutant clones seen in normal mouse skin begin to disappear after UV irradiation ceases (Berg *et al*, 1996). Conversely, if another genetic hit occurs before the previous DNA aberration, or aberrant

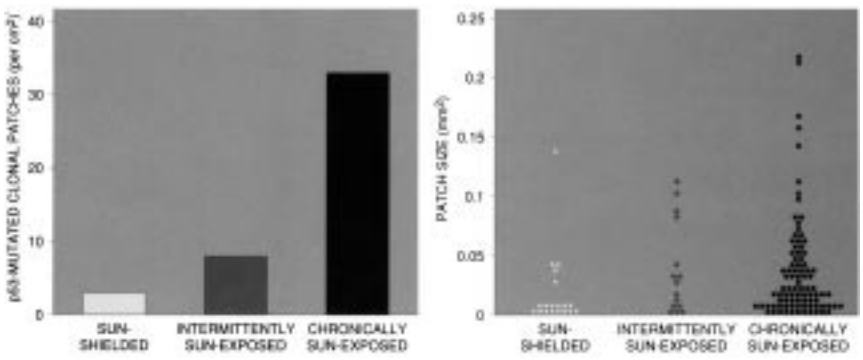

Figure 2. Frequency and size of keratinocyte patches with positive immunohistochemical reaction against TP53. Samples of normal skin from healthy individuals were collected from chronically sun-exposed, intermittently sun-exposed, and sun-shielded areas. After separating the epidermis, the presence of TP53 was studied by immunohistochemistry using anti-TP53 antibody. The epidermal sheets contained clones of cells with immunopositive nuclei at a frequency of 35 clones per cm^2 (Jonason *et al*, 1996) in sun-exposed skin. On the other hand, in sun-shielded skin, only few TP53 positive cells were found and these cells appeared in a scattered pattern (*left panel*). The size of the patches (*right panel*) showed a large variation from patch to patch; however, patches in sun-exposed skin were larger than intermittently sun-exposed or sun-shielded skin.

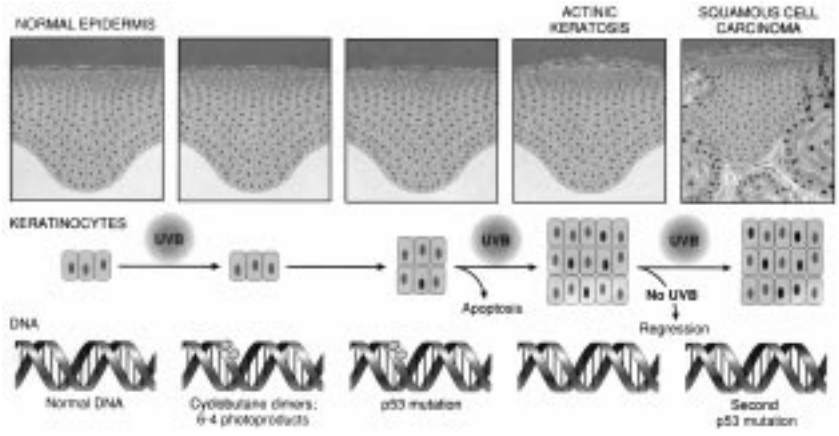

Figure 3. Proposed model of SCC formation. An unrepaired photoproduct leads to a mutation in an epidermal cell. If the mutation occurred in one allele of the TP53 gene, the cell can fail to undergo apoptosis and thereby gain a proliferative advantage over normal cells, with AK developing. Continued exposure to UV might then inactivate the remaining functional allele of TP53, which could accelerate the formation of SCC by promoting genetic instability.

cell, has been disposed of, the likelihood of tumor formation increases. For example, cells with one allele of TP53 mutated are partially deficient in apoptosis, but cells with two defective TP53 alleles begin to amplify genes and become aneuploid (Yin *et al*, 1992). Our model (**Fig 3**) begins with the observation that the UVB component of sunlight creates cyclobutane dimers and (6-4) photoproducts in the DNA molecule and some of these lesions slip past the repair system to cause mutations. If a mutation occurs in the TP53 gene, the cell involved shows one of the cellular phenotypes of TP53 mutations: resistance to sunlight-induced apoptosis. Cells that fail to die may gain a proliferative advantage over normal cells, with actinic keratosis developing. Continued exposure to UV could then inactivate the remaining functional allele of p53, further accelerating the formation of squamous cell carcinoma by promoting genetic instability. This is the point of no spontaneous return. A skin cancer appears and we next see the patient in the physician's office.

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