Sunlight and the onset of skin cancer

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The photons of sunlight precipitate a series of genetic events in skin leading to cancer. These events involve somatic mutations as well as inherited alleles. Competition between cell populations ensues, as a single mutated cell expands into a clone. Thus cancer involves both a single-cell problem and a many-cell problem; in skin cancer, sunlight appears to drive both.

How do cancers start? We would prefer to know actual events in human patients, rather than how cancers can be triggered artificially in laboratory mice. This will be a detective story – sifting through available human tissue for smoking guns. In addition to identifying important genes, we wish to know the carcinogen, the cell it entered, the structural change in the DNA, the resulting mutations, and the effect of these mutations on cell physiology. Finding a gene in which these events occurred creates a system for repeatedly asking questions at the interface of carcinogenesis and genetics. Tools for answering such questions are most powerful for skin cancer, because a great deal is known about ultraviolet radiation. Parts of this story are the subject of specialized reviews[1–4]. Here, after briefly describing the disease, we trace the skin's genetic misfortunes.

Skin cancer

Sun-exposed skin is heir to three cancers: melanoma; basal cell carcinoma (BCC); and squamous cell carcinoma (SCC)[5]. Melanomas, the most deadly, can arise in young adults. They begin as a radial proliferation of normally nonproliferating melanocytes. Danger enters when the radial lesion begins growing vertically, after which metastasis is possible. SCC and BCC are tumors of keratinocytes, which are cells that routinely proliferate, differentiate and are shed from the skin. These tumors often appear at the age of 70 years or later. They usually begin on a background of sun-damaged skin, characterized by lost elasticity and individual disordered keratinocytes[6]. Continued sun exposure leads to keratinized reddish patches of actinic keratosis, with aberrantly differentiating and proliferating cells. These pre-cancers usually regress, but one in a thousand progresses to SCC; these tumors are often aneuploid and can metastasize. In contrast to this step-wise progression, BCCs seem to arise without precursors, seemingly from keratinocytes in hair follicles. They are usually diploid and rarely metastasize, though they invade locally. These profound tissue changes are precipitated by photons, quantum packets of light.

Sunlight

All three cancers correlate with exposure to sunlight. They usually occur in individuals with light skin – blondes or red-heads who burn rather than tan – and those living in sunny climates[7]. The post-war obsession with the beach has resulted in a surge in skin cancers. In the southern US and Australia, they exceed all other cancers combined and are still increasing.

The sun emits radiation ranging from X-ray to ultraviolet to infrared. UV wavelengths all cause skin cancer in mice[8]. The most energetic of these, UVC (100–280 nm), has the right wavelength to be directly absorbed by DNA. Less energetic UVA (315–400 nm), found in tanning parlors, is absorbed by other cellular chromophores. Their excitation generates reactive oxygen species, such as the hydroxyl radical, which can cause DNA-strand breaks and chromosome translocations. UVB (280–315 nm) is intermediate, its photons weakly absorbed by the same molecules that best absorb UVC or UVA.

Because adults can reduce their risk of precancers by using sunscreen[9], some of the photons leading to cancer must act after striking the skin of adults. However, most of the critical sunlight exposure occurs before age 18. For example, people who moved from England to Australia as children, but not as adults, acquired the high Australian skin cancer risk[10,11]. Thus, some of the molecular scars left by sunlight are a half-century old. This persistence motivated a search for mutations made by sunlight; acute effects of sunlight, such as suppression of immune surveillance, would have long since disappeared.

UV photoproducts and UV-induced mutations

The most frequent DNA photoproducts made by UV join adjacent cytosines or thymines[1]. UVC and UVB photons are usually absorbed at the 5–6 double bond, allowing it to open. Where two pyrimidines are adjacent, one of two events usually occurs. If both 5–6 bonds open, a ring is formed, creating the 'cyclobutane dimer' (Fig. 1a). Alternatively, the double bond of the 5′ pyrimidine opens and reacts across the exocyclic group of the 3′ pyrimidine. After spontaneous rearrangement, a single bond is left between the two pyrimidines. This is the 'pyrimidine–pyrimidone (6–4) photoproduct' (Fig. 1b) (Ref. 12). Both photoproducts bend the DNA or rotate a base so it resembles an abasic site.

Both photoproducts cause mutations. This was shown in *Escherichia coli* using the observation that methyl groups prevent (6–4) photoproducts from forming. In *dcm*⁻ bacteria, which do not methylate cytosines at restriction sites, UVC-induced mutations increase only at the restriction sites[13]. In human cells, using a DNA repair enzyme to remove cyclobutane dimers from a UVC-irradiated shuttle vector before transfection reduces the mutation frequency[14].

In both organisms, UV-induced mutations are located where one pyrimidine is next to another. Mutations are usually C→T (cytosine to thymine), resulting from insertion of A (adenine) opposite the damaged C during subsequent DNA replication. (Thymines in photoproducts are less often mutagenic.) Ten percent of the mutations are CC→TT resulting from replacing both cytosines. This unique specificity of UV mutagenesis – about 70% C→T at dipyrimidines and 10% CC→TT – has been known for many years[15–17]. Few other mutagens involve tandem bases, and they primarily make other mutations. CC→TT, in particular, is considered to be diagnostic for UV (Ref. 18). These distinctive mutations are the smoking gun. If made by sunlight in a single cell half a century ago, they will still be present in descendants of that cell.

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Genetic changes in SCCs

To find such mutations, one first has to guess the right gene. Our guess was based on a rare skin disease characterized by lesions that contain human papillomavirus and can progress to SCC. One of the viral proteins binds to and inactivates the TP53 tumor suppressor protein. It seemed that sunlight might act by mutating the *TP53* gene directly. We now know that *TP53* is mutated in over half of all human cancers. The TP53 protein has since been found to be a transcription factor, a protein that turns other genes on and off.

Over 90% of the SCCs from the USA have a mutation somewhere in the *TP53* tumor suppressor gene[2,3,19]. The mutations are unusual, occurring only where one cytosine or thymine is adjacent to another. About two-thirds of the base changes are C→T and several are CC→TT (Table 1). Many of the same codons are mutated as in internal cancers, such as colon or bladder, but the base changes are different[20]. The mutations tend to cluster at nine mutation hotspots[3], many of which are sites of slow excision repair of DNA photoproducts[21]. UV-like *Trp53* mutations are also found in skin tumors from UV-irradiated mice[2]. Correspondingly, mice with a mutated *Trp53* gene are more susceptible to UV-induced skin cancer[22]. Not all genes are targets of sunlight: human skin cancers rarely contain mutations in members of the RAS oncogene family.

Observing dipyrimidine C→T mutations, including CC→TT, allows us to deduce that the mutagen was UV radiation directly absorbed by DNA. The culprit is not UVC, however, because the ozone layer absorbs it completely. Without an ozone layer, skin cancer would increase at least 10^8-fold[23]. UVB does penetrate the ozone layer, the degree depending on the thickness of the layer. Its weaker absorption by DNA leads less efficiently to the same UV photoproducts. Some skin tumors contain mutations other than C→T, just as seen in UV experiments using cultured cells[16,17]. While these are probably also due to UV, they are best considered 'non-informative': they are consistent with a UV origin, but are not compelling because other physical and chemical carcinogens also cause them. We have now identified the carcinogenic wavelength in humans (UVB), the DNA photoproducts (cyclobutane dimers and (6–4) photoproducts), the types of mutations made (C→T and CC→TT), and one of the genes mutated by sunlight (a tumor suppressor gene, *TP53*).

But do these mutations contribute to the tumor, or is DNA just an exposure meter for UV radiation? A crucial finding is that all mutations change the amino acid (Table 1). Because many base changes, such as C→T at the third position of a codon, do not change the amino acid, the mutations seen in skin cancers must have been selected for. Mere passengers could have been silent or could have constituted only a portion of the DNA sequencing signal. It is also fortunate that the mutations were found in a tumor suppressor gene. Because such genes require inactivation in order to contribute to cancer, most mutations will make amino acid changes that are potentially carcinogenic. Tumor mutations therefore tend to reflect the mutation spectrum of the carcinogen. Oncogenes are less free from phenotypic selection, because they must acquire a novel function for oncogenesis. But when do these mutations occur?

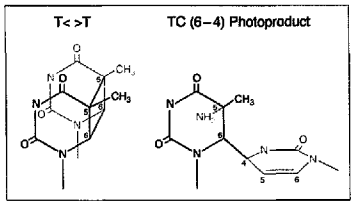

FIGURE 1. UV photoproducts. The TT cyclobutane pyrimidine dimer and the TC pyrimidine–pyrimidone (6–4) photoproduct. In the cyclobutane dimer, the angle between bases is less than the normal 36° and the DNA-strand acquires a bend. In the (6–4) photoproduct, the 90° rotation of the 3′ base makes it resemble an abasic site.

SCC pre-cancers

The first clinically apparent lesions are the actinic keratoses, SCC precancers that begin appearing about age 45 in light-skinned individuals. Sunlight-induced C→T and CC→TT mutations have been found, as well as in the next SCC stage, termed carcinoma *in situ*[3,24]. In contrast to the tumors, amino-acid substitutions in actinic keratoses are spread evenly across the gene. Some *TP53* mutations are therefore less likely to progress to SCC than others. Actinic keratoses also have allelic loss at loci in addition to *TP53* (Ref. 25), so other genes might be involved as well.

Patients with multiple actinic keratoses often have *TP53* mutations in more than one. In these cases, the mutation is different in each lesion[24], so each actinic keratosis records a separate UV photon absorption event. A patient's skin is a living 'petri dish' for the effects of photons.

TABLE 1. Mutations in the *TP53* tumor suppressor gene in squamous cell carcinomas of the skin (representative subset)[19]

Patient age (years)	Tumor site	*TP53* codon	DNA sequence	Base change	Amino acid change
86	Preauricular	7	TCT	C→G/WT	Asp→His
82	Temple	104/105	GCCT	AC/WT	Gly→Ala ... stop
69	Scalp	151	CCCCC	C→A/WT	Pro→His
69	Hand	152	CCCCC	C→T/0	Pro→Ser
80	Nose	247–248	ACGG	CC→TT/WT	AsnArg→AsnTrp
56	Side of face	258	TTCC	C→T/0	Glu→Lys
76	Cheek	278	TCCT	C→T/WT	Pro→Ser
85	Face	285–286	TCCT	CC→TT/0	GluGlu→GluLys
75	Postauricular	317	CCCCA	C→T/WT	Gln→stop

Letters in bold show mutated bases.
Abbreviations: 0, allelic loss; Δ, deletion; WT, wild type.

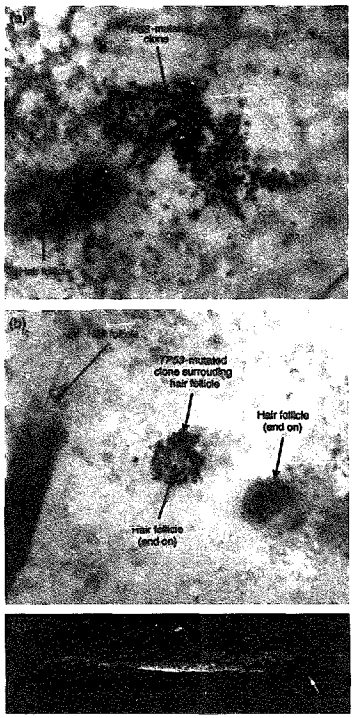

FIGURE 2. TP53 immunopositive clones in whole-mount preparations of human epidermis. The epidermis was separated from the dermis, fixed and immunostained for wild-type TP53 protein. Elevated TP53 usually corresponds to a mutation in the *TP53* gene, which was confirmed by microdissection and DNA sequencing. (a) Clone of *TP53*-mutated keratinocytes; (b) clone surrounding a hair follicle. (c) Clone viewed from the side in a three-dimensional confocal microscopy image. The apex of the clone lies at the dermal–epidermal junction (arrow). (Reproduced with permission from Ref. 27.)

Mutations in normal skin

Cancer is believed to result from multiple genetic hits. Normal individuals would then have mutated cells containing fewer hits than needed for cancer. Several *TP53* codons were found mutated in sun-exposed human skin at frequencies of one mutated gene per 10^6 cells or higher[24,26]. The three-dimensional arrangement of *TP53*-mutated cells in human epidermis was recently observed[27]. Because most *TP53* mutations lead to overly-stable TP53 protein, staining the epidermis with antibody to normal TP53 protein reveals the arrangement of mutated cells. Instead of being scattered in the epidermis, *TP53*-mutated cells are present as tiny clones 60–3000 cells in size (Fig. 2). Often, these clones encase a hair follicle or form a cone having its apex at the dermal–epidermal junction. Clones are most frequent on sun-exposed skin. *TP53* gene mutations were identified in half of 24 clones microdissected and sequenced; most of these were sunlight-induced mutations that changed an amino acid[27,28].

Because the antibody method detects most *TP53* mutations, it reveals the actual mutation frequency in skin. Surprisingly, the frequency on sun-exposed skin averages 30 clones cm^{-2}. Face and hands contain thousands, with almost one epidermal cell in 20 having a *TP53* mutation. The clonal arrangement reveals that these cancer-prone cells are not sitting still while waiting for additional genetic hits – they are proliferating. Clearly, most such clones never progress. This halt might be due to regression or to failure to mutate an additional gene. In mice, regression has been observed. Repeated UV-irradiation generates *Trp53*-mutated clones, but after irradiation ends the clones begin to disappear[29].

Apoptosis and cellular proofreading

To learn how inactivating TP53 contributes to skin cancer, we need to know its function. As a transcription factor, it turns on or off the expression of genes involved in the cell cycle, DNA synthesis, DNA repair, and programmed cell death[30]. TP53 is part of a DNA damage response, becoming more stable after a cell is irradiated with UV or γ-rays. The signal for UV-induction of TP53 and cell death was recently found to originate from photoproducts in actively transcribed genes[31]. Without TP53, cultured colon cells have a fivefold higher spontaneous mutation rate[32].

In skin, TP53 also regulates cell death. Dermatologists know that skin overexposed to sunlight contains unusual keratinocytes, dubbed 'sunburn cells'. These have pycnotic nuclei and an intensely eosinophilic cytoplasm (Fig. 3a), a morphology typical of apoptosis. More conclusively, they contain the hallmark of apoptosis, DNA-strand breaks (Fig. 3b). Generating these apoptotic cells requires TP53. In *Trp53* knockout mice, the sunburn cell frequency after UVB is an order of magnitude lower than in wild-type; the heterozygote is intermediate[24].

Therefore, the skin recognizes and eliminates aberrant keratinocytes – a supplement to the protection afforded by routinely shedding keratinocytes during differentiation. UVA may be as important for apoptosis as UVB, because it is more prevalent. The elimination mechanism has been termed 'cellular proofreading' because the mistake is erased rather than repaired[33]. The term also draws attention to the fact that apoptosis is just an effector step. The sensor that recognizes that a cell is aberrant is, perhaps, more interesting. For example, abnormal cell cycles caused by inactive retinoblastoma protein lead to TRP53-dependent apoptosis in the lens and choroid plexus of transgenic mice[34]. When pregnant mice are X-irradiated, *Trp53* is required for the apoptotic resorption of embryos that otherwise would be born malformed[35]. Capitalizing on the cell's own sensor for recognizing cancer-prone cells might be a more precise route to cancer therapy than bludgeoning the cell with toxic drugs.

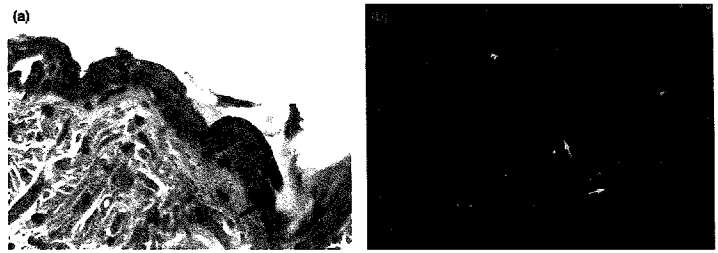

FIGURE 3. Sunburn cells are apoptotic. (a) Pycnotic nuclei and intensely eosinophilic cytoplasm of two sunburn cells in murine skin (arrows). (b) Cells with DNA-strand breaks (pink), after end-labeling with fluorescent nucleotides in the same paraffin section (arrows).

BCCs and other genetic changes

BCCs are a little different. Although nearly all BCCs have overly-stable TP53 protein, only half the tumors have mutations in the *TP53* structural gene itself[5]. These tumors often have sunlight-induced mutations in both alleles, a striking demonstration of the mutagenicity of sunlight. The mutations in the remaining tumors evidently lie in another member of the TP53 pathway.

An exciting development has been the emergence of a new, apparently BCC-specific, pathway of tumor suppressor genes and oncogenes. The starting point was Gorlin syndrome, an autosomal dominant disorder in which patients have multiple BCCs as well as jaw cysts and tiny pits in the palms of the hands. Positional cloning revealed the gene to be *PTCH* ('patched'), a homolog of the *Drosophila* gene *ptc* (Ref. 35). It is the transmembrane receptor for the Hedgehog signal transduction pathway, whose downstream targets include *TGF-β* and the *WNT* family. *PTCH* is lost not only in the BCCs of Gorlin patients, but also in their jaw cysts – evidently clonal developmental defects that arise in the same molecular way tumors do. Sporadic BCCs also have *PTCH* mutations, most of which are UV-like, indicating the gene as another genetic target for sunlight. An inhibitory ligand for the *PTCH* receptor is the secreted protein Sonic hedgehog. Transgenic mice overexpressing the *Shh* gene develop many features of Gorlin syndrome, and an *SHH* mutation has been identified in a BCC (Ref. 36).

Although melanomas rarely involve *TP53*, the tumor suppressor gene *CDKN2A* (or '*p16*') is mutated in several familial melanoma kindreds. It encodes an inhibitor of CDK4, a cell-cycle regulator. Most melanoma cell lines have mutations in *CDKN2A*, often C→T at dipyrimidine sites or CC→TT (Refs 37, 38). In primary melanomas, mutations are rarer and the relation to UV less clear[39]. The gene encoding β-catenin, a signaling protein, has mutations in cell lines that might also be UV-induced[40].

Clonal expansion: the many-cell problem

How do the *TP53*-mutated clones in normal skin arise from a single mutated cell? An apoptosis-resistant cell is

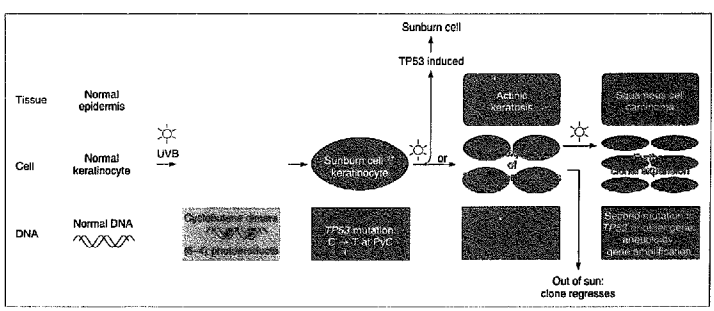

FIGURE 4. A model for genetic and cellular events in the onset of human squamous cell carcinoma of the skin. Sunlight creates cyclobutane dimers and (6–4) photoproducts in DNA, some of which cause mutations in the *TP53* tumor suppressor gene. One of the cellular phenotypes of a *TP53* mutation, resistance to sunlight-induced apoptosis, allows repeated sunlight exposure to select for *TP53*-mutated cells. One of these cells might again incur the first effect of sunlight, mutagenesis. (Adapted from Ref. 24.)

more likely to survive a DNA-damaging trip to the beach. Yet, failure of cellular proofreading has a worse consequence. Because the mutated cell's normal neighbors still undergo apoptosis when damaged, they will leave room for the mutant to expand clonally into other stem-cell compartments. Thus sunlight might act as a selection pressure favoring the clonal expansion of *TP53*-mutated cells (Fig. 4), each beach visit giving the clone a nudge. Sunlight appears to act in two ways: mutating genes and then, afterwards, selecting for clonal expansion of mutated cells.

This model is supported, but not proven, by the observation that *Trp53*-mutated clones in mice regress in the absence of UV exposure[29]. Similarly, in humans the *TP53*-mutated clones are largest in sun-exposed areas[27]. Clonal expansion actually contributes more mutant cells than the initial mutagenic effect of sunlight. In chronically sun-exposed skin, the aggregate size of large *TP53*-mutated clones (the ones most clearly dependent on exposure) exceeds the total of the more numerous small clones[27].

An expanding cell-death defective clone will be more likely to accumulate additional mutations (Fig. 4). First, it presents a larger target for future UV exposures. Second, it is a target in which a greater fraction of cells can survive UV irradiation to acquire an additional mutation in *TP53* or another gene.

Geneticists might be surprised by the numerical impact of a cellular mechanism such as clonal expansion. Mutations after low UV doses occur in mammalian genes at a frequency of 10^{-5} per cell generation or less[17]. Thus, the probability of mutating both alleles of two particular tumor suppressor genes is 10^{-20}. Because the number of proliferating keratinocytes in human skin is about 10^6 cm^{-2}, with about 0.1 m^2 of skin exposed, about 10^{-11} cells will be quadruply mutated. Even after 100 cell generations, only one in 1000 million people would have a skin tumor. For comparison, the lifetime expectancy of skin cancer in sunny climates is actually 20% or more. This discrepancy cannot easily be resolved by invoking mutator phenotypes, and would grow worse if additional genes are involved.

Clonal expansion, however, can easily increase the likelihood of each subsequent mutation a thousandfold. Apoptosis is a relatively high-frequency physiological event that can facilitate the expansion of many *TP53*-mutated cells simultaneously. The next mutation can then be quite rare, because only one cell in one of the clones must be hit. Clonal expansion makes multiple-genetic-hit cancer feasible. The lessons sunlight has taught us – about mutations and expanding clones – are, thus, likely to illuminate the remaining catalog of tumors whose onset occurs in the dark, out of sight.

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References

1 Brash, D.E. (1988) *Photochem. Photobiol.* 48, 59–66
2 Nataraj, A.J., Trent, J.C. and Ananthaswamy, H.N. (1995) *Photochem. Photobiol.* 62, 218–230
3 Brash, D.E. *et al.* (1996) *J. Invest. Dermatol. Symp. Proc.* 1, 136–142
4 Kraemer, K.H. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 11–14
5 DeVita, V.T., Hellman, S. and Rosenberg, S.A., eds (1997) *Cancer: Principles and Practice of Oncology*, Lippincott–Raven
6 Gilchrest, B.A., ed. (1995) *Photodamage*, Blackwell Science
7 Urbach, F. (1984) in *Topics in Photomedicine* (Smith, K.C., ed.), pp. 67–104, Plenum Press
8 de Gruijl, F.R. and Forbes, P.D. (1995) *BioEssays* 17, 651–660
9 Thompson, S.C., Jolley, D. and Marks, R. (1993) *New Engl. J. Med.* 329, 1147–1151
10 Marks, R., Jolley, D., Lectsas, S. and Foley, P. (1990) *Med. J. Aust.* 152, 62–66
11 Kricker, A., Armstrong, B.K., English, D.R. and Heenan, P.J. (1991) *Int. J. Cancer* 48, 650–662
12 Mitchell, D.L. and Nairn, R.S. (1989) *Photochem. Photobiol.* 49, 805–819
13 Glickman, B.W. *et al.* (1986) *Proc. Natl. Acad. Sci. U. S. A.* 83, 6945–6949
14 Brash, D.E. *et al.* (1987) *Proc. Natl. Acad. Sci. U. S. A.* 84, 3782–3786
15 Miller, J.H. (1983) *Annu. Rev. Genet.* 17, 215–238
16 Lehkowski, J.S., Clancy, S., Miller, J.H. and Calos, M.P. (1985) *Proc. Natl. Acad. Sci. U. S. A.* 82, 8606–8610
17 McGregor, W.G. *et al.* (1991) *Mol. Cell. Biol.* 11, 1927–1934
18 Hutchinson, F. (1994) *Mutat. Res.* 309, 11–15
19 Brash, D.E. *et al.* (1991) *Proc. Natl. Acad. Sci. U. S. A.* 88, 10124–10128
20 Greenblatt, M.S., Bennett, W.P., Hollstein, M. and Harris, C.C. (1994) *Cancer Res.* 54, 4855–4878
21 Tornaletti, S. and Pfeifer, G.P. (1994) *Science* 263, 1436–1438
22 Li, G., Ho, V.C., Berean, K. and Tron, V.A. (1995) *Cancer Res.* 55, 2070–2074
23 Frederick, J.E., Snell, H.E. and Haywood, E.K. (1989) *Photochem. Photobiol.* 50, 443–450
24 Ziegler, A. *et al.* (1994) *Nature* 372, 773–776
25 Rehman, I., Quinn, A.G., Healy, E. and Rees, J.L. (1994) *Lancet* 344, 788–789
26 Nakazawa, H. *et al.* (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 360–364
27 Jonason, A.S. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 14025–14029
28 Ren, Z.P. *et al.* (1996) *Oncogene* 12, 765–773
29 Berg, R.J.W. *et al.* (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 274–278
30 Levine, A.J. (1997) *Cell* 88, 323–331
31 Ljungman, M. and Zhang, F. (1996) *Oncogene* 13, 823–831
32 Havre, P.A. *et al.* (1995) *Cancer Res.* 55, 4420–4424
33 Brash, D.E. (1996) *Nat. Med.* 2, 525–526
34 White, E. (1994) *Nature* 371, 21–22
35 Guilani, M.R. *et al.* (1996) *Nat. Genet.* 14, 78–81
36 Oro, A.E. *et al.* (1997) *Science* 276, 817–821
37 Liu, Q. *et al.* (1995) *Oncogene* 10, 1061–1067
38 Pollock, P.M., Yu, F., Parsons, P.G. and Hayward, N.K. (1995) *Oncogene* 11, 663–668
39 Gruis, N.A. *et al.* (1995) *Am. J. Pathol.* 146, 1199–1206
40 Rubinfeld, B. *et al.* (1997) *Science* 275, 1790–1792

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