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p53 Alterations in Human Skin

A Molecular Study Based on Morphology

BY

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ABSTRACT

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Mutation of the p53 gene appears to be an early event in skin cancer development. The present study is based on morphology and represents a cellular and genetic investigation of p53 alterations in normal human skin and basal cell cancer.
Using double immunofluorescent labelling, we have demonstrated an increase in thymine dimers and p53 protein expression in the same keratinocytes following ultraviolet radiation. Large inter-individual differences in the kinetics of thymine dimer repair and subsequent epidermal p53 response were evident in both sunscreen-protected and non-protected skin. The formation of thymine dimers and the epidermal p53 response were partially blocked by topical sunscreen. We have optimized a method to analyze the p53 gene in single cells from frozen tissue sections. In chronically sun-exposed skin there exist clusters of p53 immunoreactive keratinocytes (p53 clones) in addition to scattered p53 immunoreactive cells. Laser assisted microdissection was used to retrieve single keratinocytes from immunostained tissue sections, single cells were amplified and the p53 gene was sequenced. We have shown that p53 mutations are prevalent in normal skin. Furthermore, we detected an epidermal p53 clone which had prevailed despite two months of total protection from ultraviolet light. Loss of heterozygosity in the PTCH and p53 loci as well as in the sequenced p53 gene was determined in basal cell cancer from sporadic cases and in patients with Gorlin syndrome. Allelic loss in the PTCH region was prominent in both sporadic and hereditary tumors, while loss of heterozygosity in the p53 locus was rare in both groups. p53 mutations found in the hereditary tumors differed from the typical mutations found in sporadic cases. In addition, we found genetically linked subclones with partially different p53 and/or PTCH genotypes in individual tumors. Our data show that both genes are important in the development of basal cell cancer.

Key words: p53, skin, mutation, carcinogenesis, microdissection, UV, Basal cell cancer (BCC).

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ABBREVIATIONS

BCC	basal cell cancer
CPD	cyclobutane pyrimidine dimer
Cdk	cyclin dependent kinase
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
EPU	epidermal proliferative unit
LOH	loss of heterozygosity
MED	minimal erythema dose
NER	nucleotide excision repair
NMSC	non-melanoma skin cancer
RB	retinoblastoma protein
SPF	sun protecting factor
SCC	squamous cell carcinoma
CIS	carcinoma in situ
UVR	ultraviolet radiation
wt	wild type
XP	xeroderma pigmentosum

Background

Introduction

Cancer arises from a cell that escapes normal growth regulation through a multistep process, involving activation of growth promoting oncogenes and inactivation of tumor suppressor genes. As proposed by Hanahan (Hanahan and Weinberg, 2000), virtually all malignant transformation of cells is through the acquisition of the same six capabilities **(Fig.1)**. These are i) self-sufficiency in growth signals, e.g. by activation of ras oncogene; ii) insensitivity to anti-growth signals, e.g. by loss of RB and p53 tumor suppressor gene functions; iii) evading apoptosis, e.g. by producing IGF survival factors and inactivation of p53 gene; iv) limitless replicative potential, e.g. by turning on telomerase;

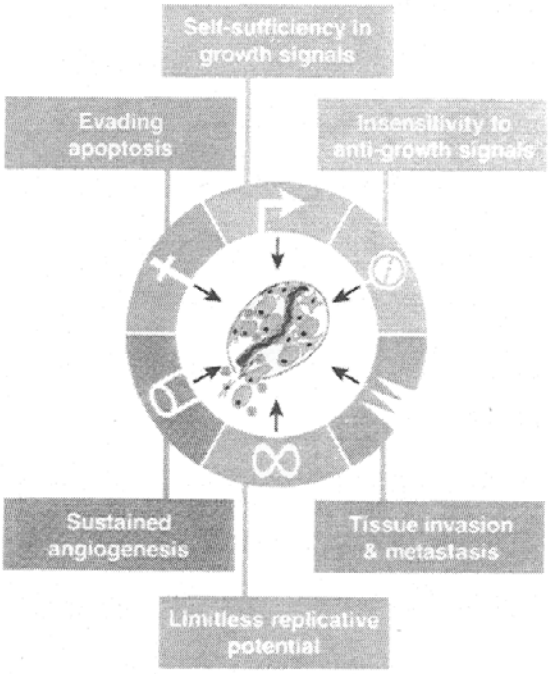

Fig.1 Acquired Capabilities of Cancer.

Reprinted from Cell, vol.100, by Hanahan D and Weinberg RB, The Hallmark of Cancer, 57-70, ©2000, with permission from Elsevier.

v) sustained angiogenesis, e.g. by producing VEGF inducer; vi) tissue invasion and metastasis, e.g. by inactivation E-cadherin. Albeit through various means, the order in which these capabilities acquired varies according to cancer types and subtypes. In certain tumors, one particular genetic change may confer several capabilities simultaneously, leading to fewer mutational steps in tumorigenesis. One example is the loss of p53 function which facilitates angiogenesis and resistance to apoptosis as well as contributes to the establishment of genomic instability.

Individuals with alterations in genes that maintain the integrity of genome are more prone to develop cancer, such as XP in xeroderma pigmentosum, p16 in

familial melanoma and p53 in Li-Fraumeni syndrome (Kinzler and Vogelstein, 1997). Other factors that may also influence the appearance of cancers are host characteristics, such as decreased immunological responses, level of antioxidant defenses and viruses infections (Yoshikawa et al., 1990) (Nishigori et al., 1996).

The multiple genetic hits model is well accepted in skin carcinogenesis. Animal experiments showed a two-step carcinogenesis model using ultraviolet radiation (UVR) and chemical compounds in mouse skin indicating that UVR is an initiator and promoting agent (Epstein and Roth, 1968). Clinical observations have established empirically that non-melanoma skin cancer is directly related to UVR. More has been known in molecular level the effects of UVR along the way from benign lesion to the malignant transformation. UVR is not only an initiator, but also a promoter and selective pressure in non-melanoma skin cancer development. This is best illustrated in the carcinogenesis of SCC in human skin. UVR acts as a mutagen, and also a promoter that drives clonal expansion resulting in precancerous lesions, e,g. actinic keratosis. As a result of continued exposure to UVR, these unstable clones accumulate genetic instability such as the mutation of the second allele of the p53 gene or loss of wide type allele, and eventually lead to SCC (Ziegler et al., 1994). Thus the mouse carcinogenesis model is converging with the human multiple genetic hit model.

Attempts are currently underway to scrutinize genetic events in the early stage of skin carcinogenesis. Non-melanoma skin cancer, basal cell cancer (BCC) and SCC, together with the sun-damaged skin as well as p53 clones as potential precursors of BCC, provide an excellent model in the studying of skin carcinogenesis. They are easily detected, diagnosed and sampled. The displayed characteristic mutations caused by a known carcinogen (UVR), serves as a molecular tracer in skin carcinogenesis. In addition, BCCs also occur at early age in patients with a known germline deficiency (Gorlin

syndrome). Alterations in PTCH and the hedgehog pathway are underlying events for BCC tumorigenesis. In addition, the p53 tumor suppressor gene is the most frequently mutated gene in all human cancers, and appears to be an early event in non-melanoma skin cancer development. The transient accumulation of p53 protein leads to cell cycle arrest, allowing time for cells to repair the damage. If DNA damage is too severe and p53 accumulation is sustained, the cell is thought to undergo apoptosis. Our knowledge regarding the p53 gene makes it one of the best-understood pathways for revealing how a cell acquires a mutation and how that mutation leads to cancer.

Human skin

The skin consists of a multi-layered epidermis overlying dermis in conjunction with subcutaneous tissues **(Fig.2)**. Keratinocytes undergo a continuous process of proliferation, differentiation and apoptosis. In this life long self-renewing process, quiescent stem cells are triggered to produce transiently amplifying cells, which then give rise to early stages of differentiation followed by terminal differentiation and death. This process is responsible for maintaining the proper epidermal thickness and barrier function of stratified epithelium. Cellular transitions take place within a few cell layers each month over a life span of several decades.

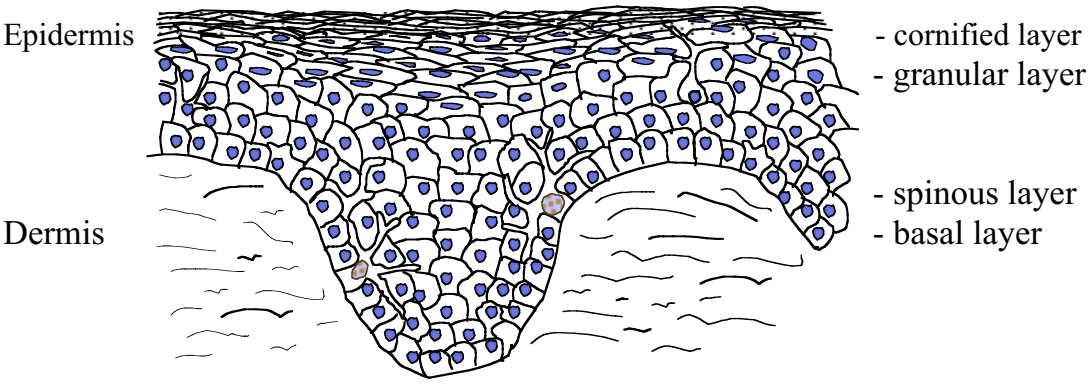

Fig.2. Schematic drawing illustrates different layers of human skin. By courtesy of Helena Bäckvall.

The epidermis is proposed as comprising by proliferative units (clones), columns of keratinocytes overlaying 10 basal cells in which epidermal stem cells are responsible for maintenance (Potten, 1974). A recent study in our group showed that normal human skin is composed of mosaic pattern sizing from 20-350 basal cells, with either the maternal or paternal X-chromosome inactivated. The data suggest that an epidermal clone is less than 35 basal cells in diameter (Asplund et al., 2001).

The epidermal stem cells reside in the basal layer of the epidermis and are slow-cycling cells, which express high levels of cell surface integrins, such as β1, α6β4 (Kaur and Li, 2000) (Jensen et al., 1999) and have the ability to rapidly adhere to type IV collagen and fibronectin (Fuchs and Segre, 2000). Epidermal stem cells accumulate β-catenin, upon external signals, such as activated Wnt signal pathways (Nusse, 1999). β-catenin is a protein that normally interacts with E-cadherin to promote intercellular adhesion. Excessive β-catenin is responsible for the activation of downstream target genes, such as c-*myc* and *cyclin D1* and leading to cell proliferation and subsequent cell differentiation (He et al., 1998); (Tetsu and McCormick, 1999). In addition to stem cells, there are transient amplifying cells and terminal differentiating cells. Transient amplifying cells are daughters of stem cells that undergo a finite number of divisions. Terminal differentiating cells are the progeny of transient amplifying cells that are destined to move upwards from the basal layer. A process involves morphological and biochemical changes including the synthesis of the differentiation specific keratins K1, K10 as well as the downregulation of integrins (Fuchs, 1990) (**Fig. 3**).

The epidermis is contiguous with the outer root sheath of the hair follicle. Hair follicles are constantly renewing, proceeding from phase of growth (anagen), through regression (catagen) to a rest stage (telogen). The shift of phases during the hair cycle involves a series of morphological and biological changes as well as epithelial and mesenchymal interactions (Paus et al., 1999). Recent

studies in mice showed that stem cells in the bulge area of the hair follicle can give rise not only to the hair follicle but also to epidermis (Taylor et al., 2000); (Oshima et al., 2001). It is proposed that the bulge area of the hair follicle contains the ultimate stem cells of the epidermis/hair follicle system. The progeny of hair follicular stem cells undergo two independent pathways of cell migration and specialization: moving upwards in the long term maintenance of the epidermis, or downwards during the anagen of each hair cycle that give rise to the lower part of a new hair follicle (Taylor, et al., 2000). Morphological observations suggest a close relationship between BCC and hair follicle.

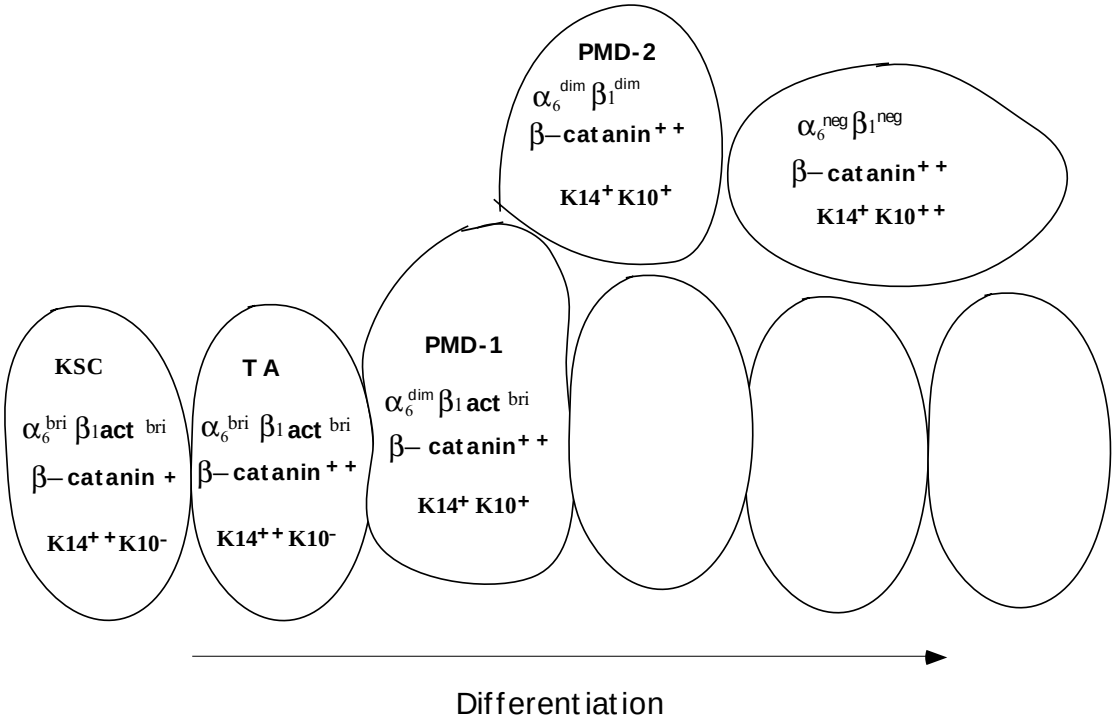

Fig.3 Expression of adhesive molecules during cell maturation.

The p53 gene

Introduction

The p53 gene is located on the short arm of chromosome 17 (Benchimol et al., 1985), is 20kb long and has 11 exons. The gene encodes a 53 KD nuclear phosphoprotein consisting of 393 amino acids. It was discovered first as a tumor antigen due to its ability to bind to the SV40 oncogene product large T

antigen. Then it was defined as an oncogene by virtue of its ability to transform cells and bind to viral oncoproteins. Subsequent studies clearly established that wild-type p53 acts as tumor suppressor gene rather than an oncogene (May and May, 1999).

Normal p53 functions

p53 protein can function as a transcriptional factor. It has been divided structurally and functionally into four domains, the transactivation domain, the sequence-specific DNA-binding domain, the oligomerization domain and the regulatory domain.

The first 42 amino acids at the N-terminus constitute a transcriptional activation domain that positively regulates gene expression. The adenovirus E1B-protein, MDM2 protein and hepatitis B virus X protein, negatively regulate the transactivation activity of the p53 gene (Yew and Berk, 1992); (Oliner et al., 1993); (Levine, 1997). The N-terminal region also contains a proline-rich region (amino acids 63-97) which is required for p53-mediated apoptosis (Sakamuro et al., 1997). A polymorphism at codon 72 resulting either in a proline or an arginine (p53 A) is thus of high interest. It has been reported that individuals homozygous for p53 A are more susceptible to human papillomavirus-associated tumorigenesis than heterozygotes due to the fact that p53 Arginine is more susceptible to E6-mediated degradation (Storey et al., 1998).

The sequence-specific DNA-binding domain of p53 is localized between amino acid residues 102 and 292, containing four conserved regions II-V. 80-90% of missense mutations reside in this central part.

In the C-terminal region, between amino acids 323 and 356, is the oligomerization domain. p53 forms a tetramer through this domain. The fourth

domain is a regulatory domain, between amino acids 363 and 393, and acts as a negative regulator of p53 sequence-specific binding (Selivanova et al., 1997).

p53 and the cellular response to DNA damage

p53 can be activated by several different types of DNA damage, including double-strand breaks and single strand breaks by γ-irradiation, cyclobutane pyrimidine dimers (CPDs) by UVR and chemicals. Following DNA damage, p53 protein rapidly accumulates and exerts various functions **(Fig.4)**.

Different types of DNA damage are recognized by different cellular proteins and by p53 itself. It has been demonstrated that p53 binds strongly to strand breaks by its C-terminal domain (Nelson and Kastan, 1994). Two pathways exist for p53 to exert its function on the cellular level. One is termed the "guardian of the genome", which the increased p53 protein after DNA damage leads to cell-cycle arrest in G1 and allows more time for DNA repair. Another function is termed "cellular proofreading", in this pathway, induction of p53 leads to apoptosis (Fisher, 1994). One example is the "sunburn cells" in epidermis representing apoptotic keratinocytes following overexposure to UVR.

In response to DNA damage, p53 is activated and turns on p21, a critical mediator of the p53-mediated G1 arrest response (El-Deiry et al., 1993). p21 binds to a number of cyclin and Cdk complexes. Elevated p21 inhibits the cyclin D1-Cdk4 complex leading to subsequent accumulation of the unphosphorylated form of RB, which in turn binds E2F resulting in cell arrest in G1 before the damage propagates in the S phase. p21 can also form a complex with PCNA which blocks PCNA as a DNA polymerase in DNA replication, thus p21 exerts its effects on cyclin-Cdk complexes and PCNA to stop DNA replication but not DNA repair.

A p21-independent pathway that contributes to p53-mediated G1 arrest is the overexpression of the *gadd45* after certain types of DNA damage. Although the precise mechanism is unclear, GADD45 also binds to PCNA and plays a backup role for p21.

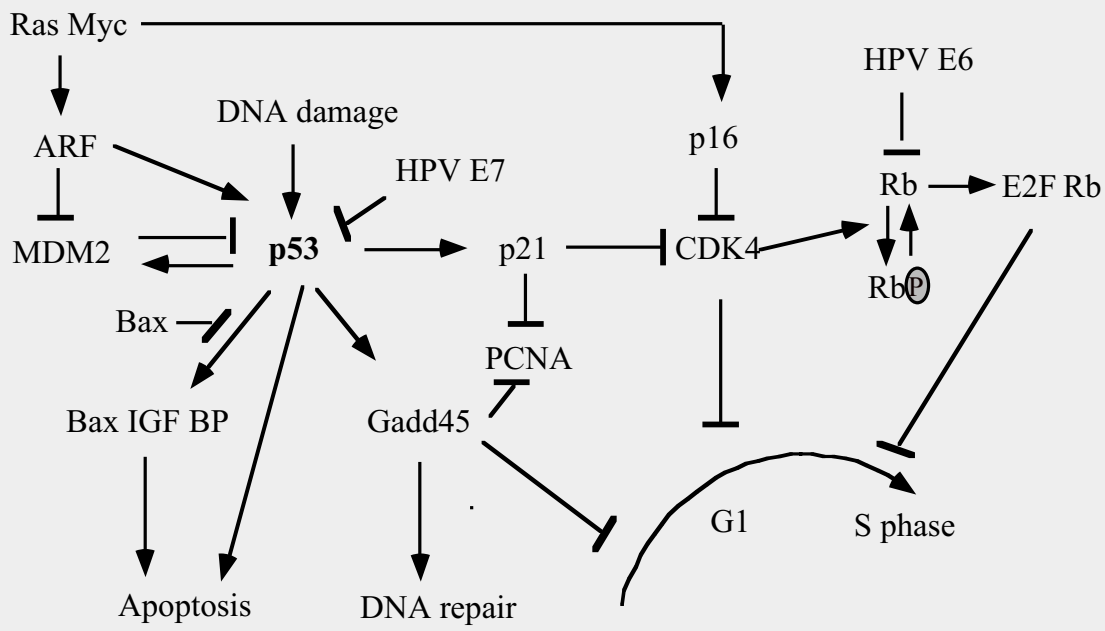

Fig.4 p53 network.

p53 up-regulates the *mdm2* gene which in turn inhibits the transcriptional activity of the p53 gene. MDM2 regulates p53 levels in the cell by inducing rapid degradation of p53 protein, thus implying a negative feedback loop between MDM2 and p53. p53 is also implicated as part of the G2/M check point preventing premature entry to S phase.

When DNA damage is severe, cellular survival factors are limiting, or an activated oncogene is forcing the cell into a replicative cycle, the apoptotic pathway prevails. Two genes are involved in initiating this apoptotic pathway: *bax* and *IGF-BP3* (Miyashita and Reed, 1995) (Buckbinder et al., 1995).

Two additional members of the p53 family, p73 and p51, share substantial sequence homology with p53 (Kaghad et al., 1997). Overexpression of p73 and p51 can activate transcription from p53-responsive promoters and induce apoptosis in a p53-like manner, irrespective of the p53 status.(Osada et al., 1998). Unlike p53, p73 is not induced by DNA damage caused by dactinomycin and UVR (Kaghad, et al., 1997). Expression of p73 can lead to a decrease in the level of p53, possibly through the increased activity of MDM2-mediated proteolysis (Wang et al., 2001).

p53 mutations and cancer

p53 has been proposed as the "guardian of the genome" (Lane, 1992). p53 is by far the most frequently mutated gene in all human cancers. Mutations are mostly found in DNA binding domains. Most of them are found in hot spots, clustering around codon 173-179, 235-250, and 273-278 (van Kranen and de Gruijl, 1999); (Wikonkal and Brash, 1999). Individuals who carry a germ-line p53 mutation, the Li-Fraumeni syndrome, are predisposed to cancer. In contrast to the combination of a point mutation at a CpG site and LOH that are common in internal cancer. Two or more point mutations are often found in basal cell cancer, which often happen at dipyrimidine sites and the majority of the mutations are C-> T transitions with typical UV signature. Eighty-five percent of mutations are single base missense substitutions that produce an altered p53 protein which is unable to bind to DNA of p53 responsive genes.

All mutations fall into two classes. Class I mutation which retain a wild-type p53 conformation, affects residues that directly contact DNA(Ory et al., 1994). Class II mutations affect residues that do not contact DNA directly. Those mutations make extensive contact with other residues in the polypeptide and have a strong stabilizing effect on the protein structure. The class two mutations exert a dominant negative effect over the wild-type p53. Class II mutations are associated with a more severe phenotype *in vitro* compared to class I mutations (Ory, et al., 1994). The heterogeneity of the structure and

function of the various p53 mutants has led to studies of their clinical implications. In breast and colon cancer, there is an implied association between mutations in the residues that stabilize the protein structure and shorter survival or poor response to treatment (Borresen-Dale et al., 1998) (Borresen et al., 1995). Null p53 mutations, such as nonsense, deletions, insertions and mutations at splice sites, result in premature stop codons and truncated protein products. Such mutations give a poor outcome of non-small cell lung carcinomas (Hashimoto et al., 1999) However, the exact functional consequence of a p53 mutations is still unclear.

p53 in normal human skin

Increased expression of p53 protein can be detected in human skin as a response to exposure of UVR (Burren et al., 1998); (Campbell et al., 1993) (Hall et al., 1993).

Induction of p53 protein can be detected as early as 30 minutes after exposure to UVR (Maltzman and Czyzyk, 1984), and is mainly due to reduced degradation. The normal half -life of wt p53 protein is short. It is almost undetectable in normal cells with immunohistochemistry. Overexpression of p53 protein is often due to either the accumulation of wt p53 or mutant p53. It can be visualized immunohistochemically as a nuclear staining.

Scattered p53 immunoreactive keratinocytes are found in epidermis after sun exposure. In addition to scattered immunoreactive cells, there are clusters of p53 immunoreactive keratinocytes, denoted epidermal p53 clones. Such p53 clones can be found as "background" in chronically sun-exposed skin and perhaps are more frequently detected in morphologically normal skin adjacent to non-melanoma skin cancers. Genetic analysis has disclosed that 70% of such clones harbored a p53 mutation (Pontén et al., 1997a). It has never been shown that p53 clones found in the vicinity of tumors share the same mutation as adjacent SCCs, CISs and BCCs (Pontén, et al., 1997a); (Ren et al., 1996a). The

density of p53 mutant clones in chronically sun exposed skin is approximately 30/cm^2 (Jonason et al., 1996); (Ren et al., 1997); (Brash and Ponten, 1998). Although the biological role of p53 clones is unclear, the incidence and location of p53 clones suggest a possible role in skin carcinogenesis. Furthermore, animal experiments have shown the early onset of epidermal p53 clones following chronic UV-irradiation in histologically normal mouse skin and such clones are indicators of tumor risk(Berg et al., 1996); (Rebel et al., 2001).

Ultraviolet radiation

UVR contains UVA (320nm-380nm), UVB (280nm-320nm) and UVC (200-280nm). UVC is completely filtered out by the ozone layer. In UVR that reaches the earth surface, 95% consists of UVA. The UVB is absorbed by epidermis and causes DNA damage, while UVA exerts its major effects in the dermis, causing photoaging of the skin. UVA is also capable of inducing genetic damage in cellular DNA. The activity of telomerase in human skin has been shown to be strongly associated with sun exposure (Ueda et al., 1997).

Sun exposure is the major environmental agent implicated in the induction of non-melanoma skin cancer. The absolute specific UVR mutations are the tandem transitions CC->TT (Hutchinson, 1994), which are more frequently found in XP patients (Williams et al., 1998). The more common and typical UVR induced mutation is C->T transition at a dypirimidine site (Miller, 1985); (Brash, 1988). Several mechanisms for such mutations have been suggested. One is the "A rule", as adenosine will be inserted to the opposite of the photoproducts e.g. CT, leading to the mutation C->T; another is that the amino group of C quickly deaminates when the C is part of cyclobutane dimers, the resulting U will code as T if not quickly repaired (Brash et al., 1996). It was accepted that UVB is primarily responsible for induction of skin cancer **(Fig.5)**.

Animal experiments show that chronic exposure to UVB can induce skin cancer in mice (Rebel, et al., 2001). However, it has also been shown that UVA induces skin tumors in animals as well as UVB (Setlow et al., 1993); (Strickland, 1986). A recent study has demonstrated that UVA induces DNA damage through an indirect mechanism by which UVR-absorbing substances (photosensitizers) are activated to produce reactive species causing DNA damage (Kawanishi et al., 2001).

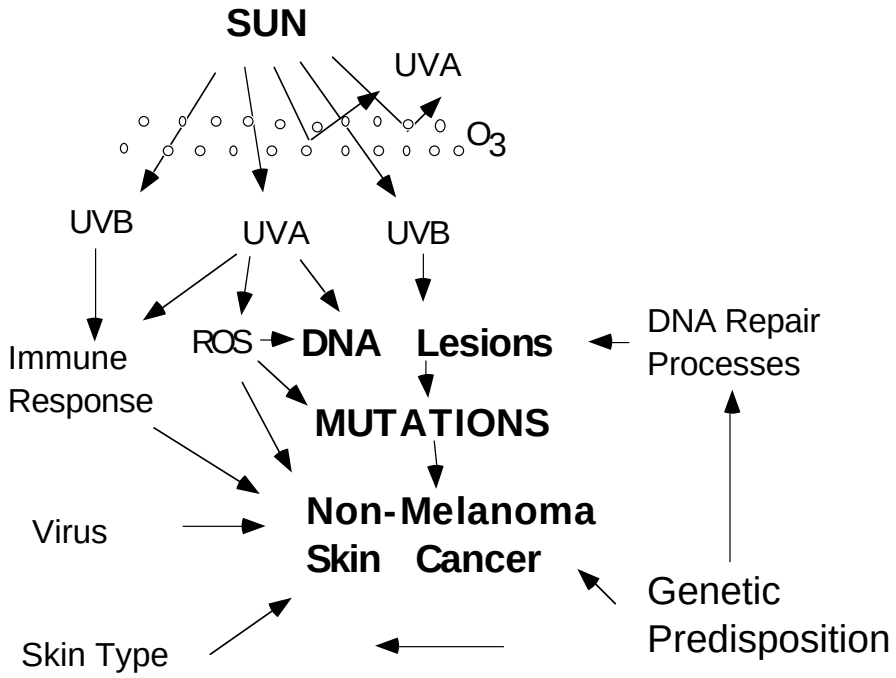

Fig.5 UVR and its effects.

DNA damage and DNA repair

The basis of any photo-process is absorption of a quantum of light-photons. DNA maximally absorbs UVR at a wavelengths of 320nm. The major UVR-induced DNA damages are cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6-4) photoproducts (Tornaletti and Pfeifer, 1996). Both photo lesions have been detected in human and animal skin, *in vitro* and *in vivo* (Rosenstein BS, 1991), and are essential with mutagenesis and carcinogenesis (Ananthaswamy and Pierceall, 1990). Cyclobutane pyrimidine

dimers are formed via a cyclobutane ring connecting the 5,6 carbons of two adjacent pyrimidine bases **(Fig.6)**. They are the dominant compounds induced by UVC and UVB.

6-4 photoproducts are formed between carbon 6 of one residue and carbon 4 of its neighbour **(Fig.6)**. They have spectral characteristics and chemical behavior that is distinct from CPDs and are assigned to a new class of photolesions. They are formed, on average 10-20% of CPDs (Mitchell and Nairn, 1989).
The induction of oxidative DNA damage has been observed in cultured cells irradiated with UVB, UVA and visible light (Taira et al., 1992). Recent studies showed that oxidative DNA damage also plays a role in mutagenesis (Kawanishi, et al., 2001).

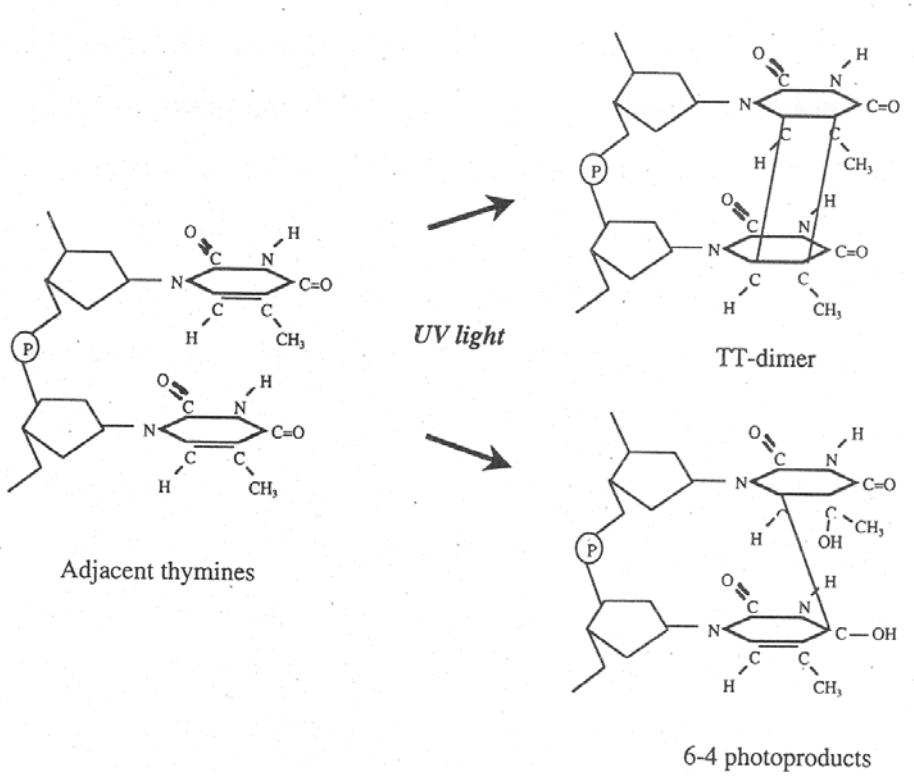

Fig.6 Structure of UV-induced photoproducts. By courtesy of Helena Bäkvall.

UVR-induced DNA lesions are primarily removed by the process of nucleotide excision repair (NER) (Sarasin, 1999). NER is one of the major DNA repair pathways, involved in the removal of a broad range of DNA lesions like UVR photolesions, bulky DNA adducts and DNA crosslinks (Hoeijmakers, 2001).

The NER process comprises five steps: recognition of a DNA lesion, incision of the damaged strand on both sides of the lesion after separation of the two DNA strands by helicases, removal of the damage-containing oligonucleotide, gap-filling by DNA synthesis, and ligation. Two NER sub-pathways exist with partial substrate specificity: the global genome repair system surveys the entire genome for distorting injuries and the transcription-coupled repair (TCR) focuses on damage that blocks elongating RNA polymerase. They are different only in the fist step, DNA damage recognition by formation of a different complex. It has been suggested that p53 modulates DNA repair by direct binding to certain NER proteins, and DNA damage encountered by the TCR machinery, especially in transcribed genes, seems to trigger the p53 response (Ljungman et al., 1999). Data from animal experiments suggest that DNA damage and the defective cellular functions in p53-deficient mice are related (Donehower, 1996) (Tennant et al., 1996).

In the case of inefficient repair, lesions remaining in the DNA can give rise to mutations during replication. This is highly pronounced in XP patients who are deficient in NER. Cells from XP patients are hypermutable following UVR, which is consistent with the high incidence of skin cancer arising in XP patients at an early age (Williams, et al., 1998).

Photo-protection

In addition to NER, there exist two other strategies of photo-protection, including endogenous and exogenous factors.

Endogenous factors

Skin pigmentation and thickening of epidermis are the two major natural protection factors against solar radiation. In response to UVR, tanning occurs due to the immediate release and *de novo* synthesis of melanin, as well as an increased mitosis of melanocytes..

Melanocytes are dendritic cells of neural crest origin. They are located in the basal layer of the epidermis and account for 10% of the cell population in the epidermis. Two variants of melanin are synthesized in the cytoplasm of melanocytes: eumelanin, which is a black-brown compound and pheomelanin, which is a yellow-reddish-brown compound. Each melanocyte is in contact with 20-40 keratinocytes and melanin is transferred via its dendrites in the form of melanosomes. Melanin absorbs, reflects and scatters UVR and may also act as a free radical quencher.

The stratum corneum consists of several layers of keratin from terminal differentiated keratinocytes. It is constantly desquamated from the surface and renewed from below. UVR is absorbed and scattered in the stratum corneum due to its content of keratin and other proteins. The increased mitotic activity following UVR results in a thickening of the skin, including the stratum corneum.

Exogenous factors

Other types of photo-protection include wearing of protective clothes, application of topical sunscreens and using systemic photoprotection.

Topical sunscreens have been widely used to protect against the harmful effects of UVR. The effectiveness of sunscreens, expressed in terms of sun protection factor (SPF), is determined by their ability to delay the development of erythema. Sunscreens give rise to protection against UVR-induced edema, but its immunoprotectivity was inferior (Wolf et al., 1993). Clinical studies have shown that topical sunscreens can prevent UVR-induced damage, including sunburn, photoaging (Boyd et al., 1995), actinic keratosis (Thompson et al., 1993) and SCCs, but not BCCs (Green et al., 1999). It has also been shown that sunscreens can decrease UVR-induced epidermal p53 induction (Berne et al., 1998). The recent concern is the question of how well sunscreens can protect genetic material, as their efficacy is largely dependent on the prevention of erythema. Furthermore the efficacy of sunscreens in delaying the formation of

erythema may encourage longer exposure and thus compromise the beneficial effects (Bykov et al., 1998b)).

Basal cell cancer

Basal cell cancer (BCC) is the most common cancer in humans, with more than 750,000 new cases per year in the United States alone (Green et al., 1996). BCC rarely occurs before the age of 30, but the incidence increases considerably in populations aged over 60 years. Exposure to sunlight, particularly UVB, is a major etiological risk factor associated with BCC development (Gallagher et al., 1995). BCCs arise only in hair bearing and mostly in sun exposed areas of the skin. Eighty-five percent of the tumors appear in the head and neck region. BCC development is associated with high childhood exposure to sunlight leading to sunburn, however BCC does not show a strong correlation with continuous exposure to UVR as does SCC, indicating that other factors are also involved (Kricker et al., 1995).

BCC shows a fairly invariable histological picture as well as an indolent clinical behavior. There is a range of histologic subtypes, including the well-circumscribed nodular subtype (45-60%), the superficial subtype (15-30%), the pigmented subtype (1-2%), and the aggressive-growth or infiltrative subtype (4-17%). The cell of origin is probably a pluripotential stem cells in the bulge area of hair follicles. BCCs are slow growing and are lacking of precursor lesions. Tumors are locally invasive but are rarely metastasized. Treatment for BCC is usually by local excision, but recurrence is commonly due to the difficulty in defining surgical margins (Miller et al., 1990). BCCs can result in extensive morbidity through local recurrence and tissue destruction.

BCC is also the most prevalent tumor reported in Gorlin syndrome. The Gorlin syndrome is an autosomal dominant disorder characterized by multiple BCCs with onset at an early age, jaw keratocysts, dyskeratotic palmar /plantar pits, skeletal malformations and soft tissue calcification. Other malignant or benign

tumors such as medulloblastoma, ovarian fibroma and fibrosarcoma, meningioma, rhabdomyoma and cardiac fibroma may also occur in this cancer-prone condition (Gorlin, 1995); (Evans et al., 1993). The gene responsible for this syndrome has been identified as the *patched* gene, PTCH, a human homologue of the *Drosophila* segment polarity gene.

In addition to PTCH alterations, p53 mutations are common in sporadic BCC (Pontén, et al., 1997a). Published data indicate that p53 mutations contribute to the malignant characteristics of tumors but are probably not in themselves sufficient for BCC development. Activating mutations of proto-oncogenes are found in many types of cancer, but there is little evidence that oncogene activation plays a major role in BCC carcinogenesis. Several studies have shown activating *h-ras* and *k-ras* point mutations in a small fraction of BCCs (van der Schroeff et al., 1990); (Anasthaswamy et al., 1989); Lieu FM et al, 1991). Limited studies of other oncogenes have shown no genetic abnormalities (Ananthaswamy et al., 1988). These data suggest that RAS activation may play a secondary role in BCC pathogenesis.

Immuno-suppression can lead to increased susceptibility for NMSC including BCC. Elevated risk of developing BCC is found in immuno-suppressed patients following renal transplantation and also in dermatological patients undergoing PUVA therapy (Gupta et al., 1986)

PTCH and hedgehog pathway

The *hedgehog* and *patched* genes were identified by analyzing mutants obtained in the early genetic screens in *Drosophila* by Nüsslein- Vollhard and Wieschaus in 1980. The sonic hedgehog/patched (SHH/PTCH) pathway plays an important role in embryonic development and has rapidly emerged as one of the most important regulators of oncogenic transformation.

The human PTCH gene maps at chromosome band 9q22.3. It encodes a 12-transmemberane protein that binds to the sonic hedgehog (SHH) ligand with

high affinity. The PTCH protein also negatively interact with a putative G-protein coupled receptor, known as smoothened (SMO). Upon association of PTCH with its ligand SHH, the inhibition of SMO is relieved resulting in up-regulation of a number of target genes, such as *WNT*, genes of the *TGF*β families and PTCH itself. Inactivation of PTCH by mutations therefore results in the constitutive activation of SMO, which is characterized by over-expression of *GLI1*, ending in uncontrolled cell proliferation.

Mammalian genomes harbor a second PTCH gene, PTCH2, and the encoded protein is capable of both SHH binding and formation of a complex with SMO (Motoyama et al., 1998a); (Carpenter et al., 1998). There are similarities between the two human PTCH proteins on the cellular level, although they have different target tissues (Motoyama et al., 1998b). PTCH2 is up-regulated in both sporadic and hereditary BCCs with PTCH mutations, suggesting a negative regulation of PTCH2 by PTCH (Zaphiropoulos et al., 1999). One report suggests that PTCH2 mutations play no major role in the etiology of BCCs and medulloblastoma (Smyth et al., 1999).

Individuals with germline PTCH gene mutations suffer from Gorlin syndrome, exhibiting a variety of developmental defects and predisposition to tumor formation. The majority of the identified alterations in the PTCH gene are deletions and insertions, which give rise mostly to truncated protein (Johnson et al., 1996); (Hahn et al., 1996); (Gailani et al., 1996b); (Undén et al., 1996); (Wicking et al., 1997). Approximately 15% of germ-line mutations are missense and are often located in highly conserved domains of the gene, resulting in significant effects on protein function. However there is no clear correlation between genotype and phenotype.

LOH at the PTCH locus has been found in more than 50% of sporadic BCCs whereas inactivating mutations in the remaining allele have been detected in 30% of these tumors (Gailani et al., 1996a). Thus PTCH mutations follow

Knudson's hypothesis that tumors in inherited cancer predisposition syndromes have mutations of the same genes as sporadic cases of the same tumors (Knudson Jr., 1971); (Sidransky, 1996). Mutations of the proto-oncogene SMO and the SHH ligand have also been detected in BCC although at a lower frequency than PTCH. A recent study has shown that, in comparison with normal skin, that BCCs have increased levels of mRNA for PTCH, *GLI1*, *HIP*, *WNT2B* and *WNT5a*; decreased levels of mRNA for *c-MYC*, *c-FOS* and *WNT4*, and unchanged levels of mRNA for *PTCH2*, *GLI2*, *WNT7B* and *BMP2* and *4* (Bonifas et al., 2001). BCCs can be induced in transgenic mice over-expressing SHH and GLI-1 (Nilsson et al., 2000; Oro et al., 1997), and also in patched heterozygous knockout mice (Aszterbaum et al., 1999). PTCH mutations have also been detected in medulloblastoma, meningioma, trichoepithelioma and breast cancer.

AIMS OF THE PRESENT INVESTIGATION

The general aim of this study was to elucidate the role of p53 in skin carcinogenesis. Specifically, the goals were to:

- investigate the kinetics of UVR-induced DNA damage and repair in relation to subsequent p53 protein expression in normal human skin and to study the effects of sunscreen (Paper I).

- develop a morphology based method for analyzing p53 gene mutations in single cells retrieved from stained tissue sections (Paper II)

- characterize p53 mutations in single cells from normal, chronically sun-exposed human skin (Paper III)

- map p53 mutations and LOH in both PTCH and p53 loci in BCCs from patients with Gorlin syndrome; and to analyze the PTCH gene alterations in microdissected fractions of sporadic BCCs (Paper IV)

Materials and Methods

Biopsies from healthy volunteers

In paper I, four healthy volunteers were irradiated on the skin of both sides their buttocks with a single dose of solar simulating ultraviolet radiation. One side was pretreated with a topical sunscreen. Sunscreen preparation (2mg/cm^2) containing both UVA and UVB absorbers, was applied to unexposed buttock skin over an area of 16 cm^2. Biopsies were obtained from buttock skin without sunscreen before irradiation as controls. Skin from both areas was biopsied at 4, 24, 48 and 120 h after irradiation. Tissue samples were fixed in 4% buffered formalin for 1-3 days, embedded in paraffin and sectioned.

In paper III, the dorsal side of forearms of 11 healthy volunteers were protected from sun by blue denim fabric (SPF 1700) during 5-10 weeks of Swedish summer time. Biopsies were taken 24 hours after the last exposure, both from protected and unprotected areas and frozen at -20°C (Berne, et al., 1998). Biopsies from sun-exposed and shielded skin from one male volunteer and shielded skin from two additional female volunteers were analyzed in this study.

Biopsies from sporadic basal cell cancers and Gorlin patients

Surgically excised fresh tumor tissues were sliced and snap frozen. Parts of the tumor tissues were stored at -70°C before subsequent analysis. In paper IV, nine sporadic BCCs were from chronically sun-exposed areas, four with solid tumor type, three with superficial tumor type and two with mixed tumor type. Twenty BCCs collected from three Gorlin patients were enrolled in the study, thirteen with solid type and seven with superficial type. Sixteen micrometer sections were made before immunostaining. Normal skin in the vicinity of the excised tumor was also collected and treated similarly to tumor tissue. In paper II, one previously characterized BCC containing two p53 mutations (codons 159 and 177), was included in the study.

HaCaT cells

In paper II, single cells from cultured a HaCaT cell line were also included. The HaCaT cell line was derived from human keratinocytes containing two heterozygous p53 mutations (exons 5 and 8) (Boukamp et al., 1988). Cells were trypsinized. A drop of cell suspension was put on a thin slide and air dried before storing at -20°C.

Simulating ultraviolet radiation

In paper II, in order to mimic daily sunlight, a SUPUVASUN 3000 (Mutzhas, Germany) equipped with a SUN-filter was used to generate a broad band of UVB (0.03 mW/cm$^{2)}$, UVA (65 mW/cm$^{2)}$ and near-infrared radiation. The minimal erythema dose (MED) on the untanned buttock skin was determined 24 h after irradiation using a monochromator (Applied Photo Physics, England) emitting a narrow band of UVB at 313 nm (slit width 3 mm, corresponding to a band width of ±4-6nm) through a 1-m liquid light guide with an aperture 9 mm in diameter). Two MED of UVB was administered to the buttock skins.

Microdissections

Scalpel microdissection

In paper IV, microdissections from immunohistochemically stained 16µm cryo-sections were performed under the light microscope, enabling careful examination of cells sampled during microdissection. Small scalpels (Alcon Ophthalmic Knife 15°) were used to microdissect and retrieve samples from the sections. The tip of the scalpel with the sampled cells was dipped into tubes containing 50µl PCR buffer (10 mM Tris-HCL pH 8.3, 50 mM KCl). Cells were incubated at 56°C and lysed by the addition of 2 µl freshly prepared proteinase K solution (20 mg/ml, dissolved in re-distilled water) for one hour. Proteinase K was heat inactivated (95ºC, 10min) and samples were stored at -20ºC prior to PCR.

Laser microdissection

In paper II and III, dissection was carried out by PALM® MicroLaser systems (P.A.L.M. GmbH, Wolfratshausen, Germany). This system (Schütze and Clement-Sengewald, 1994) allows "non-contact" optical micro-manipulation of cells on slides. Using fine focused laser beams, the surrounding tissue can be eradicated. The isolated cells were picked up by small glass capillaries (Femtotips, Eppendorf). The tips of the capillaries with attached cells were dipped in the bottom of tubes containing 10µl PCR buffer (10 mM Tris-HCL pH 8.3, 50 mM KCl) and subsequently covered with 50µl mineral oil prior to PCR. Capillaries were re-examined under the microscope to ensure that the cells had been transferred to the tubes.

Immunohistochemistry and immunofluorescence

In paper I, p53 expression and DNA damage was visualized by double immunofluorescent labelling using antibodies against p53 protein (DO-7, DAKO Ltd) and TT-dimers (KTM53, Kamiya Biomedical Ltd.). Sections were first incubated with DO-7 (1:100) overnight at 4° C. Following incubation with biotinylated rabbit anti-mouse antibody (1:100, 30 min) and TRITC - conjugated streptavidin (1:100, 30 min), slides were incubated with the KTM53 monoclonal antibody (1:1000, 45 min). Sections were then incubated with biotinylated rabbit anti-mouse antibody (1:200, 30 min) followed by FITC-conjugated avidin (1:100, 45 min). Incubation with KTM53 and rabbit anti-mouse antibodies was performed at room temperature. Nuclei were stained by immersion in a solution of 4',6-diamidino-2-phenylindole (Sigma) at a concentration of 250 ng/ml in PBS for 10 min, and coverslips were mounted onto the tissue sections in an anti-fading medium (DAKO Ltd). In paper I, proliferation was visualized on sections using a monoclonal antibody MIB-1 (Immunotech Ltd), recognizing the antigen Ki-67.

In papers III and IV, Sixteen micrometer thick cryosections were made and air dried at room temperature for 1 h. Tissue sections were incubated with a primary monoclonal anti-p53 antibody DO-7 (DAKO, code M7001), and

biotinylated rabbit anti-mouse antibody (DAKO, code E354) was used as secondary antibody. The reaction was visualized by avidin/biotin (DAKO code K355), using diaminobenzidine (DAB) as chromogen. Mayer's hematoxylin was used for counter-staining.

In situ hybridization

Two human cDNA fragments (bases 190-628 and 3625-4269) were cloned into PGEM5. They were linearised and transcribed *in vitro* to obtain a sense and two different anti-sense probes. Paraffin-embedded sections of 11 μm were treated with proteinase K (Sigma Chemical Co.) and washed in 0.1M triethanolamine buffer containing 0.25% acetic anhydride. Subsequently, sections were hybridized overnight with $2.5x10^6$ cpm of ^{35}S-labeled anti-sense or sense probe at 55°C. Autoradiography was performed for 14 days. After development of the photographic emulsion, slides were stained with H&E.

PCR and direct DNA sequencing

The amplifications were conducted in a multiplex/nested configuration (Berg et al., 1995). In paper IV, microdissected multi-cell samples were amplified in exons 2-11 of the p53 gene. Two outer PCRs were performed. Outer PCR for exons 2-3, 10 and 11 was amplified with 3 pairs of primers, and outer PCR for exon 4-9 was amplified with 6 pairs of primers. 5μl of sample lysate was added in the outer PCR mixture. After diluting the outer PCR products, a specific inner amplification for each exon was performed. In papers II and III, microdissected single cells were amplified in exons 4-9 of the p53 gene. The amplification was essentially the same as described above, except that the first four cycles of the outer PCR were run with prolonged annealing and extension time, and using *Pfu* Turbo instead of Taq polymerase in the outer PCR.

DNA sequencing was conducted in an automated fluorescent laser apparatus ALF (Amersham Pharmacia Biotech, Uppsala, Sweden) and an ABI 377 DNA sequencer (Perkin-Elmer-Applied Biosystems Inc., Foster City. CA). Samples to be analyzed on the ALF were amplified with one of the primers in a biotin-

labeled pair. The PCR products were purified and single strands were eluted by streptavidin-coated paramagnetic beads as a solid support. Direct sequencing was performed with the automated fluorescent laser apparatus (papers II and IV). Analysis on the ABI 377 DNA sequencer was carried out using the BigDye terminator cycle sequencing kit (Perkin-Elmer-Applied Biosystem) (papers II, III and IV).

Loss of heterozygosity

Loss of heterozygosity was examined for the p53 gene and PTCH gene region 9q22.3 using a microsatellite based assay in paper IV. Two *p53* microsatellites consisting of an AAAAT-repeat in intron 1 and a CA-repeat located downstream of exon 11 were co-amplified with three microsatellites (D9S280, D9S287, D9S180) in the 9q22.3 region. One primer in each pair was fluorescently labeled. A 4% denaturing polyacrylamide gel for ABI PRISM 377 (Applied Biosystems) was used and GeneScan software (Applied Biosystems) was employed for quantification and interpretation of raw data output. Cells from adjacent, morphologically and immunohistochemically unremarkable epidermis were defined as normal. The criterion for loss of heterozygosity was based on allelic imbalance in the tumor (T1:T2) divided by the allelic imbalance in the normal (N1:N2). An allele ratio (T1:T2)/(N1:N2) of less than 0.6 was scored as LOH.

Results and Discussion

Simultaneous visualization of UVR-induced DNA damage and subsequent p53 response in human epidermal keratinocytes in situ (Paper I)

It has been shown both *in vitro* and *in vivo,* that UVR can induce specific DNA damage such as cyclobutane dimers, e.g. TT-dimers (Rosenstein and Mitchell, 1991) (Bykov et al., 1998a). The formation of cyclobutane pyrimidine dimers is an important step preceding UVR-induced mutations. These DNA lesions are primarily removed by NER (Sarasin, 1999). The consequence will be deleterious if the damage is not repaired or if the damaged cells fail to undergo apoptosis. The importance of NER can be seen in patients with xeroderma pigmentosum, who develops a multitude of skin tumors due to a defective NER system. p53 protein plays a central role in mediating the cellular response to UVR-induced DNA damage (Lakin and Jackson, 1999). Elevated p53 protein transcriptionally activates a series of target genes such as p21 and Bax which facilitates DNA repair and enables cell-cycle-arrest and apoptosis. p53 has also been suggested to be directly involved in DNA repair (Ljungman, et al., 1999).

The accumulation of TT dimers and the induction of p53 following different sources of radiation have been investigated (Burren, et al., 1998); (Young et al., 1996) (Ponten 2001). However, the kinetics of p53 induction and TT dimer repair simultaneously, using a double-labelling technique, has not been studied previously. In this study, we took the advantage of double immunofluorescent labeling in an effort to investigate the formation and repair of TT-dimers, and its relation to the subsequent p53 induction. Topical sunscreens are the most common choice for protecting against harmful effects of UVR. We also try to determine the efficacy of photoprotection by topical sunscreens.

A widespread formation of TT-dimers was generated 4 h after a single dose of physiological UVR (2 MED) in the 4 volunteers. There was a wide range of positive cells (93-37%). The level of TT-dimers decreased over time and

remain above baseline at 120 h. Large variations between the volunteers with different degrees of reduction at different time points were found. Thus large inter-individual differences regarding the formation and repair of TT-dimers was evident. This is in agreement with what has been shown in earlier studies where TT-dimers persisted 7 days post-irradiation of mouse epidermal keratinocytes (Young, et al., 1996). Another study demonstrated up to 20-30 fold differences in the efficacy of repairing DNA damage on human skin (Bykov, et al., 1998b) (Bykov et al., 1999). The TT-dimer positive cells were visualized in all the layers of epidermis 4 h after radiation, but mainly in the superficial layer at 120 h. This is in line with other findings showing that undifferentiated keratinocytes are more efficient in repairing damaged DNA compared to differentiated keratinocytes (Li et al., 1997).

The pattern of TT-dimer formation and repair was mirrored by a p53 response in the same section. This is consistent with the notion that increased expression of p53 in epidermal keratinocytes is due to UVR-induced DNA damage (Hall, et al., 1993). There was a 3 to 9-fold increase of p53 immunoreactive keratinocytes 4 h after irradiation. The peak of the 53 response showed a mean of 47% immunoreactive keratinocytes. The time needed to reach peak levels of p53 immunoreactivity varied between 4 h and 24 h, as did the degrees of reduction at subsequent time points. The more persistent TT-dimer levels were followed by a more delayed p53 response in cases C and D compared to case B. The more variable p53 responses are partly due to different rates of DNA repair.

Large inter-individual differences in epidermal p53 response, independent of the type of radiation, have been reported previously (Ponten et al., 2001). The overlay images **(Fig.7)**, show a majority of the co-expressing cells in the suprabasal layers where transient amplifying and early differentiating cells are located. This suggests that perhaps only cells in a certain phase of the cell cycle or differentiation state respond to DNA damage by over-expressing p53

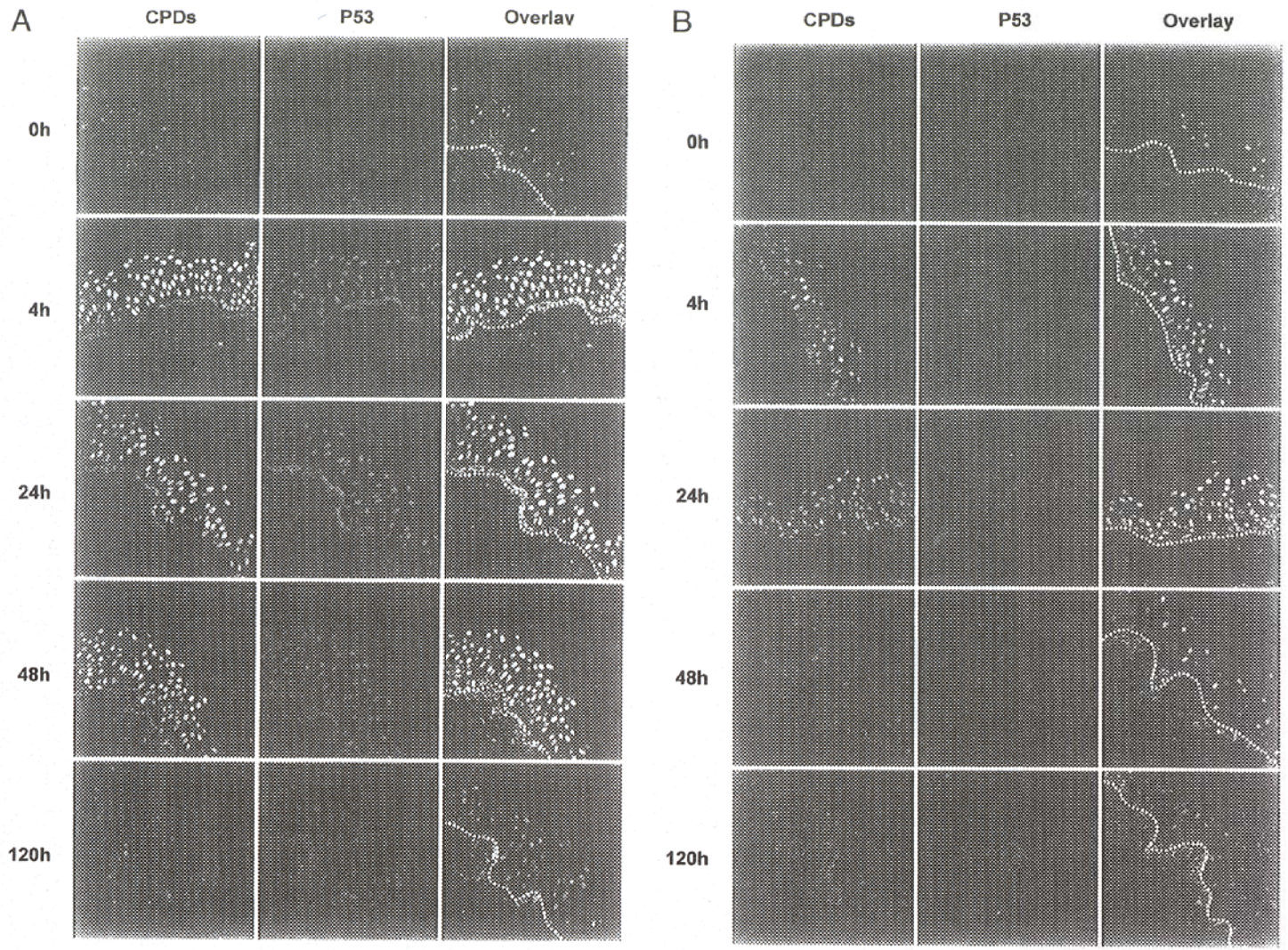

Fig. 7. Visualization of TT-dimers (CPDs) (green) and p53 protein (red) in human epidermal keratinocytes. Sections were obtained from skin biopsies of buttocks before and 4, 24, 48 and 120 h after a single dose of solar simulating UV radiation, corresponding to 2 MED. Keratinocyte nuclei show immunoreactivity for TT-dimers and p53 protein. Keratinocytes with TT-dimer and p53 coexpression (yellow colour of nuclei in overlay images) are seen in all layers of epidermis. Dotted lines outline the epidermal–dermal junction. (*A*) Skin without topical sunscreen from one volunteer (D); (*B*) corresponding skin pretreated with topical sunscreen (SPF 15) in the same volunteer.

protein. The DNA repair in proliferating cells is p53 dependent as indicated in a study by Li et al. (Li, et al., 1997). Alternatively, other factors such as MDM2 regulating intra-nuclear levels of p53, may also play a role (Ganguli et al., 2000).

Sunscreen is the most popular choice of photoprotection. The role of sunscreens in vivo is still a subject of some debate. A study in mice showed a significant reduction of DNA damage compared to unprotected skin (Wolf, et al., 1993). In human skin, Seite et al showed that an appropriate full UV spectrum product significantly reduces the UVR-induced skin damage (Seite et al., 2000a). In a study by Young et al, it was showed that sunscreens are equally adept at protecting the erythema and DNA (Young et al., 2000). Clinical studies have also indicated that the long-term use of sunscreens protected subjects from developing SCC but not BCC (Green, et al., 1999). However, recent studies by Bykov showed that sunscreen did not provide the universal protection against DNA damage as it is to erythema (Bykov, et al., 1998b).

There is a wide acceptance in its efficacy in protection against erythema formation, but recently, concern has been expressed over how well it can protect against DNA damage. The discrepancy could be due to the different endpoints applied. In our study, the formation of TT-dimers and the subsequent p53 response were partially blocked by sunscreens **(Fig.7)**. The differences in the degree of protection by sunscreens, is in line with other studies suggesting that protection by sunscreens is individual and independent of amount of DNA damage and erythema response (Bykov, et al., 1998b). Sunscreens gave a stronger protection against p53 induction than TT-dimer formation, which implied that factors such as reactive oxygen species other than cyclobutane also triggered a p53 response (Seite et al., 2000b) .

A late proliferative response as detected by the Ki-67 antibody was evident at 120 h in 3/4 subjects, suggesting that UVR is a mitogen and in agreement with increased proliferation in epidermal keratinocytes leading to thickening of the epidermis. The proliferative response was also partially blocked by sunscreens.

Although firm conclusions are difficult to draw due to the small number of subjects, the large inter-individual differences in the formation and repair of DNA damage as well as p53 response may be an indication of individual risk for developing non-melanoma skin cancer. Additional work is needed to increase our knowledge about the molecular basis for those inter-individual differences regarding repair of DNA damage and the p53 response. The double staining provides a useful tool in studying DNA damage and the subsequent response simultaneously.

p53 gene analysis in single cells (Papers II and III)

Genetic analysis in single cells has its advantage in unmasking important cellular and molecular events during different stages of tumor development. Advanced laser assisted microdissection techniques have made it possible to exploit targeted cells from histologically stained sections without contamination from neighboring cells. Many studies have been designed to dissect target cells from heterogeneous cell populations at different stages of precancerous and cancerous lesions (Emmert-Buch et al., 1996) (Suarez-Quian et al., 1999). Studies have shown that a small amount of cells and even single cells obtained from histochemically stained sections as well as cultured cells can be analyzed at the mRNA and DNA level (Becker et al., 1996); (Bernsen et al., 1998); (Schutze and Lahr, 1998). Based on our previous study (Pontén et al., 1997b), we optimized a method for the mutation analysis of p53 exons 4 to 9 in single cells retrieved by laser assisted dissection from immunostained sections. The optimized technique was applied to characterize p53 mutations on keratinocytes from chronically sun-exposed normal human skin.

In order to optimize the PCR conditions, as described in Paper II, we first took the advantage of the HaCaT cell line containing two p53 mutations (exons 5 and 8) (Boukamp, et al., 1988). In our previous study, we were able to analyze two PCR fragments (exons 7 and 8) in 14% of laser-dissected single cells from immunostained tissue sections (Pontén, et al., 1997b). The low amplification rate was due to a high frequency of exon and allele drop out (ADO). In single cell genetic analysis, preferential amplification of one allele or exons is a potential problem (Garvin et al., 1998); (Findlay et al., 1998); (Rechitsky et al., 1998). ADO can either occur during PCR due to a sub-optimal technique. Alternative explanation could be that ADO reflects an underlying biological event resulting in loss of genes. Another concern in single cell genetic analysis is errors introduced by polymerase. Taq polymerase has an error frequency of 10^{-4}-10^{-5} (Tindall and Kunkel, 1988). The recently available *Pfu* polymerase has proofreading activity and reduces the misincorporation rate by a factor of 10 (Andre et al., 1997).

By increasing the concentration of template, primer and polymerase, prolonging primer annealing time and choosing *Pfu* Turbo as polymerase, a significant improvement in exon representation was achieved (12/19, 63%). The allele dropout rate (ADO) was 50% (Table 1).

As a second step, this improved technique was also applied to single cells retrieved from frozen tissue sections of normal human skin. Due to low exon representation from fresh material (1/16, 6%) (Table 1), care was taken during sample preparations. Activated endogenous DNase which leads to DNA degradation, is presumably the major cause. A nuclease inhibitor EDTA, was introduced during every step of sample preparation (sectioning, staining and microdissection). Different concentrations of EDTA and tissue fixations were tested. Higher concentration of EDTA (100mM) gave better amplification rates but poor morphology. Formalin and acetone/methanol fixation gave better morphology however amplification was poor. The optimal analysis was thus

accomplished by using a low concentration of EDTA (10mM). By using this optimized tissue preparation method, we were able to amplify all the possible exons in 10/20cells (Table 1).

Finally, a previously characterized BCC was used to evaluate ADO in this system. Single cells were microdissected from tumor nests and normal skin nearby. The exon representation rate in samples amplified using Taq polymerase was 10/30, in contrast to 26/50 using *Pfu*. ADO was 60% in samples amplified by Taq polymerase, while ADO was 50% in *Pfu* amplified samples (Table 1). No difference was observed between tumor cells and cells from normal skin. Further studies are needed to verify the underlying molecular mechanisms for ADO.

Table 1 percentage of cells with all possible exons amplified under different conditions of treatments

Treatment	Sample source	No. of single cells	% of cells with all possible exons	ADO
Pfu Turbo	HaCaT cells	19	63%	50%
Pfu Turbo	skin	16	6.3%	-
EDTA + *Pfu* Turbo	skin	20	50%	-
EDTA+ Taq/stoffel	BCC	30	33%	60%
EDTA + *Pfu* Turbo	BCC	50	52%	50%

The laser microdissection techniques, when combined with optimized genetic analysis, has provided an insight into the biology of different diseases (Lehmann et al., 2000); (Cerroni et al., 2000); (Volante et al., 1999) and (Walch et al., 2000) **(Fig.8)**. Detailed questions relating to morphology to genetic background can be addressed. It is known that natural doses of UVR can induce p53 protein overexpression in epidermal keratinocytes (Hall, et al., 1993). Studies by Ouhtit indicated that the frequency of UV-specific p53

mutations in normal skin is a predictor of risk for basal cell cancer (Ouhtit et al., 1998). Our previous study showed a 66% decrease of p53 immunoreactive keratinocytes after complete protection from the sun during two Swedish summer months (Berne, et al., 1998). However, the genetic background underlying the remaining scattered p53 immunoreactive cells is unclear. It was therefore of interest to conduct a molecular study based on well defined morphology, together with the advent of proper technique. In paper III, the refined technique was applied to characterize p53 mutations (exons 4-9) in single cells in normal, chronically sun-exposed skin from healthy individuals. Biopsies were taken from the skin of 11 volunteers which had been subjected to daily sun during two summer months, as well as from adjacent skin which had been totally protected from the sun by blue denim fabric (SPF 1700) (Berne and Fischer, 1980).

Four biopsies were chosen, one from non-shielded skin and three from shielded skin. Biopsies were sectioned and immunostained with p53 antibody. A total of 172 single cells were retrieved by laser assisted microdissection, followed by PCR and direct DNA sequencing analysis of the p53 gene. In 99/172 (58%) cells, it was possible to amplify one or more p53 exons. In the 99 samples, which contained a template, a total of 494/594 (82%) possible exons were amplified. Fourteen mutations were identified (Table2).

Table 2 Summary of samples, amplified exons and percentages of mutations found.

Biopsy	Keratino-cytes	No. cells collected	No. amplified exons	No. different mutations	% of mutation in amplified exons
Non-shielded	scattered	14	64	4	6.2%
shielded	p53	48	134	5	2.3%
	scattered	110	296	5	
Total		**172**	**494 (82%)**	**14**	

Typical UVR signature mutations i.e. C->T or G->A transitions at dypirimidine sites were apparent in 8/14 mutation. Only one silent mutation was recorded. Thus, 26 morphologically normal keratinocytes contained one or more p53 mutations, 15 showed immunoreactivity. This is the first time to our knowledge that scattered p53 immunoreactive keratinocytes in morphologically normal human epidermis have been to carry p53 mutations.

In one sample from shielded skin (V2), a minute epidermal p53 clone was disclosed **(Fig.9)**. It had a diameter of 10-15 basal cells corresponding to an area of 0,05 mm^2. Forty-eight single cells were isolated from 12 consecutive immunostained cryosections representing this epidermal clone. Immunoreactive and non-immunoreactive basal, suprabasal and superficial keratinocytes from this area were analyzed. Five different mutations were found. Two missense mutations (codons 241 and 281) were dominant in both immunoreactive and non-immunoreactive keratinocytes of all layers of epidermis within the p53 clone **(Fig.10)**. The remaining 3 mutations (one missense, one stop and one silent) were only found in solitary keratinocytes. This is in line with other findings showing that p53 clones often contain p53 mutated keratinocytes (Jonason, et al., 1996), (Ren, et al., 1996a); (Pontén, et al., 1997a).

Our results show that p53 mutations are common in chronically sun-exposed skin. The mutated cells can be distributed in a dispersed and a clonal pattern. The role of the p53 gene mutations in tumor development is widely accepted. p53 is mutated in more than 50% of human cancers, but differences exist among different tumor types, e.g. in colon cancer, p53 alteration seems to be a late event (Fearon and Vogelstein, 1990), whereas in skin cancer, p53 mutations appear to be an early event (Ziegler, et al., 1994). Most mutations are missense mutations, resulting in amino acid substitution, which leads to abrogation of normal p53 function and consequently disrupted important

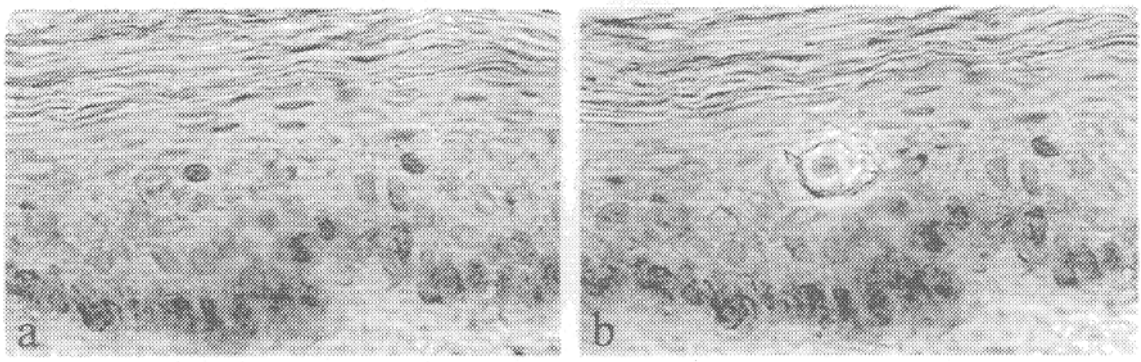

Fig.8 p53 immunoreactive keratinocytes in epidermis before microdissection (a); after isolation using laser-assisted microscope (b).

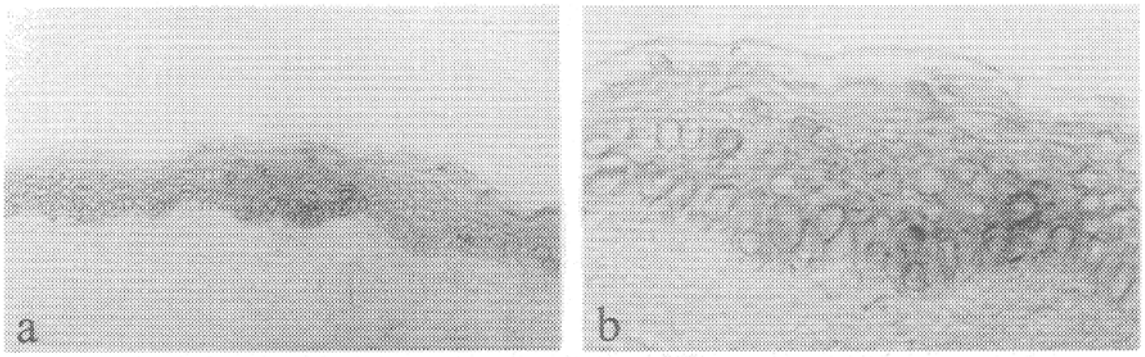

Fig.9 Immunohistochemically staining revealing a small p53 in normal skin after two months of total protection from UV-radiation. Low magnification (a) and high magnification (b)

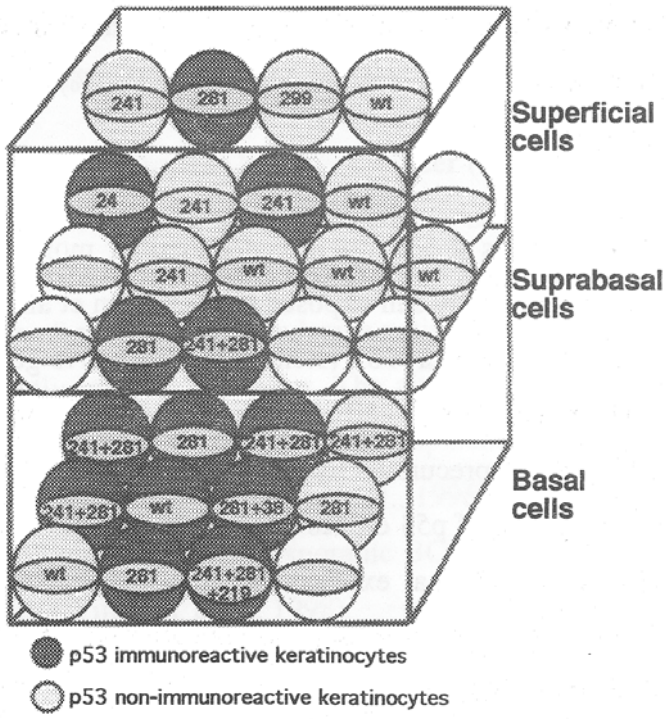

Fig.10 Cartoon illustrates topography of the detected p53 mutations in the p53 clone from B. Two missense mutations (codon 241 and codon 218) were present in all three layers of epidermis. Both p53 immunoreactive (red) and non-immunoreactive (yellow) keratinocytes show mutations.

cellular functions, such as cell cycle control, DNA repair, differentiation, genomic stability and apoptosis.

The consequence of a p53 mutation will not only depend on the type of genetic alteration but also on the target cell itself. In skin, p53 mutations in terminal differentiating cells and transit amplifying cells are probably less detrimental than that of in epidermal stem cells. Mutated differentiating and transit amplifying cells will be eventually shed as a result of the constant turnover of epidermis regardless of the mutations. In skin protected by blue denim fabric (SPF 1700), which is almost impermeable for UVR, mutated non-stem cells keratinocytes would have shed within 26-42 days of normal epidermal turnover time (Wilkinson et al., 1992). The majority of detected mutations showed a UV signature, suggesting that those mutations were acquired two month earlier. We interpret our findings to suggest mutations occurring in epidermal stem cells. The scattered p53 mutated keratinocytes showing UVR signature mutations, are most likely also the progenies of mutated stem cells. It is unclear why they have not started a clonal expansion is unclear. The frequency of mutations found in non-shielded skin was notably higher (6.2%) than in shielded skin (2.3%) (Table2).

Epidermal p53 clones are frequently detected in morphologically normal epidermis from chronically sun-exposed sites (Pontén et al., 1995); (Ren et al., 1996b);(Brash and Ponten, 1998); (Ren, et al., 1997). No genetic link has been detected between p53 clones and skin cancer, although one study suggests a role for p53 clones as precursors for BCC (Tabata et al., 1999). However, the incidence and location of p53 clones suggests a possible role of p53 clones in skin carcinogenesis. Animal experiments also indicate that p53 clones are closely related to UVR and that they are indicators of tumor risk (Berg, et al., 1996); (Rebel, et al., 2001).

Two missense (codons 241 and 281) mutations were dominant in the detected p53 clone. Both mutations show typical UV signature and result in amino acid changes affecting direct DNA binding. A selective growth advantage thus exists in the keratinocytes in this clone. Lacking the selective pressure i.e. UVR, the size of the clone might represent a regressed clone of a normal epidermal proliferative unit (Potten, 1974), (Asplund, et al., 2001), rather than an expanded clone.

In the small p53 clone, the allele dropout out (ADO) rate in the basal cells was 2/8, while in the suprabasal cell it was 5/6 and in the superficial cells it was 3/3. Although less plausible due to the pure technical artifacts, an alternative is that ADO is part of the terminal differentiation pathway, and reflecting cells losing alleles during differentiation. Our study provides a novel insight into the complexity of a self renewing tissue. The present strategy has a wide range of applications for investigating the different stages of tumor formation, as well as in studying biological events determining the morphological changes during the hair cycle, and will certainly extend our understanding of behavior of epidermal stem cells and the development of basal cell cancer.

PTCH and p53 gene alterations in basal cell cancer (Paper IV)

Basal cell cancer (BCC) is the most common type of cancer among Caucasian populations (Green, et al., 1996). It has two forms, the prevalent sporadic form and a rare hereditary form in Gorlin syndrome patients. Gorlin syndrome is an autosomal dominantly inherited disorder. The alterations in the PTCH gene have been reported to play a key role in the development of BCC (Hahn, et al., 1996); (Gailani and Bale, 1997); (Johnson, et al., 1996). In addition, p53 gene mutations are also frequently found in sporadic BCCs (Pontén, et al., 1997a); (Ziegler et al., 1993), (Williams, et al., 1998).

In the present study, we compared the PTCH and p53 gene alterations in BCCs from both sporadic and hereditary cases. Nine sporadic BCCs and 20 BCCs

from three Gorlin patients were investigated. A total of 70 microdissected samples from tumors and adjacent skin were subjected to PCR, analysis of loss of heterozygosity (LOH) and DNA sequencing. Forty-five samples were collected from 9 BCCs (9 from normal epidermis, 9 from p53 clones and 27 from tumors). Twenty-five samples were microdissected from BCCs of 3 Gorlin patients (4 form normal epidermis, one from a p53 clone and 20 from BCC tumors).

In situ hybridization was used to detect PTCH mRNA in sporadic BCCs. Overexpression of PTCH mRNA was found in all 9 BCCs, which is an indirect indication of PTCH mutation in tumors (Undén et al., 1997). Our findings are in agreement with previous studies showing that 90% of BCCs from XP patients and 50% of sporadic BCCs showed PTCH mutations (D'Errico et al., 2000); (Gailani, et al., 1996b).

As summarized in Table3, LOH in the PTCH gene locus was prevalent in tumors from sporadic BCCs (6/8) and hereditary BCCs (17/19). Taken together with *in situ* data, our findings are well in concert with the notion that BCC develops with one mutated allele, frequently accompanied by the frequent loss of the other allele (Gailani, et al., 1996b); (Taipale et al., 2001). Accordingly, the germline mutation in PTCH gene is a prerequisite for early onset of BCC in Gorlin syndrome (Knudson et al., 1975). LOH in the p53 gene locus was infrequent in sporadic BCCs (1/9) and in informative hereditary BCCs (1/12). As indicated by previous studies suggesting that LOH in p53 locus is not a frequent event in the development of BCC (Pontén, et al., 1997a) (Gailani, et al., 1996a).

A recent animal studies showed that p53 mutations were detected in 2/5 PTCH heterozygous knockout mice following UVR (Aszterbaum, et al., 1999). In our study, at least one p53 mutation was found in each of the 9 sporadic BCCs,

whereas p53 mutations were only detected in 7/20 hereditary BCCs. Subclones within individual tumor showed a partly different genotype with respect to p53 mutations in four BCCs (U-6, U-8, U-9 and U-11), i.e. additional p53 mutation(s) developed during tumor progression.

Table 3 Summary of the LOH in PTCH region and p53 locus in tumors from sporadic and hereditary BCCs. Numbers of samples with p53 mutation(s) from both groups.

Tumors	LOH 9q	LOH 17p	No. of samples with p53 mutations
Sporadic BCC	6/8	1/9	9/9
Hereditary BCC	17/19	1/12	7/20

When combining data regarding p53 mutation and with PTCH LOH data, an interesting finding appeared in two BCCs (U-4 and U-11). In U-4, one part of the tumor displayed a p53 mutation (codon 135) without LOH in the PTCH locus, while the other two parts of the same tumor showed the same p53 mutation with addition of LOH in the PTCH locus. In this case, BCC must have developed with a p53 mutation preceding LOH in the PTCH locus. A similar result was found in U-11, where two p53 mutations (codons 130 and 285) without LOH in PTCH was evident in one part of the tumor, whereas the rest of the tumor showed the same p53 mutations with an additional two p53 mutations (codon 213 and 158) and LOH in the PTCH locus **(Fig.11)**. In this case BCC appears to grow with a selection for both additional p53 mutation and LOH in the PTCH region.

Our results suggest that p53 mutations can occur prior to LOH in the PTCH region in certain tumors. The subclones found in U-4 and U-11 indicates that additional hits affecting the PTCH gene are selected during the growth of BCC. This is analogous to a previous findings showing additional p53 mutations accumulate during tumor progression (Pontén, et al., 1997a). The possibility that both PTCH alleles are already altered by mutations that abrogate the

normal function can not be excluded, however, it is very unlikely that LOH in the PTCH locus is just a random event.

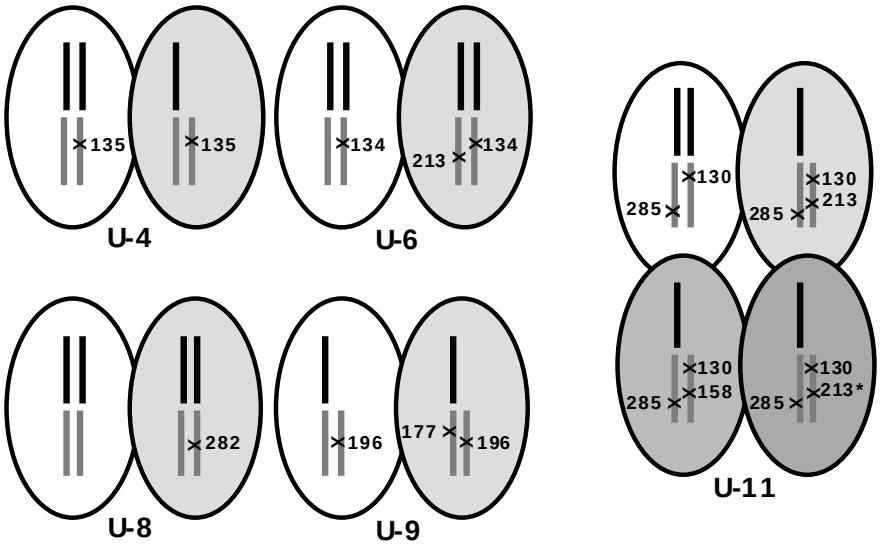

Fig.11 Cartoon schematically illustrating tumor heterogeneity in the 5 tumors where different genotypes were found within the individual tumors. Grey bars represent the *p53* gene, where mutated codons are shown. Black bars represent the PTCH gene, where LOH is shown as loss of one black bar

In sporadic cases, 12/18 detected mutations shared endemic UVR damage, i.e. C->T or CC->TT. 7/9 mutations found in Gorlin cases indicated a UVR-induced mutagen as well as deletions and unusual double base substitutions (CC-TA and GG-AC). Several mutations found in hereditary BCCs were different compared to the typical missense mutations found in sporadic BCCs. The reason for this is unclear. A possible explanation that this may be due to mutagens other than such as oxidative stress (Kawanishi, et al., 2001).

When comparing p53 protein overexpression found in sporadic and hereditary cases, 7/9 sporadic BCCs showed p53 protein over-expression in 10-100% of tumor cells, whereas this frequency was only less than 5% or less, in 5 tumors from Gorlin patients (5/20). Only one p53 clone with a wild type p53 gene was found in skin adjacent to hereditary BCC, whereas, in sporadic BCCs 6/9 clones had p53 mutations were found in sporadic BCCs. The disparity between patients with sporadic and hereditary BCCs is not clear. A possible explanation

is that Gorlin patients develop "invisible" p53 clones due to p53 mutations not resulting in protein over-expression, a scenario consistent with the low p53 immunoreactivity in BCCs from these patients (Hashimoto, et al., 1999). Another alternative is due to the fact that the Gorlin patients generally avoid solar radiation during their life, or by unknown reason.

In summary, the PTCH and p53 genes are involved in the development of basal cell cancer. Careful microdissection in tumors revealed that sporadic BCCs consist of genetically linked subclones in which as additional p53 mutations and/or LOH in the PTCH region are selected for.

CONCLUSIONS

- A single dose of UVR induces DNA damage in epidermal keratinocytes. Subsequent p53 overexpression can be detected in cells with thymine dimers. Sunscreen can block DNA damage and reduce the epidermal p53 response.

- Optimized conditions for single cell PCR and p53 gene sequencing can be obtained by inhibiting DNase activity and modifying PCR protocols.

- p53 mutations are common in chronically sun-exposed normal human skin. A small p53 clone with two dominant p53 missense mutation can prevail after complete protection from sun during two summer months.

- LOH in PTCH region is prevalent in BCCs from both sporadic and hereditary cases. p53 gene mutations and display unusual deletions and double base substitutions in BCCs from Gorlin syndrome patients compared to mutations in sporadic BCCs. Genetically linked subclones with partly different p53 and PTCH genotype exist in BCC.

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