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Benign Clonal Keratinocyte Patches with p53 Mutations Show No Genetic Link to Synchronous Squamous Cell Precancer or Cancer in Human Skin

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Ultraviolet light, which is the major etiology of human skin cancer, will cause mutations in the p53 gene. We and others have found that such mutations occur in more than one-half of nonmelanoma squamous cell cancer and precancer. Immunostaining for p53 has disclosed a characteristic compact pattern not only in cancer/precancer but also in areas of microscopically normal epidermis termed p53 patches. By microdissection, sequence analysis of the p53 gene, and analysis of loss of heterozygosity (LOH) at the site of this gene, we have now extended previous data to ascertain whether these p53 patches are precursors of simultaneously present squamous cell cancer or its morphologically recognized precancerous stages (dysplasia, carcinoma* in situ*). In none of 11 instances with co-existence of a p53 patch with dysplasia or* in situ *or invasive cancer were the mutations identical. We conclude that p53 patches, estimated to be approximately 100,000 times as common as dysplasia, have a very small or even no precancerous potential. Their common presence demonstrates that human epidermis contains a large number of p53 mutations apparently without detrimental effect. The only result of the mutation may be a clandestine benign clonal keratinocyte proliferation. The importance of p53 mutations for such benign cell multiplication on one hand and malignant transformation on the other is unclear. Although the spectrum, type, and multiplicity of mutations were similar in both types of proliferative responses, there was a clear difference with respect to LOH. No LOH was found in 17 p53 patches. By contrast 11 of 30 precancers/cancers had LOH. (Am J Pathol 1997, 150:1791–1803)

Nonmelanoma skin cancer is common in chronically sun-exposed skin, especially in people who lack protection by pigmentation.[1] Exposure to ultraviolet (UV) light is the important cause, but the events leading to invasive cancer are largely unknown, particularly at the single-cell level. This malignant disease is an excellent model for experimentally oriented studies of human cancer.[2] It is easily observed and biopsied. The characteristic mutations caused by exposure to a physical carcinogen can be detected with precision.[3] We have been particularly interested in the role of the p53 gene, which is mutated in more than 50% of the cases.[3,4]

By the combined application of immunohistochemistry, microdissection, and DNA sequencing to multiple samples from human sun-exposed skin with simultaneously present lesions, it was possible to reveal genetic relations between precancer and invasive squamous cell cancer (SCC). A salient finding was identity of mutations in the p53 gene between synchronous dysplasia (DPL), carcinoma *in situ* (CIS), and invasive cancer, a strong indication that they all derive from the same originally transformed clone. In the same study, we also observed patches of keratinocytes with intense nuclear accumulation of immunoreactive p53 (here termed p53 patches),

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which in approximately 70% of the instances also had mutations of the p53 gene but, despite these alterations, appeared morphologically completely normal.[5] We interpreted them as benign clonal expansions of cells probably driven by some aberration in intracellular handling of p53.

The discovery of these p53 patches raised some questions that we now have tried to answer by extending our material and systematically including also loss of heterozygosity (LOH) in the p53 gene area as part of the molecular analysis.

As clonal p53 patches predominated in the same sun-exposed parts of skin as cancer/precancer and were more common in the vicinity of squamous neoplasia than, for instance, nevi, there is a distinct possibility that p53 patches are early submorphological precursors of dysplasia and cancer.[6] However, no evidence in the form of identity of mutations between p53 patches and adjacent neoplasia was present in the microdissected samples.[5] As this absence of any genetic connection could be explained by a small sample size, more material may provide an answer to the question of a possible link.

Because the mutations of the p53 gene in the p53 patches seemed to be different from those in even closely apposed precancer/cancer, it was suggested that differences in appearance and tumor biological behavior could be explained by differences in the mutation spectra.[5] Although p53 point mutations in cancer/precancer could concern important DNA-binding regions of the p53 protein, mutations in p53 patches could have a milder functional effect because less critical parts of the molecule were affected. A related alternative would be if cancer/precancer was more prone to LOH than the p53 patches. These alternatives were probed by comparisons between mutation spectra of p53 patches on one hand and DPL, CIS, or invasive SCC on the other.

Materials and Methods

Specimens

Eighteen fresh specimens were obtained from seventeen patients from the Department of plastic surgery of the University Hospital in Uppsala, Sweden. From one patient, two specimens from different parts of the body (temple, 5g4 and 5g8 (two sections), and right arm, 5g1) were investigated. One case (5a8) has partially been published[5] but is now included in its entirety.

Part of the excised tissues was quickly frozen in dry ice with 99% isopentane and stored at −70°C for subsequent hematoxylin and eosin (H&E) staining, p53 immunohistochemistry, microdissection, and DNA sequencing. The remaining parts were fixed in buffered formalin for routine staining and diagnosis. Patients were from 65 to 88 years old. Fifteen of eighteen of the specimens were from strongly sun-exposed areas (head and back of the neck). The remaining three were from shoulder, arm, and back of the body.

p53 Immunohistochemistry

Immunostaining was performed as described.[7] Briefly, cryostat sections were exposed to monoclonal antibody DO-7. Reactions were visualized by avidin/biotin. Tumor tissue with a known strong reaction to the antibody served as a positive control. A consecutive section of each specimen without primary DO-7 antibody was included as a negative control.

Morphological Criteria

For exact morphological definition of areas to be microdissected, we examined an adjacent H&E-stained section as well as the immunostained cryostat section. All sections were jointly scrutinized by three or four observers (Z-P. Ren, F. Pontén, M. Nistér, and J. Pontén). For two different degrees of dysplasia (slight or moderate) and *in situ* (CIS) or invasive cancer (SCC), conventional criteria were employed. p53 patches were identified by their compact, uniform p53 immunopositivity as defined[8,9] and illustrated in Figure 1. These lesions were unambiguous and consensus was always reached, but it soon became apparent that another category, termed very slight dysplasia, also had to be taken into account. Such alterations, which primarily were spotted because of a compact immunohistochemical p53 pattern, differed from the classical picture of a p53 patch by vaguely discernible nuclear atypia with a proportion of somewhat enlarged, slightly irregular and often also hyperchromatic nuclei. Sometimes these changes were accompanied by slight basal inverted papillomatous epidermal hyperplasia with some resemblance to delicate forms of lentigo[10] but without much increase of melanin pigmentation. These lesions could imperceptibly transmute into p53 patches without atypia. The term dysplasia (DPL) will here encompass very slight, slight, and moderate dysplasia (synonym actinic keratosis), CIS will be used for severe dysplasia and classical Bowen's *in situ* cancer, and SCC will be used for invasive squamous cell cancer.

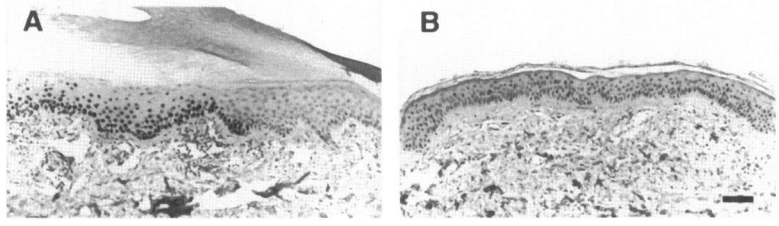

Figure 1. *Comparison between compact and dispersed patterns of p53 immunoreactivity.* **A:** *Compact pattern with strongly p53-immunopositive nuclei in the basal and suprabasal epidermal layers. Note that the cells in the granular layer are negative despite the presence of mutations of the p53 gene also in those differentiating keratinocytes. The p53 patch is abruptly demarcated against epidermis with negative immunohistochemical reaction to the right.* **B:** *Dispersed pattern. It differs from the compact p53 patch pattern by random distribution of positive nuclei among nuclei that have not accumulated immunoreactive p53. Dispersed patterns, interpreted as reactive to an acute DNA damage by UV light, do not ordinarily reflect mutations of the p53 gene. Mutations in the p53 gene were exceptionally found in cases with unusually pronounced dispersed pattern as illustrated in* **B**. *Tissue sections were immunostained with p53 antibody (DO-7) by the ABC method using Mayer's hematoxylin as counterstain. Bar, 0.1 mm*

All morphological diagnoses were irrevocably recorded before any results of gene sequencing were known. If at least one pathologist considered a lesion as borderline between dysplasia and p53 patch, it was labeled very slight dysplasia.

Microdissection

A total of 67 samples were microdissected from 19 histological sections using previous p53 immunostaining as a guide for selection of relevant areas as described.[7] Samples were transferred to tubes containing 50 μl of polymerase chain reaction (PCR) buffer (10 mmol/L Tris/HCl (pH 8.3), 50 mmol/L KCl). Cells were lysed overnight by adding 2 μl of freshly prepared proteinase K solution (40 mg/ml, dissolved in re-distilled water).

Oligonucleotides and in Vitro *Thermocycling*

The primers for amplification of exons 4 or 5 to 8 of the p53 gene and two polymorphic microsatellites used for determination of LOH have been described.[11–13] Exons 4 or 5 to 8 were amplified in a multiplex/nested configuration.[12]

Solid-Phase Sequencing

One of the inner primers was labeled with biotin to permit solid-phase DNA purification of PCR amplificates using magnetic beads as solid support.[14] By strand-specific elution with 0.1 mol/L NaOH, a clean substrate for sequencing was obtained. A semi-automatic protocol with a fluorescent-labeled sequencing primer and α-thiotriphosphate nucleotides[12] was used. Both strands were sequenced. Analysis was performed on an automated laser fluorescent apparatus (A.L.F. DNA Sequencer, Pharmacia Biotech, Uppsala, Sweden). Relative reduction of nucleotide peaks was recorded in percent separately for all analyses. The detection limit for mutations was set to 15% reduction of signal in relation to the wild-type (wt) allele.[15]

Loss of Heterozygosity

Two microsatellites consisting of a $(AAAAT)_n$ (intron 1) and $(CA)_n$-repeat (just downstream of exon 11) were used.[11,13] They were co-amplified in a single PCR 35-cycle run in a mixture containing sample (10 μl of cell lysate), 10 mmol/L Tris/HCl (pH 8.3), 2.0 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.1% Tween 20, 0.2 mmol/L dNTPs, 0.2 μmol/L of each primer, and 1.0 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, CT). The cycling profile consisted of denaturation at 94°C for 1 minute, annealing at 59°C for 1 minute, and extension at 72°C for 1 minute. PCR was initiated by a 5-minute denaturation at 94°C. The final cycle was followed by a 10-minute extension at 72°C. Solid-phase sequencing was applied as described above. A fluorescein-isothiocyanate-labeled marker ladder (50 to 500 bp) was used as size standard. For quantification and interpretation of raw data output, Fragment Manager Software (Pharmacia Biotech) was used.

Fourteen of seventeen of the patients were informative at either one or both polymorphic sites. In cases with mutations in one allele, which were noninformative because of lack of microsatellite polymorphism, we used an indirect approach to assess deletion. Theoretically, total disappearance of a nucleotide peak at a mutation site would prove LOH, ie, deletion of one allele. To compensate for statistical uncertainty, including the possibility of contamination by normal cell DNA, we

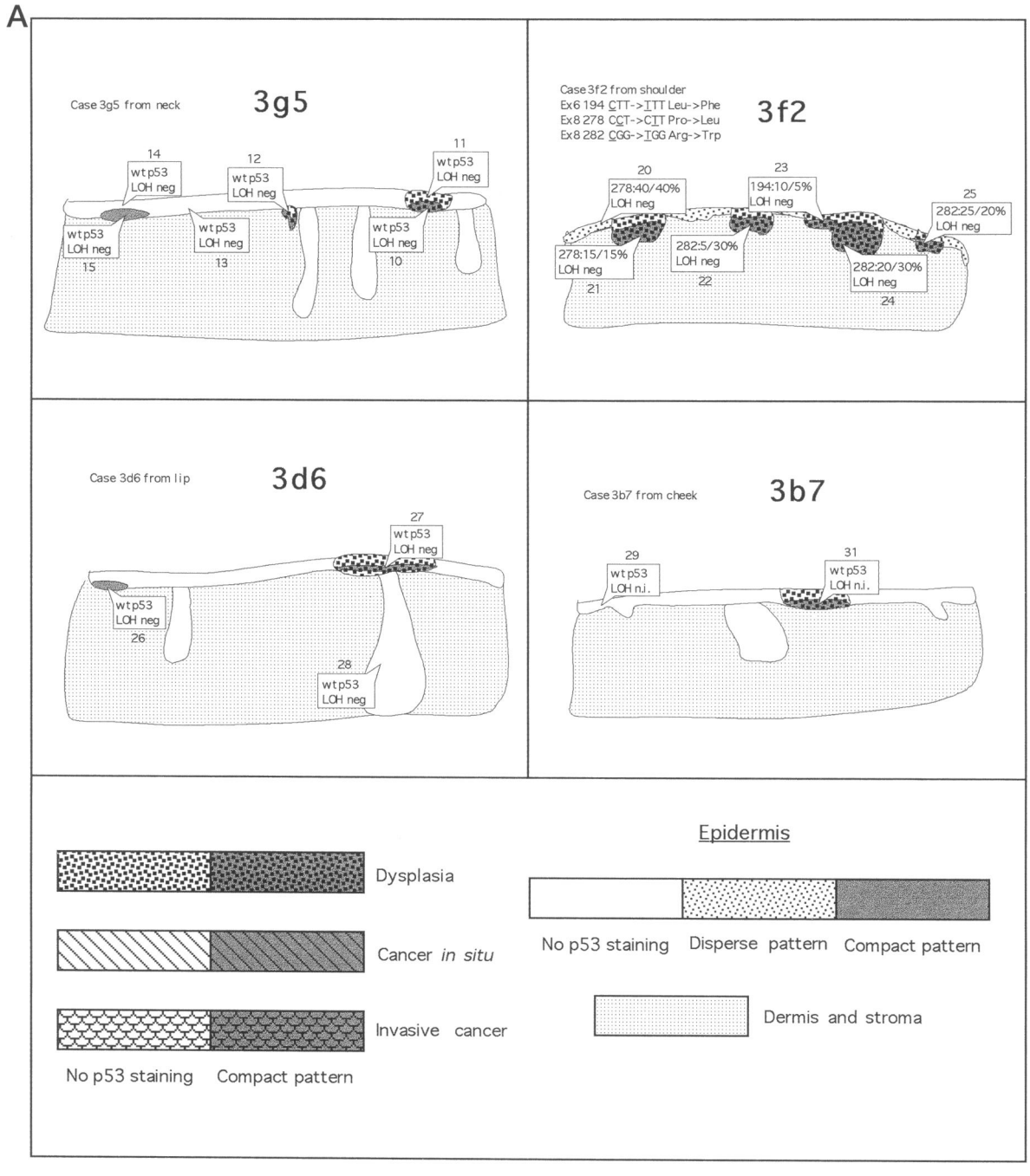

Figure 2. *Schematic drawings (also Figures 3 and 4) of sections from human skin used for microdissection, extraction of DNA, and sequencing of exons 5 to 8 of the p53 gene. The rectangles indicate the site of sampling, the location of any mutations, and the presence of LOH. The mutations obtained were confirmed in two separate analytical runs. The percent reduction of nucleotide peaks at sites of mutations are given at both sides of a slash.*

used a lower limit of 70% reduction of the signal as the cutoff level for the indirect demonstration of LOH. After application of this criterion to cases 1e8, 3b7, and 4b4, only one dysplasia (3b7) remained undecided.

Confirmation of LOH and DNA Sequence

Exons 4 or 5 to 8 of 67 samples were sequenced and analyzed for LOH. Registered mutations were confirmed by repeating the DNA sequence analyses of

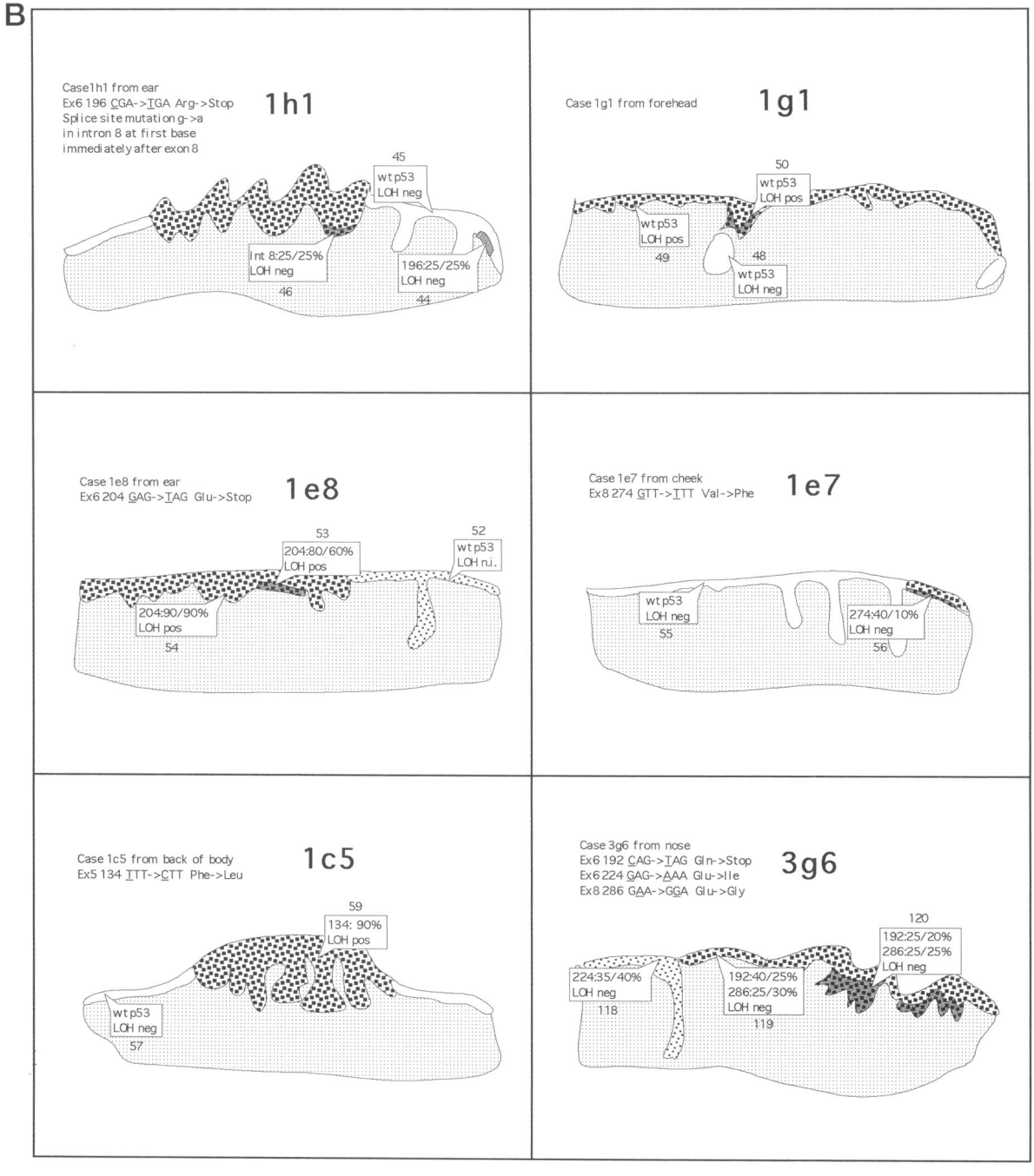

Figure 2 Continued.

the original extracts. Analyses of LOH were repeated when the primary results were ambiguous.

Cloning of PCR Fragments

Samples containing more than one mutation in the same exon were analyzed by cloning using the pGEM vector cloning kit, according to the manufacturer's instructions (Promega, Falkenberg, Sweden). Fragments of interest were amplified with the biotinylated primer replaced by unlabeled primer, ligated into the vector, and transfected into competent bacteria. Colonies were screened by blue/white selection. Single colonies containing inserts were amplified by PCR and sequenced, using vector-specific primers. An average of 10 colonies per cloned product were sequenced. One sequence containing both

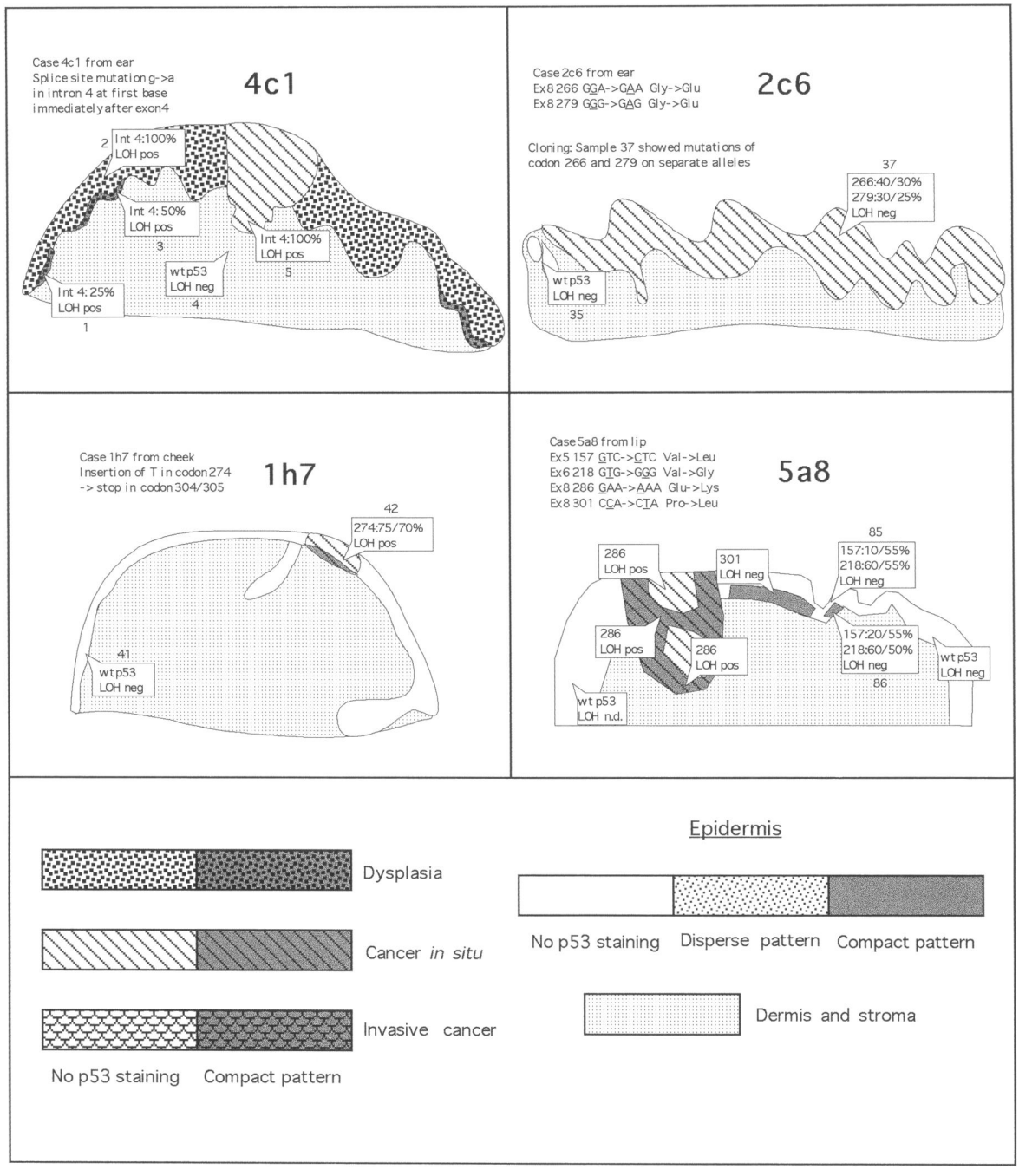

Figure 3. *See Figure 2 legend.*

mutations revealed that they were situated on the same allele. If one sequence contained one mutation and another sequence contained the other mutation(s), mutations were situated on different alleles.

Results

Detailed documentation of 19 sections from 17 patients are given in Figures 2, 3, and 4.

Probable Presence of a Constitutive (Germline) Mutation

In case 4b4, normal epidermis as well as three samples from dysplasia and invasive cancer had a mutation of codon 197 in common. It could thus not be excluded that this individual carried a constitutive p53 gene mutation. This hypothesis was strengthened by a peculiar difference in signal strength be-

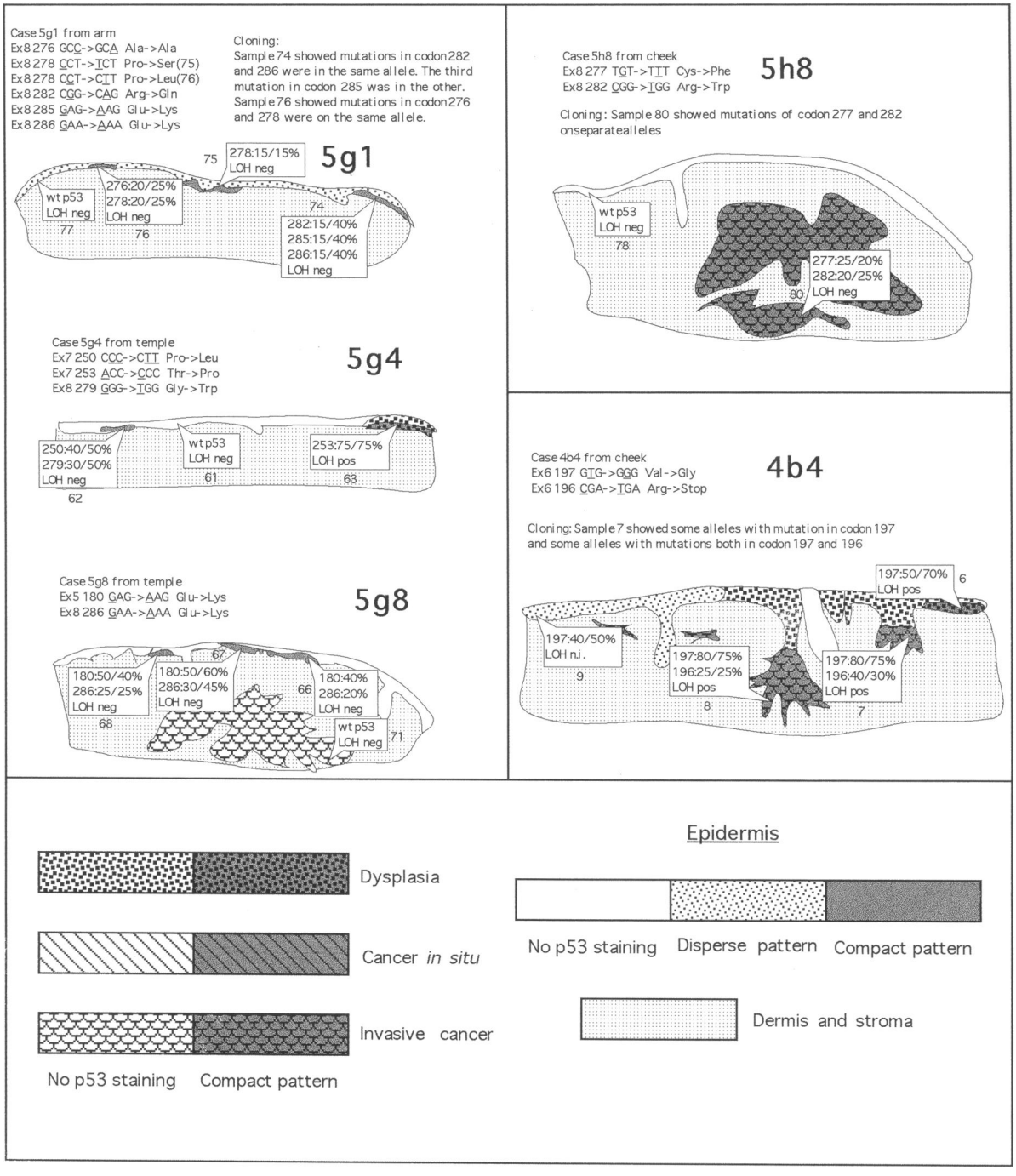

Figure 4. *See Figure 2 legend.*

tween a pair of adjacent mutated codons within the same exon in two samples from invasive cancer. Whereas the mutation signal magnitude of codon 197 was 70 to 80%, the mutation signal at codon 196 was only approximately one-half of this value. One probable interpretation is that the normal allele was deleted in the invasive cancer, leaving behind the allele containing the constitutive mutation. Thereafter, a mutation occurred at codon 196, which gave rise to a skewed pattern of reduction of nucleotide peak size. Vector cloning of this sample revealed that the tumor was heterogeneous, because some alleles contained a mutation in codon 197 only and other alleles contained mutations both in codons 196 and 197. Although illustrated in Figure 4, data from this case has been excluded from the rest of this report, because we have not obtained an op-

portunity to refute or confirm the putative germline mutation by analysis of, eg, blood lymphocytes. It is not excluded that the altered codon 197 (G*T*G→G*G*G; Val→Gly) instead represents a polymorphism, which in that case must be rare as it was only present in 4 of approximately 5000 tumors in the recent update of the p53 database.[16]

Morphologically Normal Skin (Except p53 Patches)

Fifteen samples did not show any mutations in exons 5 to 8 of p53, and LOH was negative in all informative cases. In contrast to our previous report where no p53 gene mutations were found outside of p53 patches, DPL, CIS, or SCC, samples from morphologically unremarkable epidermis of two patients (3f2 and 3g6) had a mutation in codons 278 and 224, respectively. These areas showed large numbers of immunoreactive p53 cells, which however, did not conform with our criterion for a clonal p53 patch but rather with a strong dispersed pattern (Figure 1).

p53 Patches

Ten p53 patches were sampled. In two of them, we separated the basal, strongly immunoreactive part and the superficial, negative part. Three separate samples were from one large p53 patch with lentigo (5g8). The remaining p53 patches were collected *in toto*. Two of ten p53 patches, including one sampled basally and superficially, were wt p53 and LOH negative. The remaining eight showed unique mutations, which were single in three, double in four, and triple in one p53 patch. None of the p53 patches had LOH. In one instance, the same two mutations were found superficially and basally (case 5a8).

Dysplasia

Using the same criterion as for the p53 patches, ie, to consider any lesion as a single clone if several samples had at least one mutation in common, we counted fifteen dysplasias in our material. This number was reduced to fourteen by assuming that two samples with wt p53 and no LOH in case 3g5 were from a single DPL. Eleven of fourteen DPLs had p53 gene mutations and/or LOH. Three DPLs were p53 wt and either had no LOH or were not informative.

In two DPLs (4c1 and 1g1), there was LOH together with a wt p53 allele. Six DPLs had either single (3f2, 3f2, 3f2, 1e7, and 1h1) or double (3g6) mutations without LOH. Two DPLs had one mutation and LOH (5g4 and 1e8).

Cancer in Situ *and Invasive Cancer*

Four CISs were examined. One (2c6) had double mutations and no LOH. The remaining three (4c1, 1h7, and 5a8) had one mutation and LOH. The mutations were different from case to case. 4c1 had an intronic mutation at the splice site after codon 125. 1h7 had an insertion of a T at codon 274, which caused a frame shift to a stop codon at 304/305. 5a8 had a hot spot missense mutation at codon 286.

Two SCCs were analyzed. In one of them, double mutations were found (5h8). The remaining SCC (5g8) had neither mutations nor LOH.

Genetic Relations between Benign p53 Patches (or Strong Dispersed Pattern of Immunoreactivity) and Squamous Cell Neoplasia (DPL, CIS, and SCC)

Six p53 patches co-existed with DPL, CIS, or SCC. In two of them (3g5 and 3d6), both p53 patch and DPL were wt p53 and LOH negative with no possibility to refute or confirm a genetic p53 link. Four p53 patches had different genotypes in the p53 patch and DPL/CIS. In case 5a8, there were even two p53 patches with individual genotypes and CIS with a third genotype (one mutation and LOH).

In no instance did a p53 patch and co-existing squamous neoplasia share a mutation.

In two instances (3f2 and 3g6), normal epidermis with mutations of the p53 gene and a strong dispersed pattern of immunoreactivity co-existed with dysplasia. In case 3g6, the mutations were different, but in case 3f2, there was an identical mutation of codon 278 in a focus of dysplasia and immediately bordering morphologically normal epidermis.

Mutations in Co-Existing DPL and CIS

In the only case with co-existing DPL and CIS (4c1), there was identity between the two lesions, which both showed LOH and an intronic mutation just downstream of exon 4 at the splice site.

Type of Mutations Compared between DPL/CIS/SCC and Clonal p53 Patches

Thirty-three mutations were found in p53 patches or DPL/CIS/SCC. By not taking the identical mutations in different lesions in case 4c1 into account, the true

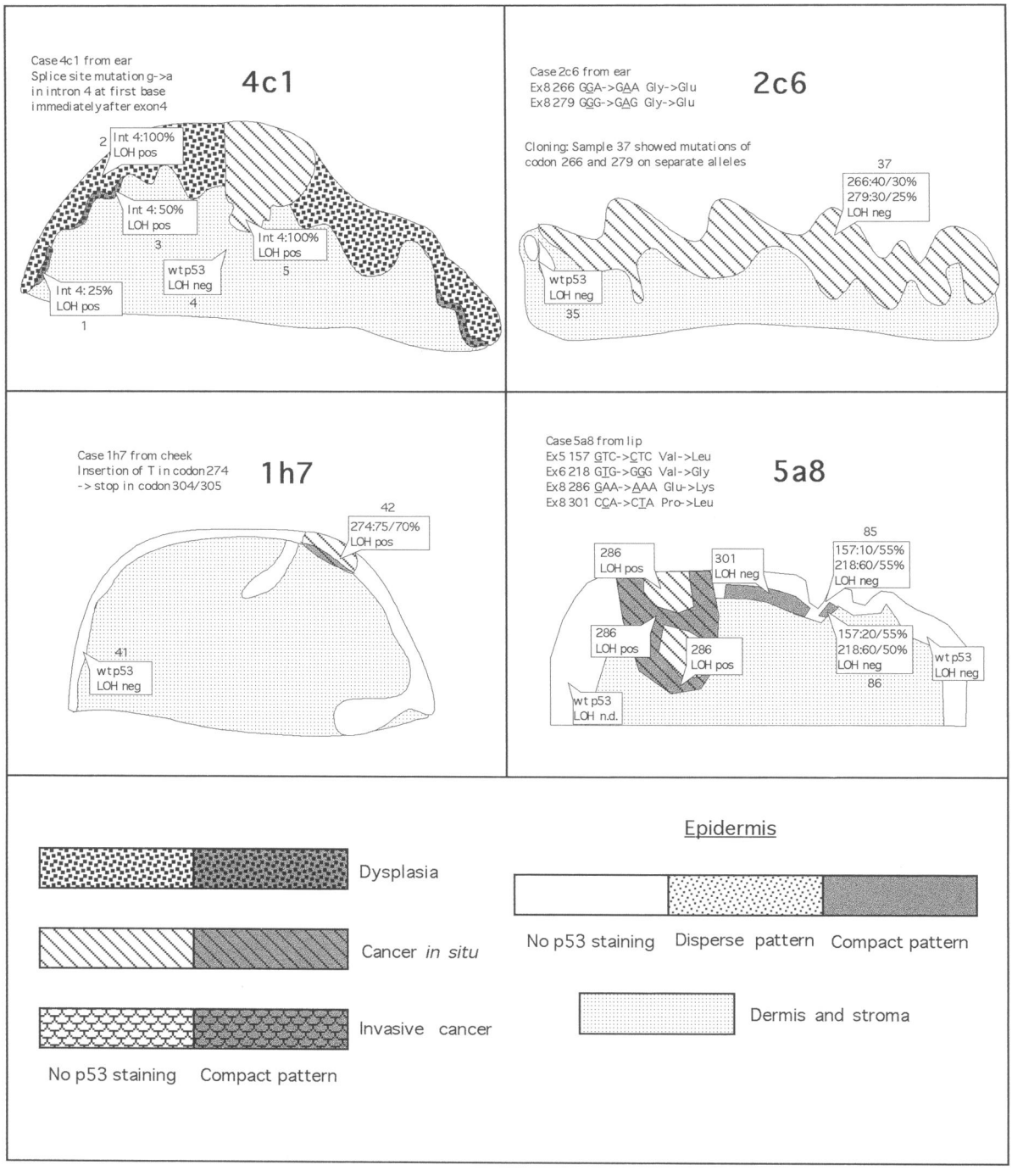

Figure 5. *Distribution of mutations in different codons of the p53 gene in exons 5 to 8 from samples (except case 4b4). The short bars at both ends of the abscissa represent intronic mutations of splice sites of codons 125 and 306.*

number of mutations was reduced to thirty-two. Twenty-five (78%) were in the domains conserved during evolution.[17,18] Twenty-nine (91%) were missense and three (9%) were nonsense mutations, which terminated transcription within the gene.

The distribution of hot spot mutations along sequenced parts of the p53 gene was similar between p53 patches on one hand and SCC and its precursors on the other (Figure 5).

Eleven of thirty-two (34%) of the mutations were C→T transitions at a dipyrimidine site. Six of eleven (54%) belonged to p53 patches. Five of fourteen (35%) p53 patch mutations were of a UV-specific type compared with five of seventeen (29%) in DPL/

CIS/SCC. No convincing difference among p53 patches, DPL, CIS, and SCC was thus established with respect to prevalence of UV-specific mutations.

LOH in p53 Patches and DPL, CIS, and SCC

LOH was not seen in any of 10 p53 patches but was present in 5 of 14 DPLs, 3 of 4 CISs, and 0 of 2 SCCs. Thus, there existed a clear difference between p53 patches and squamous cancer/precancer.

Cloning

Cloned PCR fragments from four samples (2c6, 5g1, 5h8, and 4b4) were analyzed. In one CIS (2c6), a codon 266 mutation was on one chromosome and a mutation of codon 279 on the other. In case 5g1, two samples were subjected to cloning. In one p53 patch, mutations in codons 282 and 286 were on the same chromosome and the third mutation in codon 285 on the other. In the second p53 patch, both mutations in exon 8 were on the same chromosome. In case 5h8 (SCC), a mutation in codon 277 was found on one chromosome and a mutation in codon 282 on the other. There was thus an opportunity in p53 patches as well as CIS/SCC to acquire mutations of both p53 alleles.

Discussion

The basic objective was to gain further insight into the biology of p53 patches of normal-looking epidermal cells. We could confirm that p53 patches are frequent companions of different types of squamous cell neoplasia but that genetic links that would support their status as early precursors of malignant neoplasia were still lacking. When current data are combined with previous results,[5] we have investigated six plus five p53 patches co-existing with dysplasia or *in situ* or invasive SCC without any evidence of genetic linkage. The issue is, however, not closed because morphological transitions between very slight dysplasia and p53 patches were observed in this study and more data are needed to entirely refute that p53 patches may develop into dysplasia. If such transition takes place, it will obviously be rare.

In 3f2, samples from an 87-year-old man with three dysplasias of different p53 genotype, there was evidence of a genetic p53 link between normal-looking epidermis and neighboring dysplasia. One of the dysplastic areas immediately bordering epidermis with an identical mutation and the strong dispersed pattern previously interpreted as reactive.[9] We have no certain explanation for this exceptional result. It is possible that dysplastic cells had invaded epidermis laterally to become intermingled with normal epidermal cells or that accidental contamination took place during microdissection.

In 3g6, mutations of normal epidermis were found in the absence of a p53 patch. We don't know how this has come about but suspect that the unusually strong dispersed pattern of p53 immunopositivity in 3g6 may, in fact, derive from the periphery of a p53 patch. In a separate study with extensive serial sectioning of p53-immunopositive p53 patches, we noted that some of them were, indeed, rimmed by a strong dispersed pattern but have never proved that these also contain p53 DNA mutations.[19]

Mutations of p53 have generally been regarded as strongly associated with cancer because of their high prevalence in most types of human malignancies.[20] This conjecture is obviously not true for skin where the most common lesion with a mutation in the p53 gene, instead, is a p53 patch of microscopically unremarkable epidermal cells evidently derived from a single epidermal stem cell. The same type of p53 patches were recently observed and also found to be most common in sun-exposed skin.[21] Our data from serial sections[19] and data from whole mounts of sun-exposed epidermis[21] indicated that as much as 35 to 40%, respectively, of normal skin surface contains keratinocytes with p53 gene mutations. The difference between the two estimates is not surprising given the uncertainty of the actual UV dose received and a host of other factors which tend to give rise to both over- and underestimates.[19]

Our data and other recently published data[21] begin to form a basis for a rough estimate of the prevalence of original clonogenic p53 mutations. The basic assumption is that the p53 patches are indeed clones derived from a single cell with a p53 mutation. This is supported by our present finding of identical mutations in multiple samples from the same p53 patch and the morphology of the p53 patches with sharp borders against surroundings.

The average number of p53-immunopositive patches per square centimeter was 35 in Jonason et al[21] and 40 in our own study.[19] DNA sequencing estimated 50 and 70% of the immunopositive patches to have p53 gene mutations, respectively. This similarity permits us to use an average figure of $0.6 \times 37.5 \approx 22/cm^2$ as a fairly good estimate of the average number of p53 patches (with mutations) in chronically sun-exposed human skin. This implies that at least 22 clonogenic cells/cm^2 carried a p53 mutation, which presumably gave them

the selective advantage necessary to form a p53 patch. We count 5.8×10^6 basal cells/cm^2 in our skin preparations. This implies that the fraction of basal cells carrying mutated p53 would be $22/5.8 \times 10^{-6} \approx 1$ per 3×10^5 cells/cm^2. The basal cell layer has, on uncertain grounds, been assumed to possess 1 clonogenic epidermal stem cell per 10 transit cells *en route* to terminal differentiation.[22] The result will then be that 1 in 30,000 epidermal stem cells have suffered a mutation of the p53 gene. This figure is almost certainly an underestimate because 1) p53 patches of smaller size than a few hundred cells are below the detection limit, 2) mutations outside of examined exons presumably exist, and 3) a proportion of mutations may either give rise to reversible p53 patches or no p53 patches at all. The important conclusion is that a substantial proportion of epidermal stem cells, presumed capable of malignant transformation, carry UV-induced mutations of the p53 gene in chronically sun-exposed skin.

The likelihood that a p53 patch will progress to dysplasia or squamous cell cancer is very small. We estimate that sun-exposed basal epidermis of the head and neck region has an area of approximately 1000 cm^2 and that one person in five contracts dysplasia. On a population basis, the minimal dysplasia/p53 patch ratio would then be of the order of 1/185,000. The subtleties of p53-driven clonal expansion was underscored by finding an identical mutation of a p53 patch not only in its basal immunopositive part but also in the overlying differentiating layer of keratinocytes. Consequently, mutations of the p53 gene do not have to leave any mark on execution of differentiation as revealed in the light microscope.

The observation of lentigo-like features of some p53 mutated areas may be coincidental. We note, however, that there could be a link between senile lentigo and p53 mutations. Senile lentigo is easily observed as brown spots, particularly on sun-exposed skin of practically every old fair-skinned person. Their prevalence in different age groups seems roughly identical to our analogous figures for p53 patches and they are just like the p53 patches common in xeroderma pigmentosum.[23] It would be interesting to know whether lentigo represents another form of clonal epidermal cell proliferation driven by a mutation controlling growth of stem cells.

At least three hypotheses would explain the situation in UV-exposed skin and nonmelanoma skin cancer with respect to mutations in the p53 gene. According to our first hypothesis, the basic effect of such a mutation is to enhance self-renewal among epidermal stem cells, thus permitting slow but infinite clonal expansion. Such an expansion is generally innocuous but may become important if accompanied by other yet to be discovered mutations in dysplasia or *in situ* or invasive SCC (but not in the p53 patches). Mutations of the p53 gene would then be necessary for clonal infinite growth of cancer and its precursors. There are, however, arguments against this hypothesis. First, dysplasias are apparently reversible if exposure to sun is discontinued.[24] p53 patches in mice have also been conjectured to be reversible.[25] One would not expect enhanced self-renewal of stem cells driven by a somatic cell mutation ever to revert. Second, nearly one-half of squamous neoplasias have no known alteration in p53 or its effector genes such as p21, leaving the behavior of a large proportion of the neoplasias unexplained. Third, if a p53 gene mutation is a necessary background to subsequent transforming somatic mutations, one would expect p53 patches to be common precursors of dysplasia or cancer, a conjecture not verified by our data.

A variant of this hypothesis says that mutated p53 may confer a selective advantage as long as the skin is exposed to UV, because it prevents apoptosis.[2] If exposure to the sun is interrupted, p53 patches, DPL, and CIS are expected to regress, whereas SCC will continue its growth because the malignant transformation overrides the effects of apoptosis.

A second hypothesis is that there are two classes of mutations, one mild that induces only clonal proliferation and one serious that, perhaps *via* genetic instability, promotes transforming mutations or in itself has additional effects on cellular growth control. It will be extremely hard to prove or disprove such a hypothesis. p53 has, partly as a transcription factor with capacity to bind to many promoters,[26] a pleomorphic function.[27] It also forms oligomers with wt[28] and mutated versions of p53 with possibilities of a complex net disturbance of cellular functions, particularly in cases where there is a mixture of mutated and nonmutated proteins.[29,30]

We have crudely assessed our second hypothesis by a comparison between p53 patches and neoplastic lesions. Sites of mutations were similar (Figure 5), and the mutations essentially conformed with published hot spots. Table 1 is a compilation of all our current data on the prevalence of p53 gene mutations. No differences seemed to exist between DPL, CIS, and SCC with respect to frequency, existence of multiple mutations, or combinations with LOH. When DPL, CIS, and SCC are combined and compared with the p53 patches, it may be seen that LOH was absent among 17 p53 patches but present in 11 of

Table 1. *Summary of Present and Published[5] Findings of the Distribution of p53 Mutations and LOH According to Diagnosis of Microdissected Samples**

	No LOH†			LOH	
	No mutation	1 mutation	>1 mutation	No mutation	1 mutation
Normal epidermis	27/29	2/29			
p53 patches	5/17	6/17	6/17		
Dysplasia	3/18	7/18	2/18	3/18	3/18
In situ cancer			2/5		3/5
Invasive cancer	1/7	3/7	1/7	1/7	1/7

*Except case 4b4.
†Including noninformative samples.

30 cases of DPL/CIS/SCC. We conclude that LOH is common in precancer/cancer, whereas it is absent in the p53 patches despite the fact that incidence and multitude of gene mutations did not show any significant difference between the two groups. Such a fact is superficially compatible with the hypothesis that initial disturbance of the p53 gene may determine the subsequent fate of resulting clonal proliferations. To render it more likely, it would be important to demonstrate that mutation(s) and LOH occur early and simultaneously.[4,31] As an alternative, we suggest that LOH may be part of a general chromosomal disturbance of DPL/CIS/SCC driven by some unknown factor unrelated to p53 and therefore randomly hitting also p53 alleles secondarily. This does not, of course, preclude that LOH at the p53 site will add severely to the malignant phenotype, only that LOH is not part of the initial damage and cannot, as a primary phenomenon, explain why cancers take a different path than p53 patches after a mutation of the p53 gene. We conclude that we have no strong evidence favoring the hypothesis that the nature of the initial damage to the p53 gene determines whether any given stem cell will develop into a p53 patch or DPL/CIS/SCC.

The third hypothesis is that mutations in p53 are neutral and irrelevant for carcinogenesis. They serve only as markers of degree of previous sun damage to the target cell for transformation or development of a p53 patch. This hypothesis is compatible with our vector cloning results, where secondary mutations were found both on the originally affected allele and the second allele. A multitude of mutations in the same gene would suggest that the gene is not essential for maintenance and selection of the prevalent phenotype. But the neutral p53 gene mutation hypothesis leads to serious problems, because it predicts that approximately every other stem cell capable of transformation to DPL/CIS/SCC would have a p53 gene mutation, as the prevalence of p53 gene mutations in DPL/CIS/SCC is approximately 50%. As there is no evidence that p53 is hypermutable, it follows that each other gene within stem cells would then also have a 50% chance of being mutated. Such an enormous general load of mutations seems unlikely.

The most reasonable conclusion based on current data is that mutations of p53 have important selective value by causing clonal proliferation of epidermal stem cells, which under obscure conditions, not primarily dependent on nature or type of primary p53 mutations but coupled to enhanced tendency for DNA breaks and deletion of genetic material, occasionally will be accompanied by malignant transformation, ie, the first hypothesis enumerated above. This conclusion is reinforced by the fact that the vast majority of the mutations are missense or nonsense and hardly ever concern the third base of a redundant codon, which statistically would be expected if no selective advantage of the mutated phenotypes existed.

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