# Mutation and Abnormal Expression of the p53 Gene in the Viral Skin Carcinogenesis of Epidermodysplasia Verruciformis

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**Patients suffering from epidermodysplasia verruciformis are prone to nonmelanoma skin cancers, due to an inherited abnormal susceptibility to the oncogenic human papillomavirus type 5. Genotoxic sunlight ultraviolet B radiations are likely to be a cofactor. Lesions of two human-papillomavirus-type-5-infected epidermodysplasia verruciformis patients collected during an 8 y period were retrospectively studied for p53 mutations in exons 5 through 8 by a polymerase chain reaction single-strand conformation polymorphism technique and/or by DNA sequencing of amplified exons. Mutations were detected in 11 of 26 (42.3%) specimens, including five (62.5%) squamous cell carcinomas, three (33.3%) Bowen's carcinomas *in situ,* two (40%) actinic keratoses, and one (33%) benign lesion. The nine mutations characterized by sequencing were shown to be missense and to affect mutational hotspots in human cancers. Five were C→T transitions at dicytidine sites considered as ultraviolet signature mutations. Two were transversions (C→G and C→A) at dicytidine sites and two were C→T transitions at nondipyrimidine sites. A marked p53 immunoreactivity was disclosed in 72.7% of 11 invasive carcinomas, 55.6% of nine carcinomas *in situ*, 37.5% of eight actinic keratoses, and one of three benign lesions. This includes 81.8% of 11 specimens with a p53 mutation but also 50% of 14 specimens with no mutation detected. A dysfunction of the p53 gene is thus likely to play a part in epidermodysplasia verruciformis carcinogenesis, either due to ultraviolet-B-induced p53 mutations, as in nonmelanoma skin cancers in the general population, or involving other mutagens or mechanisms. The part played by human papillomavirus type 5 proteins expressed in epidermodysplasia verruciformis keratinocytes remains to be determined.** ***Key words: genodermatosis/human papillomavirus type 5/immunohistochemistry/nonmelanoma skin cancer/PCR-SSCP. J Invest Dermatol 117:935–942, 2001***

Epidermodysplasia verruciformis (EV) is a rare genodermatosis associated with a high risk of nonmelanoma skin cancer (NMSC) that provides a model for studying interactions between potentially oncogenic human papillomaviruses (HPV) and sunlight in skin carcinogenesis (Orth, 1987; Majewski and Jablonska, 1995). EV results from a genetically determined abnormal susceptibility to infection with a specific group of related HPV genotypes (EV HPV), as well as to the oncogenic potential of some of them, especially HPV5 (Ramoz *et al*, 2000; Orth *et al*, 2001). Infection leads to the development of disseminated, persistent flat wart-like and macular lesions, usually starting in early childhood. Upon their third or fourth decade, about half of EV patients start developing multiple precancerous lesions of the skin [actinic keratosis (AK), Bowen's carcinoma *in situ* (CIS)], which evolve into NMSC [mostly squamous cell carcinomas (SCC)]. EV carcinomas harbor high copy numbers of episomal HPV genomes (usually HPV5 or occasionally HPV8, 14, 17, 20, or 47) and abundant transcripts of the E6 and E7 open reading frames (Orth, 1987). Phenocopies of EV are exceptionally observed in immunosuppressed patients. EV HPV cause widespread inapparent infections in the general population (Boxman *et al*, 1997; Antonsson *et al*, 2000) but their role in skin carcinogenesis in non-EV patients remains to be demonstrated (Kawashima *et al*, 1990; Pfister and ter Schegget, 1997).

The viral and cellular molecular mechanisms leading to the early development of skin malignancies in EV patients are still poorly understood. By analogy with the development of HPV16- and HPV18-associated anogenital carcinomas, the viral E6 and E7 proteins are likely to play a major part in tumor progression, by targeting cellular proteins that negatively regulate the cell cycle (Howley, 1996). HPV5 and HPV8 E7 proteins have been shown to associate with retinoblastoma (Rb) protein, though with reduced binding affinities compared to HPV16 and HPV18 E7 proteins (Yamashita *et al*, 1993; Schmitt *et al*, 1994). E2F transcription factors, the key regulators of cell DNA replication, may thus be released from their inactive Rb-bound forms (Hiraiwa *et al*, 1996). In contrast to HPV16 and HPV18, the E6 proteins of oncogenic EV HPV do not possess the ability to interact with p53 protein and ubiquitin-protein ligase E6-AP, which would promote p53 degradation, and to inhibit p53-mediated transactivation of cellular genes (Steger and Pfister, 1992; Kiyono *et al*, 1994; Elbel *et al*,

Manuscript received December 11, 2000; revised June 25, 2001; accepted for publication July 11, 2001.

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Abbreviations EV, epidermodysplasia verruciformis; NMSC, nonmelanoma skin cancer; Rb, retinoblastoma; SSCP, single-strand conformation polymorphism.

0022-202X/01/$15.00 · 

**Table I. Summarized data on EV tumor specimens and p53 status**

| Patient, specimen | Year of sampling | Site | Histologic diagnosis[a] | HPV type[b] | p53 exon 5–8[c] | p53 staining[d] |
|---|---|---|---|---|---|---|
| A, a | 1986 | forearm | ak | 5,8 | M7 | 2 + |
| A, b | | leg | ak | 9 | N | 3 + |
| A, c | | back | *cis* | 5,9 | M5 (8) | 3 + |
| A, d | | back | *cis* | 9 | N | 2 + |
| A, e | | thorax | ak | 5,9,36 | N (5) | – |
| A, f | | hand | *cis* | 5,9,X | M7; *M7* | + |
| A, g | | hand | *cis* | 5,8,9 | M6 (5) | + |
| A, h | | forehead | *cis* | 9,X | N | – |
| A, i | 1988 | abdomen | bl | 9,36 | M5 | 2 + |
| A, j | | finger | ak | 36 | nc (6, 8) | + |
| A, k | | forearm | µscc | 5 | M8 (6) | 2 + |
| A, l | | forearm | µscc | 5,X | N | + |
| A, m | | arm | µscc | 5,9 | M7 | 3 + |
| A, n | | forearm | SCC | 5,8,9,36 | N | 3 + |
| A, o | | wrist | ak | 5,9,X | nc (5–8) | + |
| A, p | | ear | BCC | 5,8,36 | N | 2 + |
| A, q | | hand | *cis* | 5,9,X | N | + |
| A, r | | hand | µscc | 5,9,36 | nc (5, 6, 8) | + |
| A, s | 1991 | thorax | ak | 9,X | nc (5, 6) | – |
| A, t | | thorax | bl | 9,X | N (6) | – |
| A, u | 1994 | hand | ak | 9 | N | + |
| A, v | | neck | *cis* | 5,9 | N | 3 + |
| B, a | 1986 | forehead | ak | nc | nc (7, 8); *M7* | 3 + |
| B, b | | forehead | µscc | 5 | M5 | 3 + |
| B, c | 1987 | back | µscc | 5 | nc (6, 8) | nc |
| B, d | 1988 | neck | SCC | 5 | nc (5, 8) | 3 + |
| B, e | | neck | SCC | 5 | nc (5–8); *M5* | 3 + |
| B, f | 1989 | forehead | SCC | 5 | nc (5,6,8); *M7* | 2 + |
| B, g | 1990 | back | *cis* | 5 | nc (5,7); *N* | 3 + |
| B, h | 1993 | forehead | SCC | 5 | N | – |
| B, i | 1994 | back | *cis* | 5 | N (8); *N* | 3 + |
| B, j | | back | bl | 5,49 | N (5) | nc |

[a]As defined by the most severe histologic change: µscc, microinvasive SCC; *cis*, Bowen's CIS; ak, AK; bl, benign lesion.
[b]As determined by Southern blot hybridization experiments. X, unidentified EV HPV related type; nc, not conclusive because of DNA degradation.
[c]As analyzed by PCR-SSCP. Numbers indicate mutated exons or, in parentheses, nonconclusive exons. N, normal for at least three exons; M, mutation; nc, nonconclusive. In italics, data obtained from microdissected tissue sections.
[d]p53 immunoreactivity was scored as (–) for less than 1%, (+) for 1%–30%, (2+) for 30%–50%, and (3+) for 50%–100% stained, lesional immature epidermal cells

1997). Strikingly, expression of the HPV5 E6 protein has recently been reported to inhibit ultraviolet (UV) radiation-induced G1 arrest and apoptosis, in spite of increased levels of transcriptionally active p53 protein (Jackson and Storey, 2000).

Malignant transformation of EV lesions usually occurs on sun-exposed areas of the skin, which suggests that UV radiation may act as a cofactor in the carcinogenesis process, through its mutagenic and immunosuppressive effects (Orth, 1987; Majewski and Jablonska, 1995). Sunlight, mainly its highly genotoxic UVB component, represents the major environmental factor associated with NMSC in the general population (Brash, 1997). It is well established that the p53 gene is a major target of UV-induced mutations and that inactivation of this gene is a common event in the process of skin carcinogenesis in the general population (Dumaz *et al*, 1994; Nataraj *et al*, 1995; Ren *et al*, 1996; Brash, 1997). p53 mutations have been detected in more than half of the cases of AK, Bowen's disease, and NMSC. About two-thirds of the mutations are C→T or, less frequently, CC→TT transitions that result from unrepaired cyclobutane pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts (Brash *et al*, 1991; Molès *et al*, 1993; Ziegler *et al*, 1993; 1994). In response to DNA-damaging agents, p53 protein has been shown to activate G1 and G2 cell cycle checkpoints, to participate in nucleotide excision repair, and to evoke apoptotic processes (Levine, 1997; Amundson *et al*, 1998). Dysfunction of the p53 protein, through impaired sequence-specific transactivation or protein–protein interactions, results in genomic instability and is thus likely to play a crucial role in skin carcinogenesis, as it does in more than 50% of all human malignancies (Greenblatt *et al*, 1994).

As there is no evidence for an EV-HPV-induced inactivation of the p53 protein, our aim was to analyze the status of the p53 gene at various stages of the EV carcinogenesis process. We took advantage of a collection of DNA preparations and paraffin-embedded tissue specimens from EV lesions taken over an 8 y period from two patients suffering from this very rare disease. We searched for mutations in the p53 gene or its abnormal expression and for over-expression of the Bcl-2 protein, which is able to block p53-dependent apoptosis (White, 1996; Amundson *et al*, 1998). We wanted to determine at which step of the tumor progression these events could occur, and whether the p53 mutation pattern fitted that of UV-induced mutations in NMSC of the general population.

## MATERIALS AND METHODS

**Patients** Two patients of Polish origin, a woman born in 1940 with a familial form of EV (patient A) and a man born in 1935 (patient B), were included in the study. Both patients had almost generalized typical benign flat wart-like and pityriasis versicolor-like lesions and reddish plaques, which started to appear at the age of 5 y. Patients constantly developed multiple, locally destructive malignant changes on the forehead and other sun-exposed areas of the skin, from the age of 29 y (patient A) or 39 y (patient B) to their death at the age of 56 y and 60 y, respectively. Since 1977, repeated biopsy specimens from both patients were analyzed by Southern blot hybridization of *Hind*II- or *Pst*I-restricted tumor DNA using DNA probes specific for various HPV

genotypes associated with skin warts and EV (Orth, 1987; Obalek *et al*, 1992). Patient A was found to be infected with 11 HPV genotypes (HPV types 3, 5, 8, 9, 14, 17, 19, 20, 22, 24, 36), and patient B with five HPV genotypes (HPV types 3, 5, 22, 36, 49). The detection of HPV5 DNA in an SCC specimen of patient B was the first demonstration of the association of HPV5 with EV cancers (Orth *et al*, 1980). Molecular cloning and characterization of the genomes of the HPV5 isolate of patient B and the HPV9 isolate of patient A, and the identification of HPV5 variants 5aE3 in patient A and 5aE1 in patient B, have been reported previously (Kremsdorf *et al*, 1982; Kawase *et al*, 1996).

**Polymerase chain reaction (PCR) amplification and single-strand conformation polymorphism (SSCP) analysis** Thirty-two archival total DNA preparations obtained from biopsy specimens of lesions at different stages of tumor progression (**Table I**), and stored at –20°C, were selected because of the availability of paraffin-embedded samples for histologic, *in situ* hybridization, and immunohistochemical studies. PCR amplification of exons 5 through 8 of the p53 gene was performed using primers described previously (Ahomadegbe *et al*, 1995). PCR was carried out with 100 ng of genomic DNA, 0.3 pmol of each primer, 5 μM of each deoxynucleotide triphosphate, 2 μCi of $\alpha[^{33}P]$-dCTP (Amersham; 3000 Ci per mmol), 1 unit of Taq polymerase (Perkin Elmer/Cetus), 2 μl 10 × PCR buffer (Perkin Elmer/Cetus), and 1.5 mM $MgCl_2$, in a final volume of 20 μl. The reaction mixture was heated to 94°C for 5 min and amplification was performed with 30 cycles of 94°C for 30 s, 58°C for 60 s, 72°C for 60 s, followed by a final extension at 72°C for 5 min, using an automated thermocycler (Perkin Elmer/Cetus). SSCP analysis was performed using the technique originally described by Orita *et al* (1989). Amplification products (2 μl) were diluted 10-fold in 0.1% sodium dodecyl sulfate with 10 mM ethylenediamine tetraacetic acid (EDTA), rediluted 1:2 in gel loading solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.5% xylene cyanol), heat-denatured in boiling water for 2.5 min, chilled on ice, and loaded on a nondenaturing polyacrylamide gel (0.5 × Hydrolink MDE gel, Bioprobe System, in 0.5 × TBE with 10% glycerol). Electrophoresis was performed at 50 W for 3 h or at 10 W for 13–15 h at room temperature. Dried gels were exposed to Hyperfilm β max (Amersham) at –70°C. DNAs from human cancer cell lines known to contain mutations at positions corresponding to amino acids 156, 194, 245, and 273 of the p53 protein were used as positive controls of band shifts. For each sample, at least two independent PCR-SSCP analyses were carried out. For 10 of the 32 specimens, PCR amplification was also performed in the absence of $\alpha[^{33}P]$-dCTP using a 4-fold higher concentration of primers. Heat-denatured amplification products were electophoresed through 12.5% polyacrylamide Genegel Excel gels using a Genephor apparatus (Pharmacia, Biotech). Running conditions were 600 V, 25 mA, 6 W, for 2 h at 18°C. The bands were visualized using the PlusOne DNA silver staining kit (Pharmacia, Biotech).

**PCR amplification from microdissected tumor sections** Sections 5 μm thick were prepared from six paraffin-embedded specimens (A, f; B, a; B, e; B, f; B, g; B, i), changing the knife for each specimen. Ten sections were mounted on slides, deparaffinized, and lightly stained with hematoxylin, and tumor tissue was dissected with a small scalpel under a Zeiss stereomicroscope. Samples were digested in 150 μl of 50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.5% Tween-20, in the presence of 50 μg of proteinase K (Eurobio, Paris, France), for 24 h at 37°C, and heated at 95°C for 10 min. Aliquots of 10 μl were used for each PCR. Exons 5–8 of the p53 gene were amplified by a seminested PCR. The primers used in the first step PCR were as above, except for the exon 5 antisense primer (5′-CAACCAGCCCTGTCGTCTCTC-3′). Internal primers used in the second step PCR were: 5′-ATCTGTTCACTTGTGCC-CTG-3′ (exon 5, sense), 5′-GGGTCCCCAGGCCTCTGATT-3′ (exon 6, sense), 5′-CACAGCAGGCCAGTGTGCAG-3′ (exon 7, antisense), and 5′-GCATAACTGCACCCTTGGTC-3′ (exon 8, antisense).

The reaction mixtures in AmpliTaq Gold buffer (Perkin Elmer-Roche) contained 2.5 mM $MgCl_2$, 200 μM dNTPs, 15 μM or 50 μM of each primer, and 1 U or 2.5 U of AmpliTaq Gold DNA polymerase (Perkin Elmer-Roche) for the first or second step PCR, respectively. For the first PCR, DNA was amplified for 45 cycles (1 min at 95°C, 1 min at 55°C, 2 min at 72°C) with a final extension of 5 min at 72°C. For the second PCR, 6 μl of the first step PCR products were used as template and amplified for 35 cycles (30 s at 95°C, 30 s at 55°C, 1 min at 72°C). Seminested PCR products were separated by electrophoresis in a 3% NuSieve GTG (BMA, Rockland, ME) agarose gel and purified using the NucleoSpin Extract 2 in 1 kit (Macherey-Nagel, Düren, Germany).

**DNA sequencing** The shifted bands were cut out from SSCP gels and eluted in distilled water (100 μl) at 4°C overnight and 65°C for 2 h. For two SSCP-positive DNA preparations (specimens A, a, and A, m), the amplification products were cloned into p-Bluescript II phagemid (Stratagene) before sequencing. Eluted and cloned DNAs were amplified with the Expand High Fidelity PCR System (Boehringer, Mannheim) using the primers described for PCR-SSCP (Ahomadegbe *et al*, 1995) and containing universal forward or reverse M13 sequences added to their 5′ ends to allow direct sequencing. Amplification products were sequenced on both strands by the dideoxynucleotide termination method, using fluorescent forward and reverse M13 primers and the ABI Prism Dye-Primer Cycle Sequencing Kit (PE Applied Biosystems) under the conditions described by the manufacturer. Purified seminested PCR products were sequenced using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Sequence products were analyzed with an ABI Prism 377 DNA sequencer (Perkin Elmer).

**Immunohistochemistry** A part of each biopsy specimen was fixed in 10% neutral formaldehyde and/or in Carnoy's fluid (chloroform 30%, acetic acid 10%, absolute ethanol 60%), and then embedded in paraffin. Five micron sections mounted on poly-L-lysine slides were submitted to a microwave treatment three times for 5 min in 10 mM citrate buffer, pH 6, at 750 W. Endogenous peroxidase activity was quenched by an 8 min incubation in 3% hydrogen peroxide. After a 20 min preincubation in 5% normal horse serum, two mouse monoclonal antibodies were applied as primary antibodies: anti-p53 DO7 (Dako, Glostrup, Denmark), 1:50 dilution, 60 min at 37°C, or anti-Bcl-2 clone 124 (Dako), 1:60 dilution, overnight at 4°C. Sections were then incubated for 30 min with a biotinylated rabbit antimouse antibody (Dako) at a 1:300 dilution, and processed with a streptavidin-biotin-peroxidase detection system (Dako) as recommended by the manufacturer, using diaminobenzidine as the chromogen. Counterstaining was performed with hematoxylin. Sections from a breast, an ovary, and a nasopharyngeal carcinoma known to express aberrant p53 protein and from a breast and a tonsil carcinoma known to express high levels of Bcl-2 protein were used as positive controls. Negative controls were by omission of the primary antibody.

***In situ* detection of HPV DNA** *In situ* hybridization was done on biopsy specimens found to contain a mutated p53 gene. Tritiated DNA probes (specific activity 3–8 × $10^6$ cpm per μg) were prepared by nick-translation of purified, excised HPV type 5, 8, 9, or 36 DNAs, and were used at a concentration of 0.2 μg per ml after denaturation at 95°C for 3 min. Processing of formalin-fixed and Carnoy's fluid-fixed tissues, hybridization, washings, and autoradiographic hybrid detection were performed as described previously (Bergeron *et al*, 1992). Sections were counterstained with hematoxylin.

## RESULTS

**P53 mutations are detected at different stages of tumor progression in EV** PCR-SSCP analysis of exons 5 through 8 of p53 was performed on archival DNA preparations obtained from 32 biopsy specimens. These were taken from two EV patients over an 8 y period, 39–47 y (patient A) or 44–52 y (patient B) after the onset of the disease (**Table I**). Most biopsies contained more than one (up to three) histopathologic entities**.** The majority (77.3%) of the specimens from patient A had previously been found to contain more than one EV HPV genotype by Southern blot hybridization, with HPV5 being detected in 11 of 13 (84.6%) *in situ* and invasive carcinomas. HPV5 had been found in all specimens of patient B (**Table I**). Conclusive results, i.e., band patterns with sufficient labeling intensity to allow interpretation, were obtained for 87.5% (exon 7), 71.9% (exon 6), and 68.7% (exons 5 and 8) of the 32 specimens (**Table I**). Bands showing a mobility shift compared with wild-type p53 were observed for eight of the 22 samples that showed conclusive PCR-SSCP results for at least three exons (**Fig 1**)**.** There was no evidence for a loss of the normal allele as wild-type and mutant bands were codetected, with the shifted bands showing a weaker labeling intensity in most instances (**Fig 1**).

Of the 10 specimens with conclusive PCR-SSCP results for less than three exons, four could be further analyzed by direct sequencing of PCR products of DNA extracted from micro-dissected sections of paraffin-embedded tissues. Mutations were

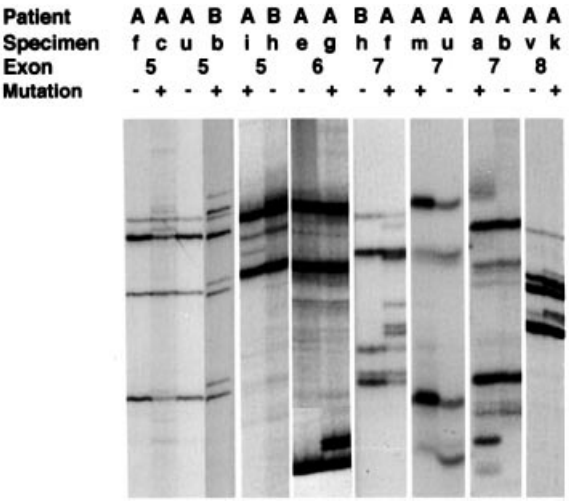


**Figure 1. SSCP analysis of amplified p53 exons from EV biopsy specimens**. Exons 5 through 8 were PCR amplified in the presence of [α33P]-dCTP. PCR products were heat-denatured and single-stranded DNA molecules were fractionated on nondenaturing polyacrylamide gels and detected by autoradiography as described in *Materials and Methods*. Capital and lower case letters given on the top of the lanes correspond to the patients and specimens described in **Table I**. Aberrant p53 DNA migration patterns (+) show a band or bands with altered migration compared with the wild-type p53 DNA migration patterns (–).

identified in three of them (**Table I**). Two specimens found to harbor a mutation (A, f) or to be normal (B, i) by PCR-SSCP analysis were taken as controls in this experiment (**Table I**).

On the whole, mutation rates were 15.4% for exon 5, 4% for exon 6, 16.1% for exon 7, and 3.8% for exon 8. Mutations were detected in seven (38.9%) of 18 conclusive specimens from patient A and four (50%) of eight specimens from patient B. Taking into account the most severe histopathologic change, mutations were detected in one (33%) specimen with features of benign lesion, two (40%) AK, three (33.3%) Bowen's CIS, and five (62.5%) SCC. Evidence for vegetative HPV5 DNA replication was obtained for seven of the nine specimens of premalignant or invasive lesions showing a mutation, as tested by *in situ* hybridization (**Fig 2**). HPV8 replication was detected in a benign lesion adjacent to an HPV5-positive AK. Replication of HPV9 or HPV36 was observed in the two lesions found within the mutation-positive specimen with benign features (**Table II**). Ten of the specimens yielding a mutation were taken from sun-exposed or occasionally sun-exposed skin (**Table I**).

**P53 mutations in EV are mutational hotspots in human cancers** Nine mutations were identified by sequencing PCR products obtained from microdissected tissue sections (three specimens) or from shifted bands eluted from the polyacrylamide gels of SSCP analysis (six specimens) (**Table II**). The nine mutations were single-nucleotide missense substitutions, and all mutations affected GC base pairs (**Table II**). The same mutations were detected after direct sequencing of two PCR amplification products or after sequencing several recombinant plasmids. Six of the nine mutated cytosine residues were located on the transcribed strand. Seven mutations were observed at sites of adjacent cytosines. Five of them corresponded to a C→T transition, fitting with the UVB fingerprint p53 mutations found in the majority of NMSC in the general population (Dumaz *et al*, 1994; Nataraj *et al*, 1995; Brash, 1997). The two others involved C→G and C→A transversions. Two mutations did not involve a dipyrimidine site or CpG sequences and were G:C→A:T transitions (**Table II**). Most mutated amino acid residues (codons 130, 135, 173, 248, 249, 258) were located in the conserved regions II–V of the DNA-binding core domain of the p53 protein. All affected codons were located at mutational hotspots found in human cancers (Walker *et al*, 1999). Only two (codon 248 found mutated twice and codon 258) corresponded to mutation hotspots reported previously for NMSC (Ziegler *et al*, 1993).

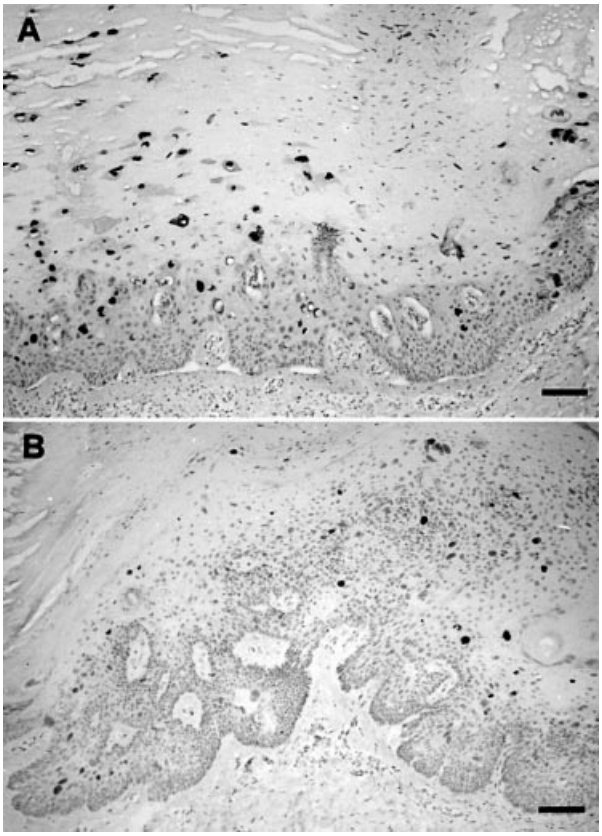


**Figure 2. *In situ* detection of HPV5 DNA in lesions with a mutated p53 gene.** Sections of an AK (*A*; specimen A, a) and a CIS (*B*; specimen A, f) were hybridized with a tritiated HPV5 DNA probe. Hybrids are detected in the nuclei of upper differentiating cells, where vegetative viral DNA replication occurs (Orth *et al*, 1980), and in differentiated cells. *Scale bar*: 100 μm.

**The p53 protein is aberrantly expressed in EV lesions** Immunoreactive p53 protein was detected in 83.3% of the biopsy specimens, using a monoclonal antibody (DO7) recognizing both wild-type and mutated p53 proteins (**Table I**). Nuclear staining involving 30%–50% (2+) or 50%–100% (3+) of the immature lesional keratinocytes was observed in 72.7% of the specimens diagnosed as microinvasive or invasive carcinoma, 55.6% of the CIS, 37.5% of the AK, and one of three benign lesions (**Table III, Fig 3**). Overall, this corresponds to 45.5% and 87.5% of the specimens collected from patients A and B, respectively. Terminally differentiating keratinocytes were variably stained. No staining or only few scattered positive nuclei were observed in the normal epidermis, except for two specimens (B, d, and B, e) that showed a compact staining pattern, either restricted to the basal layer or extending throughout the apparently normal epidermis adjacent to an immunoreactive SCC (data not shown). A marked (2+ or 3+) p53 staining of the immature keratinocytes was observed in nine (81.8%) of the 11 specimens with a p53 mutation (**Fig 3*A*, *C*, *D***), but also in seven (50%) of 14 specimens for which no mutation could be detected (**Fig 3*B*, *F***) and one (20%) of five specimens for which the PCR-SSCP analysis was not conclusive (**Fig 3*E***). When a biopsy contained more than one lesion, the same level of immunostaining was often observed for adjacent lesions, which most probably represent different stages of the tumor progression (AK and CIS or CIS and microinvasive SCC) (**Table II**).

**Table II. p53 mutations in EV lesions**

| Patient, specimen[a] | p53 exon | Codon | Sequence[c] | Base change | Amino acid change | *In situ* detection | |
|---|---|---|---|---|---|---|---|
| | | | | | | p53 protein[d] | HPV DNA[e] |
| B, e | 5 | 130 | cCtc | C:G→T:A | Leu→Phe | *cis*: 3+ SCC: 3+ | 5 |
| B, b | | 135 | tGc | G:C→A:T | Cys→Tyr | μscc, *cis*: 3+ ak: 2+ | – |
| A, c | | 173 | tGtg | G:C→A:T | Val→Met | *cis*: 3+ | 5 |
| A, i | | nd[b] | nd | nd | nd | bl: 2+; bl-; ns: – | 36, 9 |
| A, g | 6 | nd | nd | nd | nd | *cis*: –; ak: +; ns: – | 5 |
| B, f | 7 | 232 | cCac | C:G→T:A | His→Tyr | SCC, 3+ | nd |
| A, a | | 248 | cCgg | C:G→T:A | Arg→Trp | ak: 2+; bl: + | 5, 8 |
| B, a | | 248 | cGg | G:C→A:T | Arg→Gln | ak: 3+ | – |
| A, m | | 249 | aGg | G:C→C:G | Arg→Thr | μscc, *cis*: 3+; ak, ns:– | 5 |
| A, f | | 258 | gGaa | G:C→A:T | Glu→Lys | *cis*, ak: + | 5 |
| A, k | 8 | 266 | gGa | G:C→T:A | Gly→Val | μscc: 2+ | 5 |

[a]Described in **Table I**.
[b]nd, not determined; sequencing not done after PCR-SSPC positive results.
[c]The sequence is written 5′→3′ for the coding strand. The capital letter indicates the mutated base.
[d]ak, AK; bl, benign lesion; *cis*, CIS; μscc, microinvasive SCC; ns, normal skin. p53 immunoreactivity was scored as indicated in legend to **Table I**.
[e]Viral DNA replication as detected by *in situ* hybridization. The HPV36-associated benign lesion was p53-positive. The negative specimens had been found to be HPV5-positive by Southern blot hybridization (**Table I**).

**Table III. Immunoreactivity of p53 protein in EV tumor specimens**

| Histology[a] | No. of specimens | p53 staining[b] | | | |
|---|---|---|---|---|---|
| | | – | + | 2+ | 3+ |
| Basal cell carcinoma | 1 | 0 | 0 | 1 | 0 |
| Squamous cell carcinoma | 10 | 1 | 2 | 2 | 5 |
| Carcinoma *in situ* | 9 | 1 | 3 | 1 | 4 |
| Actinic keratosis | 8 | 2 | 3 | 1 | 2 |
| Benign lesion | 3 | 2 | 0 | 1 | 0 |

[a]Most severe histologic change.
[b]Two specimens (one benign lesion, one microinvasive carcinoma) were non-conclusive. One specimen contained two benign lesions. – corresponds to less than 1%, and +, 2+, and 3+ to 1%–30%, 30%–50%, and 50%–100% stained immature lesional epidermal cells, respectively.

**The Bcl-2 protein is not expressed in EV SCC and their precursor lesions** As half of EV lesions for which there was no evidence for p53 mutations showed high p53 protein immunoreactivity, we analyzed all specimens showing features of AK, CIS, or NMSC for antiapoptotic Bcl-2 protein expression, which is able to block p53-dependent apoptosis (White, 1996; Amundson *et al*, 1998). High levels of cytoplasmic Bcl-2 protein were detected in the only specimen of basal cell carcinoma (BCC), and immunostaining of consecutive sections showed that Bcl-2 and p53 proteins were congruently expressed (**Fig 3*F*, *G***). No p53 mutation was detected in this tumor (**Table I**). In contrast, no immunohistochemical evidence for the expression of Bcl-2 was obtained for the other EV preinvasive or invasive lesions tested.

## DISCUSSION

Inactivation of the p53 gene resulting from UVB-induced mutations is a major event in skin carcinogenesis in the general population (Brash, 1997). In contrast, p53 mutations are seldom found in genital cancers (Park *et al*, 1994). Abrogation of the function of p53 protein through its interaction with the viral E6 oncoprotein, however, plays a major part in the carcinogenesis associated with genital HPV genotypes (Howley, 1996). Patients suffering from EV are at high risk of NMSC due to their genetically determined predisposition to infection with the oncogenic HPV5. Analyses of the p53 status in EV carcinogenesis have been hampered by the rarity of the disease. To address the question, we took advantage of the availability of a collection of total DNA and paraffin-embedded tissues prepared from lesion specimens from two HPV5-infected EV patients taken over an 8 y period. The data presented here support the hypothesis of a dysfunction of the p53 gene in EV tumors, and provide clues concerning the mechanisms that could contribute to the development of NMSC in EV patients.

Mutations in the p53 gene were detected at different stages of tumor progression, i.e., in one (33.3%) of three benign lesions, two (40%) of five AK, three (33.3%) of nine Bowen's CIS, and five (62.5%) of nine SCC. HPV5 replication, as detected by *in situ* hybridization, was observed in most preinvasive and invasive lesions harboring a mutation in the p53 gene. Mutation rates were within the range reported for premalignant lesions of the skin and NMSC in the general population (Brash *et al*, 1991; Molès *et al*, 1993; Ziegler *et al*, 1993; 1994; Nataraj *et al*, 1995) and in immunosuppressed patients (McGregor *et al*, 1997). It is likely that mutation rates were underestimated because our study involved only exons 5–8, which encode the DNA-binding core domain of the p53 protein. Moreover, the occurrence of several types of lesions within most specimens along with the presence of normal tissue must have decreased the sensitivity of detection of mutated alleles and precluded the disclosure of loss of the normal allele in SSCP patterns with band shifts. The nine mutations identified by sequencing are all missense, affect highly conserved amino acid residues essential for p53 structure and function, and correspond to mutational hotspots found in human cancers (Walker *et al*, 1999). For example, arginine 248, which was changed to glutamine or tryptophan in two AK, plays a crucial role in sequence-specific DNA binding of the p53 protein (Cho *et al*, 1994). Thus, p53 mutations are likely to play a role in EV carcinogenesis, by counteracting the tumor-suppressive apoptotic and growth-arresting responses to various p53-activating stresses encountered during tumor progression (Evan and Vousden, 2001).

Further support for a role of a dysfunction of the p53 tumor suppressor gene in EV carcinogenesis is provided by the marked p53 immunoreactivity observed in 82% of the lesions with a mutation and in 50% of the specimens with no detected mutation. A p53 immunostaining had previously been reported for EV warts showing variable degrees of atypia in the absence of detected p53 mutation (Pizarro *et al*, 1995). This contrasts with the lack of p53 staining in warts of non-EV patients (McGregor *et al*, 1994; Pizarro

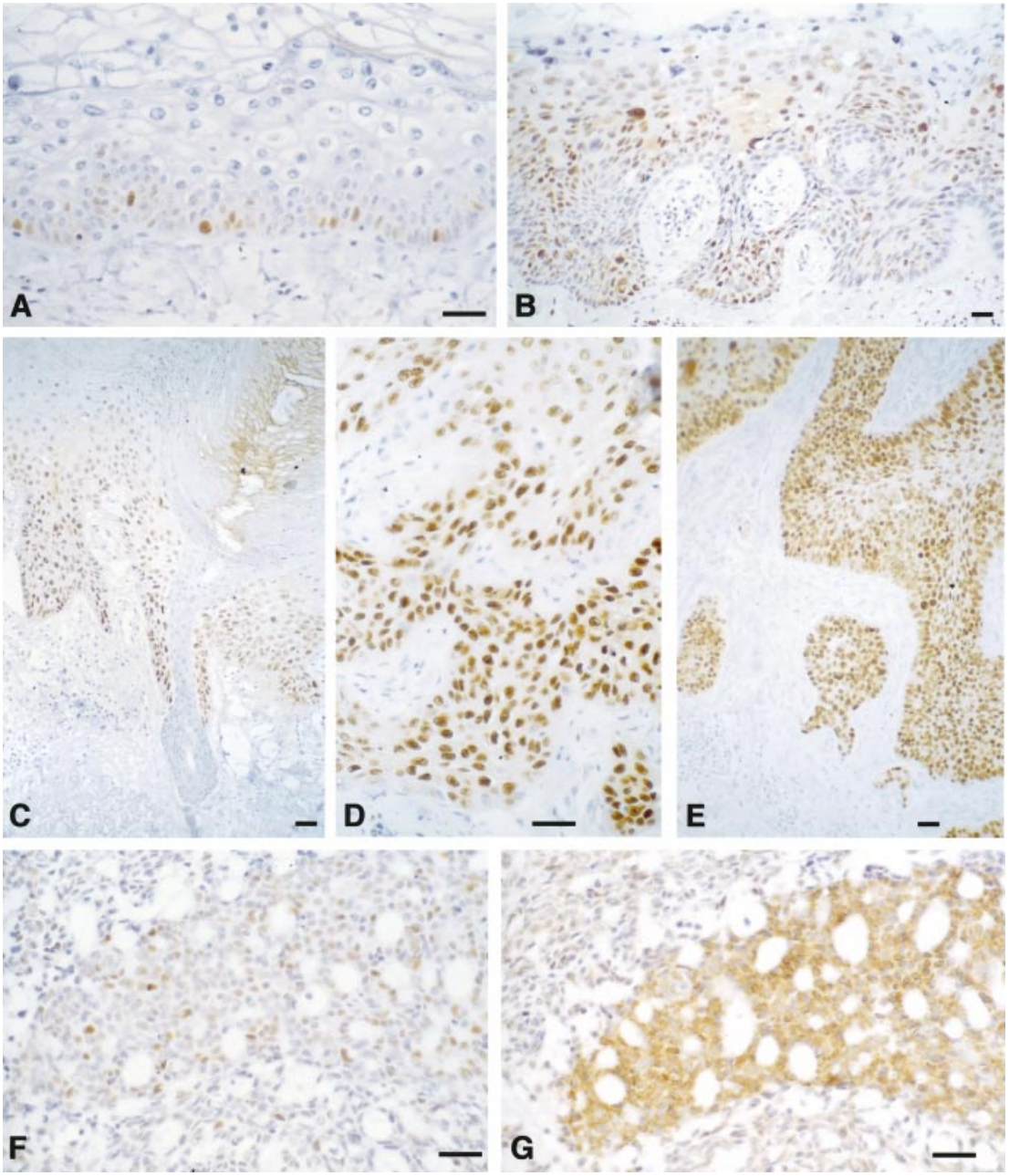


**Figure 3. Positive p53 immunostaining of lesions from two EV patients.** Six of the EV specimens described in **Table I** are illustrated. (*A*) Benign EV macular lesion (A, i) with a nuclear staining confined to basal cells; (*B*) CIS adjacent to a positive SCC (A, n) with a labeling sparing the superficial differentiating cells; (*C*) AK (B, a) with a staining of almost all immature keratinocytes and lack of staining of a hair follicle; (*D*), (*E*) microinvasive (A, m) and invasive (B, d) SCC with a strong staining of almost all undifferentiated cells; (*F*), (*G*) consecutive sections of a BCC (A, p) showing a congruent moderate p53 immunoreactivity and strong Bcl-2 immunostaining. *Scale bar*: 30 μm.

*et al*, 1995). It has been reported that the p53 immunoreactivity frequently observed in skin neoplasias in the general population does not always correlate with the detection of a mutation (Campbell *et al*, 1993; Ziegler *et al*, 1993; Ren *et al*, 1996). This may correspond to missed mutations or to a stabilization of the wild-type p53 protein. Inhibition of MDM2-mediated ubiquitin-dependent degradation of p53 protein may occur in response to endogenous stresses, such as an inappropriate growth-promoting signal resulting from the expression of viral oncoproteins (Lohrum and Vousden, 1999; Vogelstein *et al*, 2000). A stabilization of the p53 protein is induced in human keratinocytes immortalized by the HPV16 E7 gene (Demers *et al*, 1994). HPV5 E7 protein binds Rb protein, and this is likely to generate a proliferative signal (Yamashita *et al*, 1993; Hiraiwa *et al*, 1996). The E2F-1 transcription factor released from its inactive Rb-bound form may activate the expression of the p14 ARF protein, which binds to the MDM2 protein and inhibits its activity (Vogelstein *et al*, 2000). Although the E6 proteins of oncogenic EV HPV genotypes do not promote p53 degradation (Steger and Pfister, 1992; Elbel *et al*, 1997), HPV5 E6 protein has been shown to interfere with p53-induced G1 arrest and apoptosis in UV-irradiated cell lines (Jackson and Storey, 2000). The antiapoptotic Bcl-2 protein is expressed in BCC but rarely in SCC in the general population (Wikonkal *et al*, 1997). This protein was not detected in EV lesions, with the exception of the only BCC studied, suggesting that it is not involved in EV carcinogenesis. It has recently been proposed that HPV5 E6 protein could exert an antiapoptotic effect by promoting the proteolytic degradation of the UV-induced pro-apoptotic Bak protein (Jackson

*et al*, 2000). Whether the activity of p53 protein is affected *in vivo* by HPV5 E6 and E7 oncoproteins expressed from their own promoters remains to be established.

All nine p53 mutations identified in EV lesions affect GC base pairs. Five were C→T transitions at dicytidine sites, including three found at mutation hotspots unique for NMSC (codons 248 and 258) (Ziegler *et al*, 1993). These can be considered as UV signature mutations (Dumaz *et al*, 1994; Brash, 1997). Interestingly, both patients had been found to have a normal UV-induced DNA repair in the epidermis (Proniewska and Jablonska, 1980). The four other base substitutions occurred at mutational hotspots identified in internal malignant tumors (Walker *et al*, 1999). Two were C→T transitions at a nondipyridimine site. The two transversions occurring at GG:CC sequences are not informative because they can be generated by many different mutagens (Ziegler *et al*, 1993). None was found at a CpG dinucleotide, however, which excludes the deamination of a methylated cytosine, a frequent endogenous mechanism of DNA damage (Greenblatt *et al*, 1994). These four mutations could correspond to DNA replication errors or could result from unrepaired DNA lesions caused by exogenous or endogenous mutagens other than genotoxic UVB radiation (Hollstein *et al*, 1998), as has already been suggested for skin tumors of Japanese patients (Takata *et al*, 1997). Mutagenic agents could be reactive oxygen species generated from oxidative metabolism, or resulting from exposure to the UVA component of sunlight or to ionizing radiation (Perchellet *et al*, 1995; Wang *et al*, 1998; Pourzand and Tyrrell, 1999). Interestingly, patient B received X-ray therapy for forehead SCC in 1972 and 1977.

In conclusion, a dysfunction of the p53 gene is likely to play a part in EV carcinogenesis, either due to UVB-induced p53 mutations, as in NMSC in the general population, or involving other mutagens or mechanisms. Whether the genetic defects specific to EV (Ramoz *et al*, 2000), and the resulting high level of expression of the E6 and E7 proteins of HPV5 and related genotypes in EV keratinocytes, lead to increased mutation rates or to a dysfunction of the wild-type p53 protein remains to be established.

*KP was a recipient of fellowships from the Ministère des Affaires Etrangères. The study was supported in part by grants from the EEC (Copernicus Contract C1PA-CT-93–0246), the French-Polish Cooperation Project (No. 6559), the Association de la Recherche pour le Cancer, and the Institut National de la Santé et de la Recherche Médicale (U. INSERM 190). We are grateful to M.-L. Le Bihan, D. Fortin, and P. Flamant for their expert technical assistance, M. Favre and L. Daya-Grosjean for advice, F. Breitburd and K. Kean for critical reading of the manuscript, and D. Senlecques for preparation of the manuscript.*

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