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Relation of p53 Tumor Suppressor Protein Expression to Human Papillomavirus (HPV) DNA and to Cellular Atypia in Male Genital Warts and in Premalignant Lesions

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Functional disturbance of p53 tumor suppressor protein contributes to uncontrolled cell growth. Human papillomavirus (HPV) E6 oncoproteins bind to wild-type p53 and abrogate its function. Our objective was to elucidate the relation of aberrant p53 protein expression to HPV DNA and cellular atypia in male genital warts and premalignant lesions.

Immunohistochemically detectable p53 protein expression was studied in 35 male anogenital warts with low-level or no keratinocyte atypia (histologically confirmed condylomata acuminata), in 25 lesions with bowenoid papulosis (BP; carcinoma *in situ*) histology, and in 10 non-condyloma lesions using immunostaining with three established antibodies recognizing full-length wild-type accumulated p53 protein, or its conformational mutants. HPV DNA specific for HPV 6/11, 16/18, or 31/33/35 was identified by *in situ* hybridization or by polymerase chain reaction (PCR) – based amplification.

Both nuclear and cytoplasmic keratinocyte immunostaining for p53 protein was detected in 41% of condylomata with no keratinocyte atypia and in 42% of condylomata with slight nuclear atypia or with bowenoid papulosis histology. No association of aberrant p53 expression with any specific HPV type or with HPV DNA was observed. Normal skin and some other penile dermatoses were negative for p53 immunostaining. In the follow-up biopsies of 16 BP patients, treated with CO_2 laser, recurrence of atypia was seen exclusively in lesions initially positive for both HPV DNA and p53 protein.

Our results show that a few cells in male genital warts even with no cellular atypia may express abnormally sequestered or loss-of-function p53 protein, and that concomitant presence of any type of HPV DNA is associated with recurrencies or progression of premalignant changes. *Key words: bowenoid papulosis; immunohistochemistry; growth regulation.*

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Human papillomavirus (HPV) is an important co-factor in the development of genitoanal cancers (1), although HPV infection *per se* does not explain cell transformation and uncontrolled cell growth (2). HPV types 16 and 18 have been linked to genital cancers since their oncoproteins E6 and E7 form a complex with tumor suppressor protein (TSP) p53 and the retinoblastoma gene product, respectively, and this, in turn, results in the loss of TSP function (3, 4). The tumor suppressor gene (TSG) for the nuclear phosphoprotein p53 is the most commonly mutated gene yet identified in human cancers (2, 5). Under the normal cell cycle, the wild-type p53 protein controls cell entry from the G1 phase to the S phase (6–8). It was recently shown that p53 protein inhibits cell division by acting as a transcription factor and turns on a gene coding for a second protein (cyclin-dependent kinase-interacting protein), which suppresses cell division (9–12). Uncontrolled cell growth can result, upon current knowledge, either from somatic mutations in the p53 gene (and subsequent co-operation with oncogenes) or from the complexing of p53 with the transforming protein of tumor viruses (13). Especially the E6 oncoproteins of the high-risk HPV types 16 and 18 stimulate the degradation of p53 protein (14), and the viral-host protein interaction thus results in the loss of the negative growth control exerted by p53 protein.

The most common mutations in p53 TSG are missense mutations which lead to an altered protein conformation (5). This prolongs the half-life of p53 protein so that it is possible to demonstrate a mutated protein with immunohistochemical methods (15, 16). Also, wild-type p53 protein, abnormally sequestered in the cytoplasm or complexed to some cellular proteins, can be detected immunohistochemically (17). Normal p53 protein cannot be visualized with immunohistochemistry, although *in vitro* newly divided non-transformed human keratinocytes may show intense staining for p53 protein (18).

It was recently shown that a clonal p53 mutation in primary cervical cancer is associated with HPV-negative tumors and that HPV-negative cervical cancers show a worse prognosis (19–21). However, alterations of p53 protein have not been previously studied in male genital carcinogenesis. As the prevalence of HPV infection is high among young adults and as premalignant carcinoma *in situ* findings (bowenoid papulosis) are often detected, we looked for the association of abnormal p53 protein expression with histological atypia and with the presence of HPV DNA in 70 biopsies of male genital warts or acetowhite lesions, and in the follow-up biopsies of 16 BP patients after CO_2 laser treatments.

METHODS

Patient material and histopathological findings

Altogether 70 biopsies of macroscopic genital warts, BP or acetowhite lesions were obtained from 46 male patients attending the venereal outpatient clinic of the Department of Dermatology and Venereology, Helsinki University Central Hospital in Helsinki, Finland, during the years 1987–1992. The biopsies were randomly selected for the present study, with the exception for that all patients ($n = 18$) with the histological diagnosis of bowenoid papulosis during this time period were included. The patients attended the clinic on their own initiative or were referred to the clinic because of therapy-resistant warts. The patients had suffered from genital warts (or lesions suspected to be such) for 0.5–5 years (range) before the first biopsy was obtained. Clinically, 50% of the lesions were located in the preputium, 17% in the shaft of the penis, 23% in the sulcus, and the remaining 10% in the glans penis,

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Table I. *Relation of the clinical type of the genitoanal warts to the histopathological diagnosis*

Histological diagnosis	Clinical type		
	Acuminatum	Papular	Flat[a]
Condyloma – no keratinocyte atypia ($n=23$)	9 (39%)	2 (9%)	12 (52%)
Condyloma – keratinocyte atypia ($n=12$)	7 (58%)	2 (17%)	3 (25%)
Bowenoid papulosis ($n=25$)	8 (32%)	7 (28%)	8[b] (32%)
Other: scar, psoriasis, lichen sclerosus et atrophicus ($n=10$)	2 (20%)	1 (10%)	7 (70%)

[a] includes acetowhite lesions.
[b] in addition, two had erosive balanitis (erythroplasia of Queyrat).

scrotum or perianal area. Sixty biopsies were routinely fixed in 4% neutral formalin and embedded in paraffin, and 10 biopsies were snap-frozen in liquid nitrogen and stored at –80°C.

Two of the following criteria were taken as a prerequisite for the histological diagnosis of condyloma acuminatum: proliferation of the keratinocytes including papillomatosis, parakeratosis and vacuolization of the superficial keratinocytes. In some lesions, referred to as condylomata with atypia, nuclear atypia was seen in single and scattered keratinocytes in limited areas of the epidermis. In bowenoid papulosis (= carcinoma *in situ*) keratinocyte atypia (enlarged, hyperchromatic nuclei or mitotic figures) and disarrangement of keratinocyte differentiation were seen throughout the epidermis. Histologically, 35 initial biopsies were diagnosed as condylomata acuminata, and 12 (34%) of these showed scattered atypical keratinocytes but did not fulfill the criteria for bowenoid papulosis, i.e. carcinoma *in situ*. In 25 biopsies, the full thickness of the epidermis consisted of atypical cells, and a histological diagnosis of bowenoid papulosis was made. In 10 biopsies, the histological diagnosis was scar tissue, psoriasis, lichen sclerosus et atrophicus (LSA) or normal skin.

Sixteen of the BP patients were treated with CO_2-laser evaporization twice to six times, and were followed up with repeated yearly biopsies for 2.6 (± 1.5, mean ± SD) years. At the time of the last control visit, a biopsy from the original lesion area was obtained if there were any acetowhite areas or other clinically suspect lesions. The acetowhite lesions were detected by the application of 10% acetic acid to penile skin for 5–10 min. Two patients with complete clinical healing did not give their consent for a follow-up biopsy.

Ten additional formalin-fixed biopsies of normal skin from our laboratory files served as control tissue for p53 immunostaining.

HPV DNA in situ hybridization

To demonstrate DNA specific for HPV types 6, 11, 16, 18, 31, 33 and 35, we used both digoxigenin (DIG-HPV) – and biotin-labeled HPV DNA probes in *in situ* hybridization of paraffin-embedded sections, as previously described in detail (22). For our DIG-HPV method, DNA clones of HPV types 6, 11, 16 and 18 were kindly provided by professor H. Zur Hausen, Heidelberg, Germany. ViraType *in situ* HPV detection kit (Life Technologies Inc, Gaithersburg, MD) included full-length biotin-labeled probes for HPV 6/11, HPV 16/18 and HPV 31/33/35. We have previously shown (23) that these methods give results comparable to each other. The hybridization with DIG-HPV probes was performed at 42°C overnight, and the hybridized product was detected with anti-digoxigenin-AFOS conjugate and X-phosphate (BCIP)/nitroblue tetrazolium (NBT) substrate, yielding a purplish blue product (Fig. 2a). ViraType *in situ* hybridization was performed at 37°C for 2 h, and the hybridization product was detected with streptavidin-biotin-AFOS conjugate and the same colorigenic substrate as for DIG-HPV.

Immunohistochemical demonstration of p53 protein

The immunohistochemical demonstration of p53 protein was performed on paraffin sections of formalin-fixed biopsies and, in addition, on frozen sections of 10 biopsies fixed briefly with cold acetone. As specific antibodies, we used polyclonal antibody CM-1, which has been shown to recognize both wild-type p53 (also in its complexed form) and most mutant forms of p53 protein (16, 24), and three established monoclonal antibodies (MoAbs), PAb240 (25, 26), DO-1 and DO-7, which recognize diverse epitopes on conformationally mutated p53 (27). PAb240 only works on frozen sections and recognizes a normally cryptic denaturation-sensitive epitope (aa 161–220). DO-1 and DO-7 recognize both wild-type and mutant p53 (epitope aa 1–45; 26). CM-1, DO-1 and PAb240 antibodies were a generous gift from Professor D. Lane (Department of Biochemistry, University of Dundee, U.K.), and DO-7 antibody as well as later aliquots of CM-1 and PAb240 were purchased from Novocastra Laboratories Ltd, (Newcastle Upon Tyne, U.K.). Immunohistochemistry was performed on deparaffinized, pepsin-treated (0,4% pepsin, at 37°C for 60 min) sections, as previously described in detail (28). The working dilutions for the primary antibodies were as follows: CM-1 1:500–1:1000, DO-1 and DO-7 1:100–1:200, and PAb240 1:5 (supernatant). The bound antibody was visualised with the avidin-biotin-peroxidase method (Vectastain kit, Burlingame, CA) and with diaminobenzidine, (DAB, Sigma, St. Louis, MD) as colorigenic substrate.

Some biopsies were also stained with a monoclonal antibody (PC10, Dako) against the proliferating cell nuclear antigen (PCNA, polymerase delta accessory protein) to demonstrate proliferative activity in the keratinocytes (see Figures).

PCR-based amplification of HPV-16 DNA

PCR-amplification of HPV 16/E6-specific DNA and subsequent Southern blot was performed on biopsies negative for HPV16/18 in *in situ* hybridization. One of the *in situ* hybridization-positive samples always served as a positive control. Genomic DNA was extracted from 10–20 paraffin sections (10 μm) with non-ionic detergents (0.45% Nonidet P40, Sigma, and 0.45% Tween 20) in PCR buffer (Perkin-Elmer Cetus, Norwalk, CT), and with proteinase-K (200 μm/ml) digestion. HPV 16/E6-specific oligonucleotide primers, genomic location 421–440 and 521–540, were used (29). Amplification was carried out in 50 μl volume in an automatic thermal cycler (Techne PHC-2, Cambridge, U.K.) in a mixture consisting of 2.5 U Taq polymerase (Gibco BRL, Gaithersburg, MD), 200 μM each dNTP, 1 μM each primer and 1.5 mM $MgCl_2$. Cycles of 95°C 30", 55°C 30" and 72°C 1' were repeated 30 times. The amplicon was then subjected to hybridization in liquid, with a ^{32}P-labeled specific oligonucleotide probe at 55°C for 30 min. The hybridized product was electrophoresed through a 10% polyacrylamide gel, which was then autoradiographed for 3 days. To control the quality of the DNA, primers specific for human β-globin (30) were used in parallel amplification cycles, and the product was visualized by ethidium bromide staining of 1% agarose gels.

Statistical analysis

For statistical analyses, the chi-square test (Statistica, Vol. I, Stat Soft Inc., Tulsa, OK) was used. The values were also tested with Fisher's exact test, but the results were similar to those by the chi-square test.

RESULTS

The present tissue material, analyzed for p53 immunostaining and the presence of HPV-specific DNA, consisted of 70 biopsies of genitoanal warts or acetowhite lesions. Histologically, 35 were diagnosed as condylomata acuminata, 25 were diagnosed

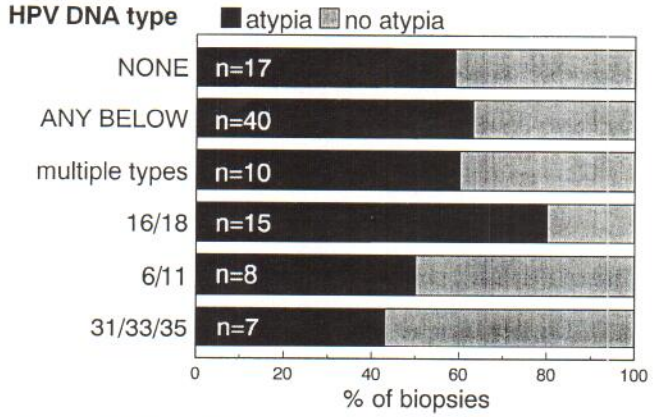

Fig. 1. Relation of histological keratinocyte atypia, found in male condylomata acuminata and in bowenoid papulosis, to various HPV DNA types detected with *in situ* hybridization. No atypia refers to the histological diagnosis of condylomata acuminata with no atypical keratinocytes (see also Methods for definition). The *n* values refer to the total number of biopsies in each category of the *y* axis.

as bowenoid papulosis (carcinoma *in situ*), and in 10 biopsies some other dermatosis or only scar tissue was found. All main clinical types of condylomata were represented (Table I), and no clinical type predicted the histopathological diagnosis.

In the initial biopsy material with condyloma or bowenoid papulosis histology, cellular atypia was mostly found in biopsies positive for HPV 16/18 (Fig. 1). Altogether, 61% (24/39) of biopsies with histological atypia (including BP) were positive for HPV DNA types 6/11, 16/18 or 31/33/35 by *in situ* hybridization. Eleven of the 18 (61%) BP patients were positive for HPV 16/18 DNA and 4 showed other HPV types. Three BP samples were HPV-negative by *in situ* hybridization but one of these was positive for HPV-16 E6-specific DNA by PCR-based hybridization. Thus, HPV DNA was detected in all but 2 BP biopsies.

In immunohistochemistry, antibodies CM-1, DO-1 and DO-7, which recognize both wild-type and mutant p53 protein, in accumulated or complexed form, showed mostly nuclear but occasionally also cytoplasmic immunostaining in the keratinocytes in condylomata (Fig. 2) and in BP lesions (Fig. 3). In our control biopsies with normal epidermis, none of the above antibodies gave a positive reaction, which is consistent with the concept that normal, functionally active p53 protein cannot be detected immunohistochemically due to its short half-life. In condyloma lesions, p53-positive staining was detected in scattered areas, usually in a cluster of about 5 to 10 near by keratinocytes (Fig. 2). In some BP lesions, the p53-positive cells were clearly in basal keratinocytes (Fig. 3b), while HPV DNA detectable by *in situ* hybridization resides in the suprabasal epidermal layers (Fig. 2a).

Equal proportions of p53-positive biopsies were detected among BP lesions and in condylomata (Table II). There was no association of p53-positivity with histological atypia in the condyloma group (data not shown). The least p53-positive keratinocytes were found in lesions positive for HPV 16/18 (Fig. 4), but there was no statistically significant association with HPV positivity. p53-positive keratinocyte nuclei were found in one scar tissue biopsy but not in psoriatic or LSA lesions. The positive biopsy was obtained after laser therapy of a previous BP lesion, which had been positive for HPV 16/18 by ISH.

In 6 of 15 lesions positive for both HPV 16/18 and abnormal p53 protein, HPV-16 was detectable with PCR amplification but not with ISH. However, of all HPV 16/18-positive lesions, whether demonstrated by ISH or PCR method, 38% were p53-negative with all three antibodies.

With the monoclonal antibody PAb240, recognizing mutant p53 protein on frozen sections, we detected p53-positive keratinocytes (nuclear staining) in 1 of 6 condyloma acuminatum lesions, in none of 2 BP lesions, and in 1 of 2 postlaser scar tissue biopsies from BP patients. Both of these PAb240-positive samples turned out to be HPV-16-positive by PCR analysis.

We finally looked for the outcome of the 16 BP patients with repeated CO_2-laser treatments and biopsies. As shown in Table III, the recurrence or persistence of cellular atypia was confined to lesions with both HPV DNA and p53 protein positivity. However, 43% of such lesions were confirmed to be histologically healed during the follow-up of 3 years.

DISCUSSION

We have shown that aberrant p53 protein is detected not only in premalignant penile lesions, like BP, but also in 41–43% of genital warts, depending on the rate of cellular atypia, but only rarely in scar tissue or other penile lesions. We did not find any statistically significant association between abnormal p53 immunopositivity and the presence of HPV DNA *per se* or with histological atypia. As the E6 proteins of both oncogenic and benign HPV types bind to p53, but only the E6 of oncogenic HPV types targets p53 for degradation (31), we have probably detected complexed wild-type p53 protein with the CM-1, DO-1 and DO-7 antibodies. Also, the cytoplasmic localization of the p53 protein in some biopsies would suggest a disturbed nuclear transport (17), possibly due to binding to some cellular proteins, such as those controlling transcription of heat shock protein Hsp70 genes (32) or the mdm-2 oncogene (33). However, it was not possible, in retrospect, to study the formalin-fixed biopsies with PAb240 antibody, which has been shown to stain p53 protein associated with Hsp70 in the cytoplasm (24). Our finding of a few PAb240-positive keratinocytes in fresh samples of a condyloma acuminatum and a scar tissue lesion was surprising, as this conformation has been shown to gain dominant transforming activity (27). Cooper et al. have recently suggested that PAb240-positive keratinocytes may alternatively represent a promoter form of wild-type p53 protein, present in proliferating basal cells (34). The elevated levels of wild-type p53 protein may modulate the expression of other genes controlling the cell cycle (35) and may thus contribute to the susceptibility of the cell to malignant transformation.

The reliability of immunostaining for p53 protein in relation to true genomic mutations has recently been questioned. Campbell et al. (36) reported discrepancy in both directions between p53 immunostaining with DO-7 antibody (corresponds to DO-1 used in this study) and mutations in exons 5–8 of p53 gene in non-melanoma skin cancers. Molés et al. (37) found false negative and Kubo et al. (38) false positive immunostaining with CM-1 and DO-7 antibodies in relation to p53 gene mutations in

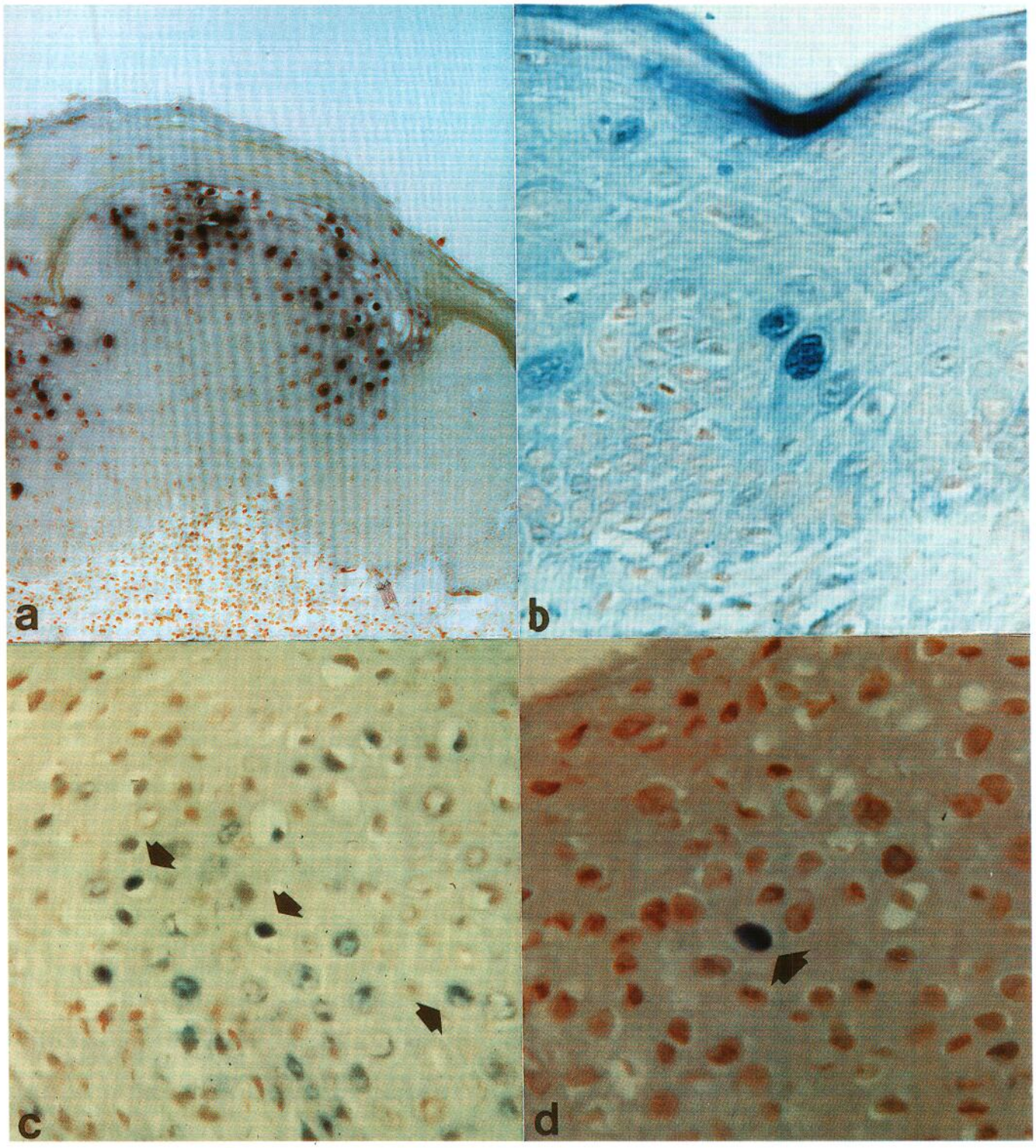

Fig. 2. Immunostaining for aberrant p53 protein and the presence of HPV DNA in male genital warts. (*a*) HPV 6/11-specific DNA (bluish-black colour) as demonstrated by *in situ* hybridization with a digoxigenin-labeled DNA probe in a condyloma acuminatum lesion. (*b*) Typical staining for p53 protein (reddish-brown reaction product) with polyclonal CM-1 antibody recognizing full-length p53 protein in a condyloma acuminatum lesion. This lesion was also positive for HPV 31/33/35 by *in situ* hybridization. (*c*) Keratinocyte nuclei positive for p53 protein (*arrow*; antibody DO-1) in a cryostat section of HPV 6/11-positive condyloma acuminatum with atypia. (*d*) A keratinocyte positive for PAb240 (*arrow*), recognizing the mutated form of p53 protein in a frozen section of condyloma acuminatum (magnifications: *a* = × 124, *b* and *d* = × 690, *c* = × 495; hematoxylin counterstain).

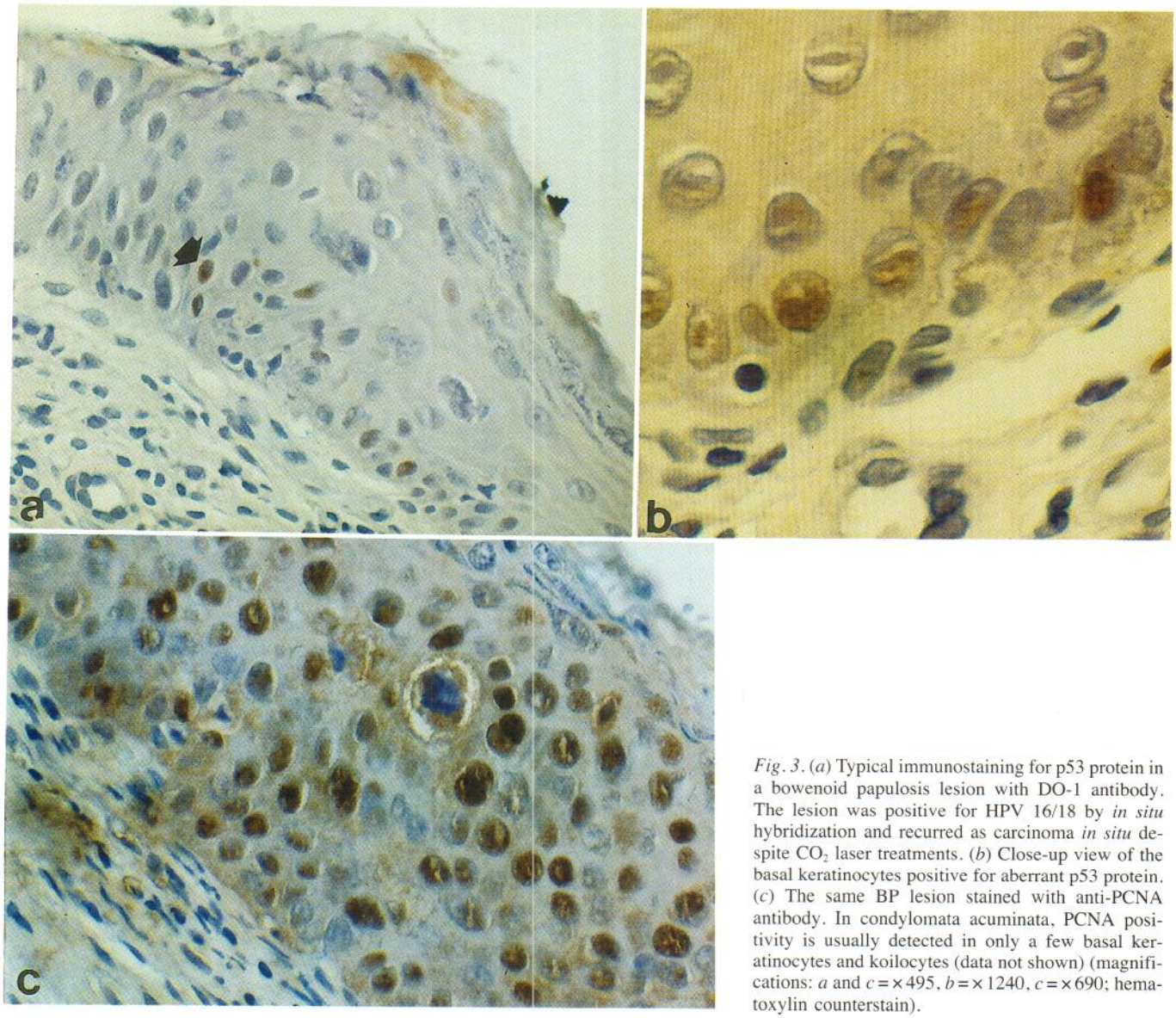

Fig. 3. (*a*) Typical immunostaining for p53 protein in a bowenoid papulosis lesion with DO-1 antibody. The lesion was positive for HPV 16/18 by *in situ* hybridization and recurred as carcinoma *in situ* despite CO_2 laser treatments. (*b*) Close-up view of the basal keratinocytes positive for aberrant p53 protein. (*c*) The same BP lesion stained with anti-PCNA antibody. In condylomata acuminata, PCNA positivity is usually detected in only a few basal keratinocytes and koilocytes (data not shown) (magnifications: *a* and *c* = × 495, *b* = × 1240, *c* = × 690; hematoxylin counterstain).

Table II. *Immunohistochemically detectable expression of p53 protein in male condylomata and bowenoid papulosis before treatment*

Histological type of lesion	Aberrant or mutant p53 protein[a]	No p53 protein detected
Condyloma acuminatum		
HPV DNA + ($n=22$)	12[b]	10
HPV DNA − ($n=12$)	2	10
Bowenoid papulosis (carcinoma in situ)[c]		
HPV DNA + ($n=14$)	6	8
HPV DNA − ($n=7$)	3	4

[a] indicates positivity for antibodies PAb240 and DO-1.
[b] chi-square, $p<0.10$.
[c] biopsies obtained from 18 patients.

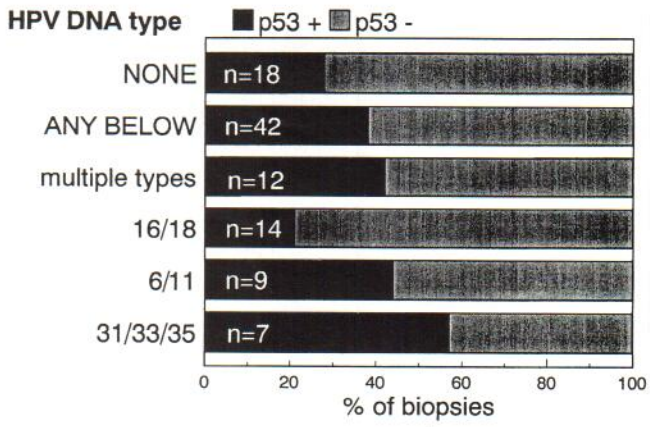

Fig. 4. Expression of aberrant p53 protein in relation to various HPV DNA types in male genital warts, including bowenoid papulosis lesions.

Table III. *Follow-up data of 16 bowenoid papulosis (BP) lesions in relation to aberrant or mutant p53 protein and HPV DNA in the diagnostic biopsy*

Outcome of lesions[a]	Detection of HPV DNA/p53 protein			
	HPV/p53 +/+	HPV/p53 +/−	HPV/p53 −/+	HPV/p53 −/−
Recurrence of premalignant findings (2–3years)	4[b]	0	0	0
Histologically cured (2–4 years)	3[c]	3	1	2
Clinically healed	0	3	0	0

[a] Fifteen patients were treated with CO_2-laser once to 6 times after the BP diagnosis, and one patient with cryotherapy.
[b] All biopsies were immunopositive for CM-1 antibody, and two also for DO-1 antibody.
[c] One biopsy showed keratinocytes positive for PAb240, too.

skin tumors. No immunostaining in tumors with a wild-type p53 gene was found (37), however, and no clear discrepancy between the two methods was found by Moll et al. in breast cancer biopsies (17). The small size of the penile biopsies in the present study did not allow for p53 sequencing, but we would like to point out that immunohistochemistry may better detect small foci of cells with abnormal p53 protein, as was the case in our samples, than molecular biological methods on extracted DNA.

Mutant p53 forms hetero-oligomers with wild-type p53 and thus, in a dominant-negative fashion, inhibits the ability of wild-type protein to bind DNA and to control adjacent genes (39). We found that the cellular atypia recurred or progressed, in 4 of 7 (57%) BP patients expressing both aberrant p53 protein and HPV 16 or 18 DNA despite CO_2-laser treatments, while all 8 patients not expressing aberrant p53 protein at any time point were completely cured. The fact that similar proportions of biopsies, positive for both HPV DNA and p53 protein, were found among condylomata and BP samples would warrant a closer follow-up of such doublepositive genitoanal warts.

The finding that HPV 16 was detectable only by PCR amplification in 40% of samples positive for both HPV DNA and p53 protein indicates that in these cases the HPV genome was in an integrated form with lower copy numbers than in the productive, episomal form. In such an integrated HPV genome, the E6 gene remains intact (1, 14). We would like to suggest that in BP, a selection of mutant p53 alleles as well as HPV 16/18 E6-protein-promoted degradation of wild-type p53 may occur.

The expression of aberrant p53 protein in the keratinocytes of condylomata acuminata might also be a result of DNA damage caused by external agents, like local cytodestructive drugs (e.g. podophyllin and podophyllotoxin). Wild-type p53 protein expression is vital for the DNA repair event following DNA damage after UV-light, irradiation or DNA-damaging agents (40, 41). In case of excesssive DNA damage, apoptosis of the cell ensues with the aid of p53 protein (42, 43). Thus, we may speculate that the sporadic keratinocytes with abnormal p53 protein that we detected in histologically benign lesions may have avoided apoptosis due to functionally deficient p53 protein. Such cells may be susceptible to uncontrolled cell proliferation, which renders them susceptible to accumulation of further mutations. However, as we found recurrence of cellular atypia only in lesions with HPV 16 or 18 together with aberrant p53 protein, it is likely that some additional, yet uncharacterized, factors may be required for clonal cell transformation.

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