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Penile/anal condylomas and squamous cell cancer

A HPV DNA hybridization study*

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Summary. Acuminate condylomas from the penis ($n=17$) and anus (six cases), three anal/penile giant condylomas, anal Bowen's disease (four cases), and intraanal squamous cell carcinomas with associated condylomatous changes (10 cases) including two verrucous carcinoma were studied for human papillomavirus (HPV) infections with nick translated, biotinylated cDNA probes for HPV 6, 11, 16 and 18. In addition, six cases of flat white penile lesions designated as lichen sclerosus et atrophicus were examined.

Reannealed complementary DNA strands were detected in situ with either immunoenzyme or immunogold protocols.

The in situ hybridizations resulted in 1/6 positive penile lichenoid lesions, 12/17 positive penile acuminate condylomas, 6/6 positive anal acuminate condylomas (including two condylomas with cellular atypias), 2/3 positive giant condylomas, 1/4 positive anal bowenoid lesions, and 4/10 positive keratinized squamous cell carcinomas, two of them being verrucous carcinomas. All penile/anal condylomas and two giant condylomas harboured HPV 6 and/or 11 DNA.

The five positive carcinomas (carcinoma in situ/invasive cancer) contained HPV 6 and/or 11 in two cases (including the verrucous carcinomas), and HPV 16 and/or 18 in three cases (one carcinoma in situ, two invasive carcinomas).

Recurrent malignancies were seen in one case to harbour the same HPV type as the primary lesions (HPV 16). In one particular patient, a double infection with HPV 16 and HPV 18 was demonstrated in distantly located malignant tumours.

Our study confirms the restrictions and the value of non-isotopic hybridization methods applied to archival tissues, and extends the knowledge on the presence and distribution of HPV infections at anogenital sites.

Key words: HPV DNA – In situ hybridization – Condylomas – Carcinomas – Anus – Penis

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Introduction

Condylomatous lesions of the penis, vulva, and anus have been classified according to their macroscopical appearance into acuminate, flat and giant (also known as "Buschke-Löwenstein tumors") condylomas. Classification based on histological and cytological criteria divides them into typical, proliferative and atypical condylomas, and distinguishes them from their truly malignant counterparts (carcinoma in situ and verrucous carcinoma with condylomatous change) (Schmauz and Owor 1980; Prioteaux et al. 1980; Bogomoletz et al. 1985; Balasz 1986). The benign nature of acuminate condylomas is widely accepted by clinicians and histopathologists, although these 'harmless' squamous epithelial proliferations are known for their tendency to persist and recur. In very rare cases, progression is seen leading to intraepithelial and invasive carcinomas (Boxer and Skinner 1977; Syrjänen 1987). With respect to prognosis and therapy, a precise pathological interpretation is of extreme importance in the sometimes intricate differential diagnosis between proliferative giant condylomas and highly differentiated verrucous squamous cell carcinomas (Bogomoletz et al. 1985).

New insights into the biology of these epithelial disorders has emerged from recent findings con-

cerning the presence and physical state of papillomavirus (HPV) DNA and the distribution pattern of different HPV types and subtypes in relation to the various lesions in question (Gross 1987; Oriel 1987). Among the more than 50 HPV types identified to date (Pfister 1984; Broker and Botchan 1986), the 'anogenital virus types' HPV 6, 11, 16, 18, 31, and 33 are particularly associated with lesions in this area and have been observed to segregate in two groups, predominantly affiliated with benign (HPV 6, 11) diseases or more frequently with higher grades of dysplasia, carcinoma in situ and invasive carcinoma (HPV 16, 18, 31, 33; Gissmann et al. 1982; Gissmann 1984; Zur Hausen 1985; De Villiers et al. 1986; Stoler and Broker 1986; Gupta et al. 1987a, b). The relevance of these findings has been increased by epidemiological studies, which have brought to light the mode of transmission of the virus and its related diseases among sexual partners (Gross 1987).

The majority of studies on the presence and diversity of HPV in anogenital lesions have been done using Southern transfer techniques to nitrocellulose filters followed by identification with radiolabelled nucleic acid probes (Broker and Botchan 1986). The manual workload and the expenses of this procedure will substantially hinder its application in most surgical pathology services. The answer to the resulting technical and logistical drawbacks was provided by the construction of non-isotopic biotinylated or chemically modified nucleic acid probes and the development of in situ detection protocols applicable to fixed and paraffin-embedded cells and tissues. These are also suited to addressing questions concerning the topographic distribution of infectious agents in neoplastic lesions and their spatial relation to areas with particular features of differentiation or dedifferentiation.

We have made use of this technique in order to compare condylomatous lesions of penis and anus for the presence and distribution of human papillomavirus types 6, 11, 16, and 18. Our studies also included giant condylomas, verrucous carcinomas, and highly differentiated squamous cell carcinomas with associated condylomatous changes collected during the years 1981–1988.

Materials and methods

This report is based on a study of 17 penile acuminate condylomas (mean age 20 y), six cases of flat white penile lesions designated as lichen sclerosus and atrophicus (six males, mean age 28 y), six intraanal acuminate condylomas (five males, one female, mean age 37 y), two intraanal giant condylomas, one giant condyloma from the penis, four cases of intraanal Bowen's disease, and ten intraanal squamous cell carcinomas with associated condylomatous changes (two of them being verrucous carcinomas), selected following histological review from the files of the Department of Pathology, University of Hamburg. All penile condylomas derived from a young age group of men routinely checked during their military service. All lesions were located at the inner lining of the prepuce. The penile condylomas were devoid of cellular atypia in contrast to two out of the six intraanal condylomas. Further clinical informations for giant condylomas and carcinomas are summarized in Table 1. The tissue material consisted of excisional biopsies or surgical specimens and had been routinely fixed in buffered formalin. Serial sections of 4–6 μ in thickness were cut from the paraffin blocks.

Table 1. Clinical data of the hybridized giant condylomas and carcinomas

Case No	Age	Sex	Diagnosis	HPV			
				6	11	16	18
1	57	m	giant condyloma*	+	−	−	−
2	34	m	giant condyloma	+	+	−	−
3	51	m	giant condyloma	−	−	−	−
4	37	f	Bowen's disease	−	−	+	−
5	58	f	Bowen's disease	−	−	−	−
6	38	f	Bowen's disease	−	−	−	−
7	29	f	Bowen's disease	−	−	−	−
8	41	m	verrucous carcinoma	+	+	−	−
9	66	f	verrucous carcinoma	+	−	−	−
10	58	m	SCC	−	−	−	−
11	63	f	SCC	−	−	−	−
12	28	m	SCC	−	−	+	+
13	53	m	SCC	−	−	−	−
14	78	f	SCC	−	−	−	−
15	68	f	SCC	−	−	−	−
16	77	f	SCC	−	−	−	−
17	71	f	SCC	−	−	+	−

* giant condyloma of the penis, SCC Squamous cell carcinoma

For DNA hybridization cloned DNA of the entire genome of HPV types 6b, 11, 16, and 18 was used. These probes, cloned into the BamH1 (HPV 18: EcoR1) restriction cleavage site of the plasmid pBR322 were a kind gift of Prof. Dr. H. zur Hausen and Prof. Dr. L. Gissmann (Deutsches Krebsforschungszentrum, Heidelberg, FRG). After propagation in E. coli, plasmids were harvested by cesium chloride centrifugation. Isolated probes were labelled employing a nick-translation procedure with biotinylated deoxyuridintriphosphate (Bio-11-dUTP) and a nick-translation reagent kit (Gibco/BRL).

For hybridization, each section was covered with 20 μl of the following, freshly prepared hybridization solution: 2 × SSC (1 × SSC = 0.15 NaCl, 0.015 trisodium citrate, pH 7.2), 20% (v/v) deionized formamide (or 50% in cases of stromal reactions), 10% (w/v) dextran sulphate, 0.1 mg/ml herring sperm DNA, and 1.0 μg/ml biotinylated HPV DNA. Washing solutions prewarmed to 34° C contained always 1 × SSC and 45% formamide corresponding to 17° C below the melting temperature Tm. Further details including the consecutive detection systems (immunoenzyme and immunogold protocols) have been described previously (Henke et al. 1987; Löning and Milde 1987; Löning et al. 1987). Sections were counterstained with haematoxylin, dehydrated and permanently mounted for light-micro-

Table 2. Frequency and distribution of HPV types in all lesions examined

Diagnosis	Number	HPV						
		6/11	6	11	16/18	16	18	neg
Lichen sclerosus	6			1				5
Penile condylomas	17	7	2	3				5
Anal condylomas	6	4	1	1				–
Giant condylomas	3	1	1					1
Bowen's disease	4					1		3
SCC	10	1	1		1	1		6

SCC Squamous cell carcinomas
HPV 6/11 (designates positive cases for two virus probes,
HPV 16/18 hybridizations conducted separately for the respective HPV DNA's)

scopic evaluation. Interference reflection microscopy (Verschueren 1985) was employed in cases of low signal intensity.

Specificity of in situ hybridization was verified by hybridizations with biotinylated plasmid DNA, by omitting specific probe from the hybridization mixture, or by applying probes to slides which were passed directly to 37° C without preceding denaturation.

Results

The results of in situ hybridizations are shown in Table 2. Lesions of the lichen sclerosus et atrophicus type (six cases) consisted clinically of ivory papules, and showed the morphology of an atrophic regularly layered epithelium without any koilocytosis and dysplasia. There was a varying extent and arrangement (band-like, zonal) of stromal lymphocytic infiltrates. Surprisingly, HPV 11 DNA was found focally in one of these cases, the nuclear label starting immediately above the basal cell layer (Fig. 1).

From the 17 cases of penile condylomas two reacted with the HPV 6 probe, three were positive for HPV 11, and seven hybridized to both HPV 6 and HPV 11 (Fig. 2). Neither HPV 16 nor HPV 18 could be demonstrated in these lesions which were free of cellular atypias. With the exception of one case the degree of koilocytosis was notably lower in the HPV-negative specimens than in the 12 positive cases.

All six anal condylomas including two condylomas with cellular atypia were positive for HPV-DNA: four cases for both HPV types 6 and 11, two cases for HPV 11 and HPV 6 either.

Two anal and one penile giant condyloma were included in this study. The penile case progressed to an invasive (verrucous) carcinoma and was positive for HPV 6, while only one of the anal giant condylomas hybridized with the HPV 6 (Fig. 3) and 11 DNA probes.

Among anal cases with Bowens's disease (four patients, see Table 1), HPV 16 was identified only in one individual (case No 4, Figs. 4, 5). This patient suffered from recurrent tumours over a period of five years. Hybridizations yielded always and exclusively positive signals with the HPV 16 probe.

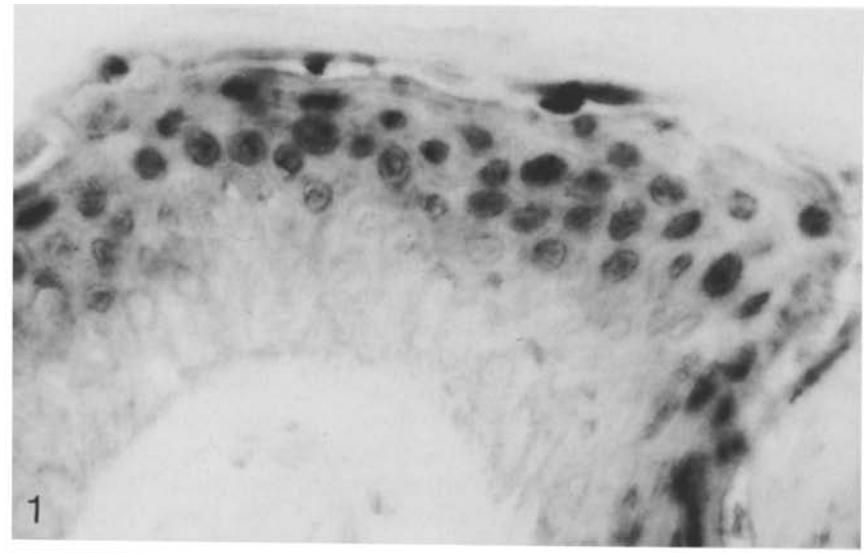
A total of ten anal squamous cell carcinomas was studied. Of these, only four cases harboured HPV-DNA to a detectable amount: One case was positive for HPV type 16, one for HPV types 16 and 18 (case no. 12). The verrucous carcinomas were positive for HPV 6 and HPV 11, the signal achieved with HPV 11 being considerably poorer in case no. 8 in comparison with the strong response to HPV type 6. From case no. 12, a total of eight biopsies from different sites of the anal canal had been obtained. In five of the eight biopsies, only HPV type 16 could be demonstrated (Figs. 6, 7), while two biopsies harboured only HPV type 18 DNA. One biopsy was negative with the probes applied. As a rule, hybridization signals in the carcinoma-bearing tissue specimens were found in condylomatous superficial zones and/or in frankly invasive regions. At the invasive front of the tumour, staining was most prominent in well differentiated areas.

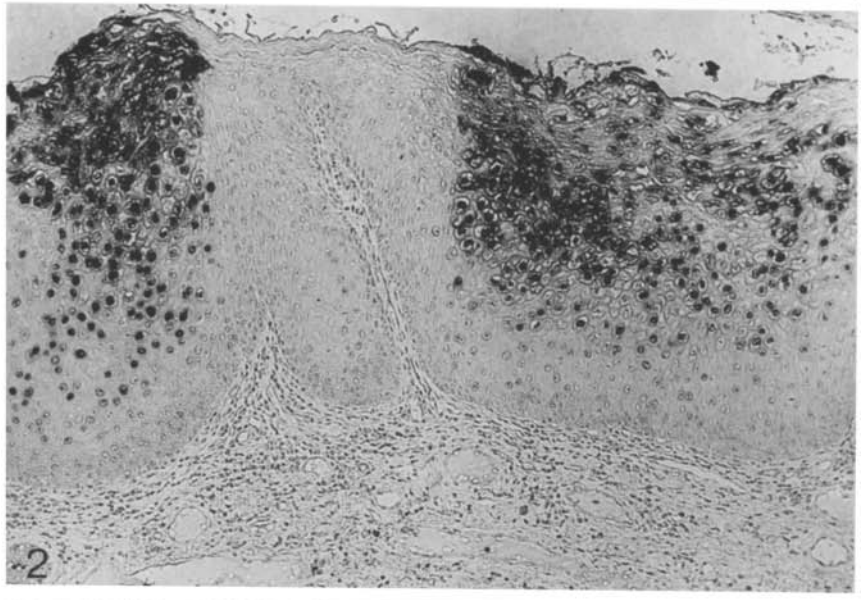
A focal accentuation of the hybridization signal was a constant finding in all HPV-positive tissue samples, with heavily stained nuclei sometimes lying in close vicinity to unlabeled ones. Staining was most intense in nuclei of superficial cell layers, in fields of koilocytosis or in dyskeratotic cells. In strongly positive cases staining could also be seen in suprabasal cell layers of the epithelium.

Hybridization signals in stromal fibroblasts were noted only rarely. These reactions, however, could be simply abolished by increasing the concentration of formamide (up to 50%) within the hybridization mixture. Controls were performed as described and were negative in all cases.

Discussion

The phenotypic spectrum of condylomatous lesions of the penis and anus is becoming more and more complex and includes the "classical" acuminate and giant condylomas, Buschke-Löwenstein tumours, and the mysterious Ackerman carcinomas (verrucous carcinomas) in addition to newly





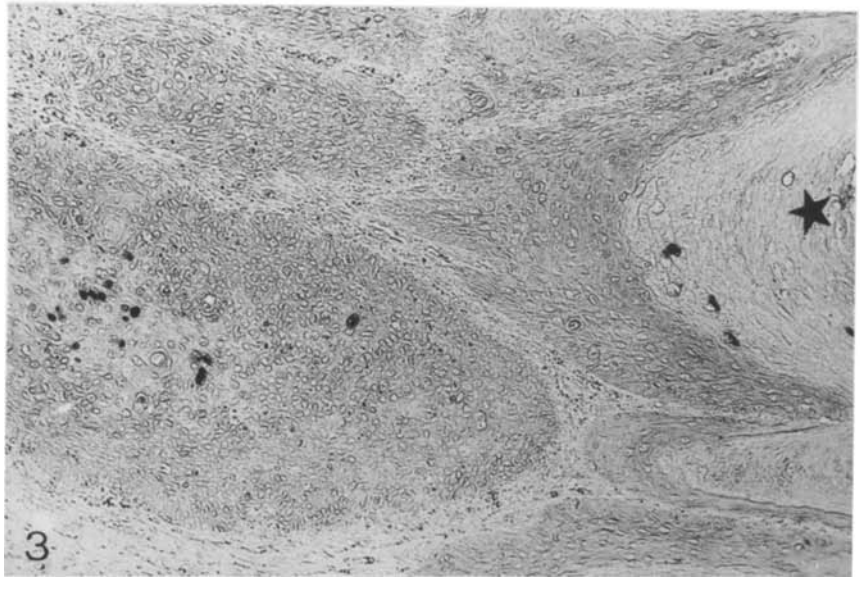
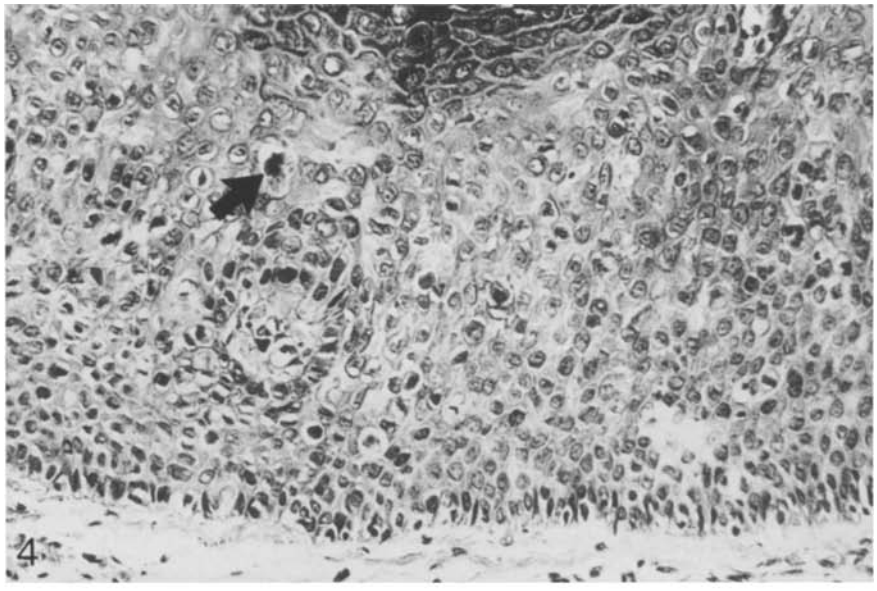
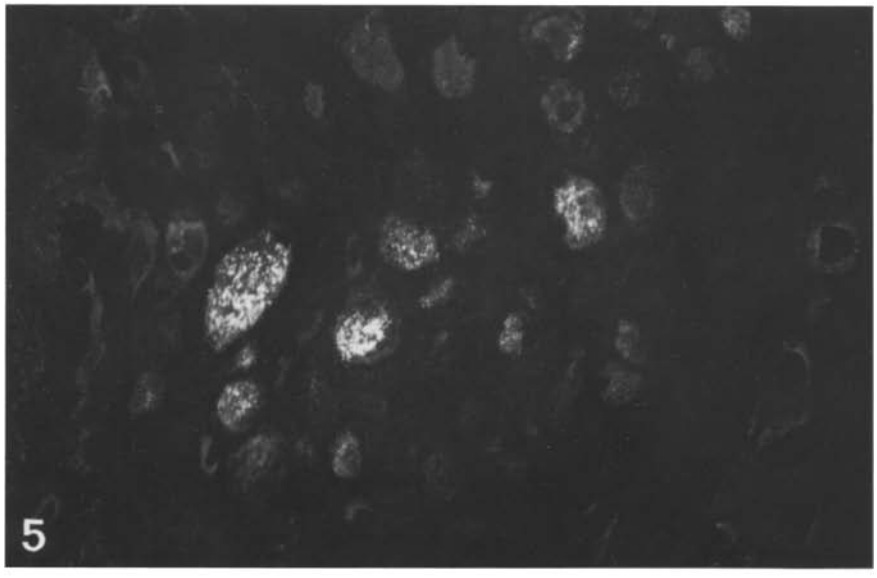


Fig. 1. Lichen sclerosus et atrophicus. In situ hybridization with HPV 11 probe. Strong suprabasal nuclear hybridization. Mag. × 500

Fig. 2. Penile condyloma. In situ hybridization with HPV 6 probe. The label starts above the basal cell layer with increasing intensity at superficial cell layers and koilocytic fields. Mag. × 80

Fig. 3. Anal giant condyloma. In situ hybridization with HPV 6 probe. Sparse staining of keratinocytes at the surface (*asterisk*) and within differentiated zones of enlarged rete pegs. Mag. × 80





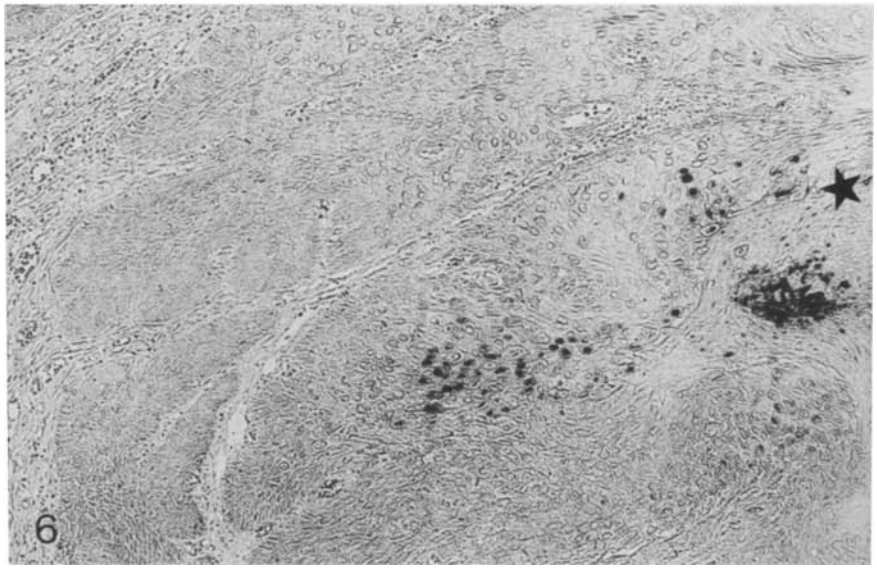


Fig. 4. Intraanal carcinoma in situ. In situ hybridization with HPV 16 probe. Immunogold-silver detection. *Arrow* points to one labeled keratinocyte. Mag. × 320

Fig. 5. Same case as in Fig. 4. Interference reflection microscopy facilitates the visualization and interpretation of the nuclear label. Mag. × 500

Fig. 6. Invasive highly differentiated squamous cell carcinoma (case no 12). In situ hybridization with HPV 16 probe. The label is concentrated in keratinized tumour zones (*asterisk* = surface). Mag. × 80

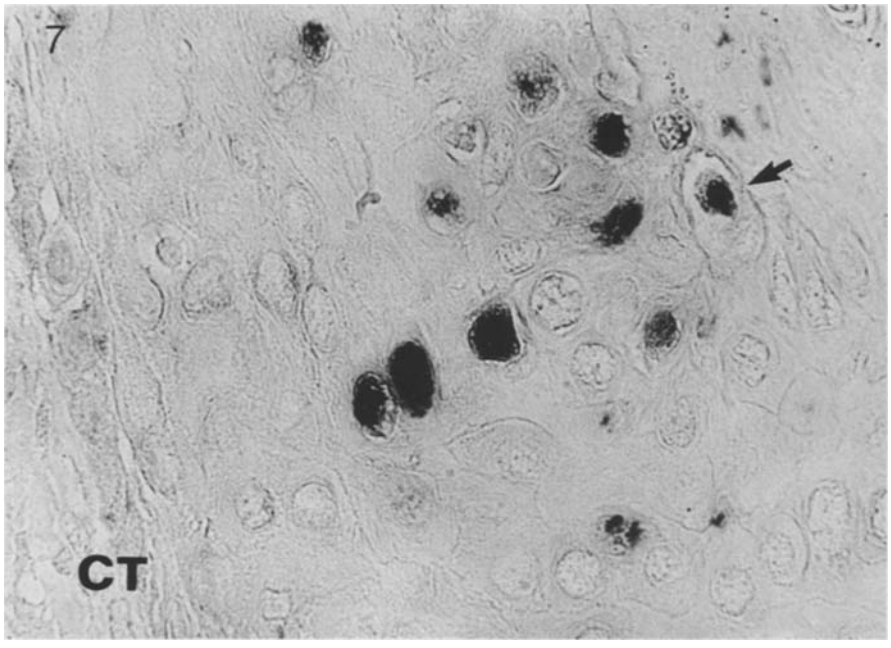


Fig. 7. Same case as in Fig. 6. Invasive front of the tumour (CT = connective tissue-tumour-interface). Polymorphous and in part dyskeratotic (*arrow*) keratinocytes with nuclear hybridization. Mag. × 500

distinguished flat, plaque-like and papular, pigmented and non-pigmented lesions (Gross 1987; Oriel 1987). The enthusiasm to subdivide and reclassify anogenital lesions has been vigorously stimulated by the past findings of the morphological diversity of skin warts related to particular papillomavirus infections (see for review: Broker and Botchan 1986). In contrast to the complex situation in skin warts, most anogenital lesions segregate rather simply into the categories of HPV 6/11 (Gissmann et al. 1982; Beckmann et al. 1985; Del Mistro et al. 1987; Syrjänen et al. 1987) and HPV 16/18 associated disorders (Ikenberg et al. 1983; Gissmann 1984; De Villiers et al. 1986; Wells et al. 1987).

The close association of HPV 6 and/or 11 with benign lesions and of HPV 16 and/or 18 with malignant tumours has been repeatedly demonstrated in different countries (reviews: Zur Hausen 1985; Broker and Botchan 1986). Nevertheless, exceptions to the rule have been recognized soon, and especially with respect to Buschke-Löwenstein and Ackerman tumours. A number of cases have been recorded over the years which constantly demonstrate the presence of HPV 6 or 11 in an episomal and/or integrated state (Zachow et al. 1982; Okagaki et al. 1984; Rando et al. 1986; De Villiers et al. 1986). These investigations were primarily pushed forward by molecular virological analysis, and prompted us to submit our cases to the morphological approach of in situ hyridization. Although this approach gives an answer only on the presence and distribution of cells containing high copy numbers, HPV was demonstrated in cells with a morphologically malignant phenotype in addition to koilocytes at the surface of the lesions. In situ hybridization clearly yielded variable results dependent on the lesions under study. Detection rates declined from approximately 80% (18/23 penile/anal condylomas) to 35% (5/14) in the case of intraepithelial and invasive cancer. The number and intensity of the nuclear label increased with epithelial differentiation. Replication and maturation of HPV is assumed to be under the control of cellular ("suppressor") genes which may be far more active in proliferating than in differentiating keratinocytes (Zur Hausen 1987). Absence of hybridizing nuclei in basal keratinocytes or absence of any signal on tissues or even filters does not exclude the presence of HPV. In this regard, further insights are to be expected from newly developed, highly sensitive gene analysis techniques (polymerase chain reaction; Scharf et al. 1986; Maitland et al. 1988).

Successful in situ hybridizations could even not always be predicted from the number of koilocytic cells. Buschke-Löwenstein tumours hybridized poorly in spite of the frequency of koilocytes, and -vice versa- some "classical" condylomas and the single positive case of lichen sclerosus et atrophicus hybridized although koilocytes were rare or absent. As yet, we cannot exclude that other HPV types not specifically looked for might be present in the negative lesions. Viral strain specific stringent hy-

bridizations revealed condylomas ("classical", giant, atypical) and the two verrucous carcinomas to contain HPV 6 and/or 11 DNA, while one intraepithelial and two invasive anal cancer harboured HPV 16 and/or 18 DNA. Our observations on giant condylomas/verrucous carcinomas reconcile virological data with the common pathology of giant condylomas/Buschke-Löwenstein tumours/verrucous carcinomas and the concept that these lesions represent a biological continuum (Bogomoletz et al. 1985). Buschke-Löwenstein tumours are probably highly differentiated carcinomas from the onset (Oriel 1987), a view which gained support from the history of one HPV 6 positive penile tumour of our series which evolved into a verrucous carcinoma. Persistent presence of the same HPV type (HPV 16+) was further consistently observed in one recurrent carcinoma in situ, which occurred within the anal canal, the vulva and vagina. Multiple HPV infections, however, have also been documented in multifocal malignancies (McCance et al. 1985; Schneider et al. 1987), and apart from the coexistence of HPV 16 and 18 – as in our case no. 12 – HPV 6 and 16 infections or even triple infections (HPV 6/16/18) have been recorded recently (Winkler et al. 1986).

Some controversy exist whether or not closely related HPV types with homologies in the order of 85% can be distinguished on the basis of in situ hybridizations (Gupta et al. 1987a; Del Mistro et al. 1987). As subgenomic probes are not available to us, we consider HPV 6/11 infections together and do not label them as double infections. Conflicting data have also been reported with respect to stromal hybridizations (Ostrow et al. 1985; Del Mistro et al. 1987). These reactions, however, could be abolished under high formamide concentrations, thus giving support to cross-hybridizations possibly unrelated to HPV.

The question of the frequency of subclinical and submorphological papillomavirus infections (Ferencyz et al. 1985; MacNab et al. 1986; Schneider et al. 1987) and the pathogenetic implications was not the subject of this study, and should be left to the rapidly evolving field of gene technology. The positive case within the lichen sclerosus and atrophicus group illustrates at least the existence of HPV DNA in normal looking epithelia and the current potential of non-isotopic in situ hybridization.

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