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Human papillomavirus type 13 and focal epithelial hyperplasia of the oral mucosa: DNA hybridization on paraffin-embedded specimens

Rolf-Peter Henke [1], Karin Milde-Langosch [1], Thomas Löning [1], and Hanna Strømme Koppang [2]

[1] Institute of Pathology, University of Hamburg, Martinistr. 52, D-2000 Hamburg 20, Federal Republic of Germany
[2] Department of Oral Pathology and Section for Forensic Odontology, University of Oslo, Norway

Summary. 16 cases of focal epithelial hyperplasia (Heck's disease) were studied for the presence of human papillomavirus DNA by means of nucleic acid hybridization. Hybridization was carried out in situ with biotin-labelled probes of HPV 1, 6, 11, 13, 16, and 18 DNA under stringent and non-stringent conditions. Under non-stringent conditions, 6 of 16 cases (38%) hybridized to a mixture of HPV 1, 6, 11, 16, and 18 DNA. When these probes were applied under stringent conditions, only one case could be shown to be weakly positive for HPV 6/11 DNA. Further stringent hybridizations, which were conducted with a HPV 13 probe on 12 of our 16 cases, revealed a positive result in 9 of 12 cases (75%). The results of our study strongly substantiate the concept that HPV 13 or a closely related HPV type is associated with lesions morphologically presenting as focal epithelial hyperplasia.

Key words: Human papillomavirus-Heck's disease – In situ hybridization

Introduction

The term focal epithelial hyperplasia (FEH) was coined for a certain type of mucosal lesions among American Indians in New Mexico, the southwestern United States, the Mato Grosso district in Brazil, and an Alaskan Eskimo published by Archard et al. (1965). Since then, an increasing number of cases has been reported from other parts of the world, including Nigeria (Sawyer et al. 1983), Iraq (Perriman and Uthman 1971), Israel (Buchner and Mass 1973; Buchner et al. 1975; Buchner 1978), South-Africa (Van Wijk et al. 1977a, 1977b; Van Wijk 1977), Sweden (Bergenholtz 1965), and the Federal Republic of Germany (Orfanos et al. 1974; Knoth and Boepple 1978; Petzoldt and Dennin 1980).

The clinical and macroscopic aspects of FEH have been described in detail elsewhere (Reichart et al. 1982; Löning 1984a; Praetorius et al. 1985). Microscopic examination reveals a hyperplastic epithelium with acanthosis. The rete ridges are elongated showing clubbing and anastomosing horizontal outgrowth. In addition to an increased cellular density, parakeratosis is found. Individual cells may stand out due to the enlarged cytoplasm and nuclear fragmentation, giving rise to so called 'pseudomitotic figures'. Koilocytosis, that is, vacuolization of the cytoplasm which is also frequently found in other human papillomavirus (HPV) related lesions of the oral cavity, appears in the superficial cell layers. The stratum proprium is normal in most cases.

The possibly infectious nature of this disease has been proposed on epidemiological grounds in the original paper. Subsequently, HPV was demonstrated in similar lesions by means of electron microscopy (Praetorius-Clausen and Willis 1971; Kuhlwein et al. 1982), immunohistochemistry (Löning et al. 1981; Lutzner 1982; Praetorius et al. 1985), and molecular biological methods (Syrjänen et al. 1985; Lang et al. 1984; Petzholdt and Pfister 1980), thus giving support to the original hypothesis.

Detection and typing of HPV DNA has been achieved by filter hybridization techniques; to our knowledge, no in situ hybridization study of the presence and distribution of HPV DNA in focal epithelial hyperplasia has yet been published.

We had the opportunity to study paraffin-embedded tissue specimens from a number of Norwegian patients who were admitted to hospital with

Offprint requests to: R.-P. Henke at the above address

different clinical diagnoses and whose oral lesions were diagnosed as FEH by light microscopy.

In addition to detection of HPV DNA we tried to evaluate the specific HPV types involved in lesions of FEH. In order to obtain this information, in situ hybridization experiments were performed under conditions of reduced and high stringency. The term 'stringency' relates to the reaction conditions which influence the rate of matching between complementary parts of nucleic acid strands. To distinguish closely related DNA sequences, hybridizations should use a stringent criterion, while for detection of more distantly related members of a family of nucleic acids permissive reaction conditions are chosen.

Thus, our investigation represents an example of the value of in situ hybridization studies for re-evaluation of rare and singular lesions, which have often been carefully collected over the years, in the paraffin files of pathological institutes.

Materials and methods

Tissues. The material consisted of 19 formalin fixed, paraffin-embedded specimens from 16 patients (12 female/4 male) with microscopically verified FEH, collected at the Department of Oral Pathology and Section for Forensic Odontology, Oslo. 15 patients were Caucasians, one patient was of Finnish-Laplandish origin.

All specimens were sectioned and routinely stained with haematoxylin-eosin in addition to nucleic acid hybridization.

Viral probes and specificity controls. For DNA hybridization cloned DNA of HPV types 1, 6, 11, 13, 16, and 18 was used. HPV 1 DNA was a commercially available biotinylated probe (Enzo, New York, USA). HPV 6, 11, 13, 16, and 18 harbouring plasmids (pBR322) were kindly provided by H. zur Hausen and L. Gissmann (German Cancer Research Center, Heidelberg, FRG). After propagation in E. coli, plasmids were harvested followed by cesium chloride gradient centrifugation. Isolated probes were labelled by a nick-translation procedure with biotinylated deoxyuridin triphosphate (Bio-11-dUTP) and a nick-translation reagent kit (Gibco/BRL, Eggenstein).

Hybridization was performed with a mixture of HPV 1, 6, 11, 16, and 18 DNA under non-stringent in addition to hybridization with HPV 1, HPV 6/11, HPV 13, and HPV 16/18 under stringent conditions (see below) in 12 of the 16 cases.

Controls were performed with biotinylated pBR322 DNA (Amersham-Buchler, Braunschweig, FRG) or by omitting specific HPV DNA from the hybridization solution. As further negative controls hybridization was carried out on tissues which never has been reported to harbour HPV 13 DNA so far (normal cervical tissue).

In situ hybridization. Paraffin sections were adhered to poly-D-lysin-coated glass slides and hybridization was performed according to Brigati et al. (1983) with some modifications:

Sections were dewaxed in xylene, rehydrated through a series of alcohol, soaked in phosphate buffered saline (PBS, pH 7.4) for 2×5 min and placed in 0.02 N HCl for 10 min. After two washes for 3 min each in PBS, the slides were incubated for 1.5 min in PBS containing 0.01% Triton X-100 (Fluka Inc., Buchs, Switzerland) and, after an additional two washes in PBS, were treated for about 6 min with pronase (1 mg/ml; Calbiochem, Frankfurt, FRG) dissolved in 0.05 M Tris-HCl, pH 7.6 containing 5 mM EDTA. Prior to use, the pronase was preincubated for 4 h at 42° C in order to digest contaminating nucleases. Pronase treatment was stopped by washing the slides twice for 3 min in PBS containing 2 mg/ml glycine, followed by a 5 min postfixation step in 4% paraformaldehyde. After 2 washes in PBS containing 2 mg/ml glycine, slides were dehydrated through a graded series of alcohol and air-dried.

For hybridization, each section was covered with 20 µl of the following, freshly prepared hybridization solution: $2 \times$ SSC ($1 \times$ SSC $\triangleq$ 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.2), 20% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 0.1 mg/ml herring sperm DNA, and 1.5 µg/ml biotinylated HPV DNA.

Sections were covered with silanized cover slips and after being sealed with rubber cement were denatured by heating in a 90° C water bath for 10 min, followed by hybridization at 37° C overnight.

After hybridization, coverslips were carefully removed and sections were washed twice for 10 min in $1 \times$ SSC, 20% formamide, 37° C (non-stringent conditions, $T_m - 35°$ C), or $1 \times$ SSC, 45% formamide, 37° C (stringent conditions, $T_m - 17°$ C), followed by three washes for 5 min each in double strength SSC at room temperature.

Labelled DNA was detected as follows: Sections were incubated for 1 h at 37° C with a rabbit anti-biotin-antiserum (Enzo), diluted 1:250 in $2 \times$ SSC + 0.75% bovine serum albumin (BSA). After meticulous washing in $2 \times$ SSC, sections were incubated for 1 h with biotinylated goat-anti-rabbit-IgG (Dianova, Hamburg, FRG), diluted 1:250 in $2 \times$ SSC + 0.75% BSA, followed by washing and incubation with 1:1000 diluted streptavidin-alkaline-phosphatase-complex (Gibco/BRL) in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl for 1 h at 37° C. After three washes in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, nitro blue tetrazolium (75 mg/ml in 70% dimethylformamide) and bromo-chloroindolyl-phosphate (50 mg/ml in dimethylformamide; 11 µl respectively 8 µl/2.5 ml of the latter Tris-buffer) were added as substrates. The colour reaction was stopped by soaking the slides in 20 mM Na_2-EDTA. After mounting in glyceryl gelatine the sections were photographed without counterstaining.

Results

Examination of the haematoxylin-eosin stained sections showed that the aforementioned morphological criteria of FEH were fulfilled to a high degree (Tables 1, 2, Fig. 1). Four specimens (cases no. 5, 13, 14, 16) showed mild hypergranulosis which is more typically seen in oral warts and condylomas. Orthohyperkeratosis of moderate degree could be noted in two (cases no. 13, 14) of the four cases with hypergranulosis. Parakeratosis, acanthosis, pseudomitotic figures, and koilocytotic cells were found in all specimens, although to a variable degree. Koilocytotic changes were usually very marked and present already in suprabasal cell layers in some of the cases.

Hybridization under non-stringent conditions with a mixture of HPV 1, 6, 11, 16, and 18 probes revealed a positive reaction in 6 of 16 cases (38%).

Table 1. Clinical data of patients and results of nucleic acid hybridization

Case	Age	Sex	Localization	Hybridization HPV 1/6/11/ 16/18	HPV 13 (strin gent)
1	49	m	Lower lip	–	nd
2	28	f	Upper/lower lip	–	+
3	48	f	Border of the tongue	–	+
4	60	f	Lower lip	+	–
5	47	f	Tip/border of the tongue	+	+
6	45	m	Buccal mucosa	–	–
7	55	f	Upper/lower lip	–	–
8	53	f	Buccal mucosa	–	+
9	85	f	Tip of the tongue	+	nd
10	32	f	Labial angle	–	+
11	67	m	Border of the tongue	–	nd
12	53	f	Labial angle/lower lip	–	+
13	55	f	Buccal mucosa	+	nd
14	22	m	Labial angle	+	+
15	33	f	Border of the tongue	–	+
16	75	f	Lower lip/labial angle	+	+

nd: not determined; f: female; m: male

Table 2. Spectrum and relative frequency of light microscopical changes observed in 16 cases of FEH

Tissue changes	
Acanthosis	16/16
Parakeratosis	16/16
Hyperorthokeratosis	2/16
Hypergranulosis	4/16
Cellular changes	
Koilocytosis	16/16
Mitosis-like figures	16/16
Dyskeratosis	8/16

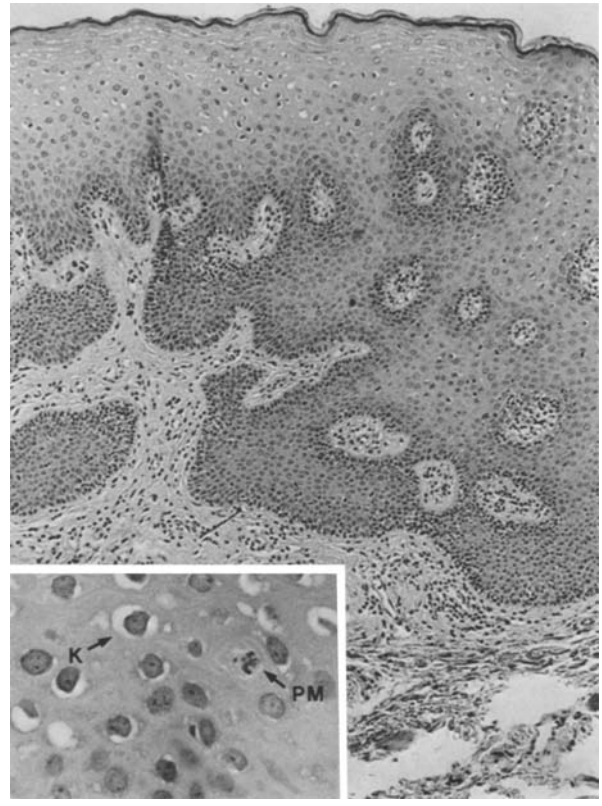

Fig. 1. Focal epithelial hyperplasia (case no. 16). Acanthosis, elongation and broadening of epithelial ridges. At intermediate and superficial cell layers koilocytotic cells. HE, Magnification ×80. *Inset:* Higher magnification of the upper part of the epithelium. Arrows point to koilocytotic cells (*K*) and to mitosis-like figures (*PM*). HE, Magnification ×320

In order to identify more specificially the HPV types involved, hybridization procedures were also carried out under stringent conditions. Due to the lack of tissue material, consecutive stringent hybridizations were performed only on 12 of the 16 cases. Here, 9 of 12 cases (75%) hybridized to the HPV 13 probe under stringent conditions. Hybridizations with HPV 1, HPV 6/11, and HPV 16/18 under stringent conditions were negative in all but one case (case no. 16), which showed a weakly positive result with HPV 6/11.

Detection of HPV DNA was most prominent in nuclei of superficial cell layers, although staining of nuclei in deeper cell layers of the stratum spinosum could be noted in some specimens (Fig. 2). In comparison with superficial keratinocytes, staining of koilocytotic cells was more the exception than the rule. The extent of hybridization in positive cases varied greatly, ranging from a strong reaction in a large number of epithelial cells in some specimens to a patchy distribution of stained cells or very sparse labelling in others.

Nuclear staining was confined to the hyperplastic parts of the biopsies. There was no signal in the adjacent normal mucosa. Specificity controls were performed with biotinylated pBR322 DNA or by omitting specific HPV DNA from the hybridization solution, and were negative in all cases, as were hybridizations with HPV 13 on normal cervix mucosa.

Discussion

Although, as yet, only the first of Koch's postulates is fulfilled, evidence points towards a viral genesis of FEH. Attempts to isolate the causative agent and to propagate it in vitro have failed to date, probably due to the lack of terminal keratino-

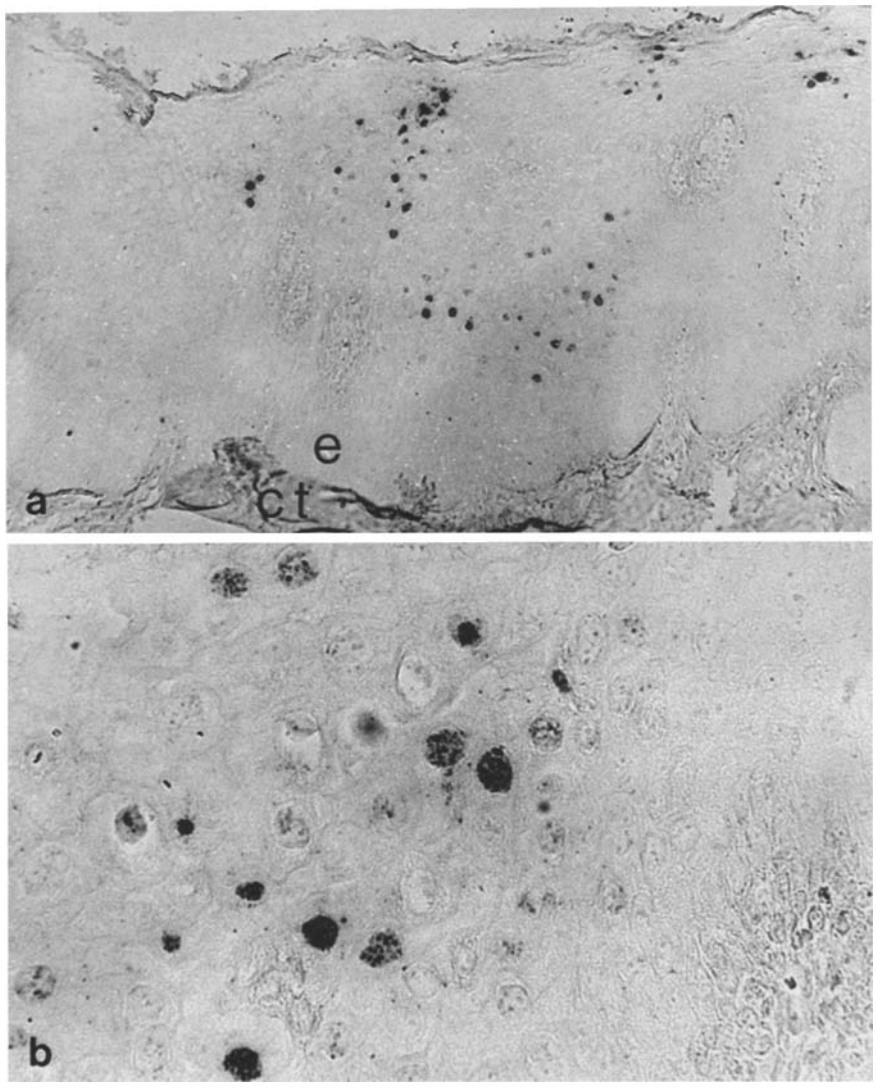

Fig. 2. a FEH, same case as Fig. 1. In situ hybridization with biotinylated HPV 13 probe under stringent conditions. At low magnification, clusters of hybridizing cells are seen at intermediate and superficial cell layers. ct: connective tissue, e: epithelium. Magnification ×80. **b** Higher magnification. In situ hybridization with biotinylated HPV 13 probe under stringent conditions. Note hybridization to be strictly confined to nuclei. Magnification ×500

cyte differentiation in culture systems (Staquet et al. 1981). Maturation of HPV is dependent on differentiation of the genome harbouring cell (Zur Hausen and Gissmann 1980; Staquet et al. 1981).

In 1969, Clausen described morphological changes in epithelial cells in FEH suggesting that these represent host cell reactions to viral infection. Later, papillomavirus-like particles in human FEH were described by Praetorius-Clausen and Willis (1971) and confirmed by studies of Hanks et al. (1972); Kuffer and Pérol (1976); Sattgast and Limmer (1976), and other groups (Van Wijk et al. 1977; Goodfellow and Calvert 1979; Petzoldt and Dennin 1980; Kuhlwein et al. 1982).

In our study, 16 cases of oral focal epithelial hyperplasia were examined by means of light microscopy and nuclei acid hybridization. The data presented in Table 2 indicate that the spectrum of morphological changes which is considered to be diagnostic of FEH (Löning 1984a, 1984b) was present in the great majority of cases. Nevertheless, four cases were included showing hypergranulosis with hyperorthokeratosis in two of them; changes which are more typically found in oral warts and condylomas. In such cases an exact classification based on light microscopic criteria alone is too imprecise. In order to overcome these limitations, increasing attention has been paid to the advanced techniques of immunocytochemistry and nucleic acid hybridization.

Immunocytochemistry, however, does not provide additional information since specific antibodies for the HPV types in question are not available. In contrast, preparation of biotinylated viral probes of defined type and the development of hybridization strategies on paraffin-embedded samples opens the door for a further diagnostic approach to FEH.

Detecetion of papillomaviruses in lesions of FEH by means of nucleic acid hybridization has been performed on a limited number of cases. In these studies, HPV 1 (Petzoldt and Pfister 1980) and later HPV-13 related DNA (Pfister et al. 1983; Syrjänen et al. 1984) were demonstrated in FEH. Lang et al. (1984) isolated DNA from lesions of FEH which reacted both with HPV 1 and HPV 18. In three and five cases examined by de Villiers et al. (1986a, 1986b) HPV 6 and HPV 13-related sequences were found.

In our in situ hybridization study, HPV 13 DNA was detected under stringent conditions in 9 of 12 cases, compared with 6 of 16 positives when a mixture of HPV 1, 6, 11, 16, and 18 probes was used under non-stringent conditions. Under stringent conditions, all but one case were negative when sections were hybridized to HPV 1, HPV 6/11, and HPV 16/18. According to sequence homology, HPV 6 and 11 are grouped together with HPV 13 (Pfister et al. 1986). Therefore, some cross reactivity between HPV 6/11 and HPV 13 could explain the weak positivity of case no. 16 for HPV 6/11. Interestingly, from the four cases which were initially regarded as doubtful FEH lesions on the basis of light microscopic criteria (cases no. 5, 13, 14, 16), three could be shown to hybridize stringently to HPV 13 (Table 1; for lack of material, the presence of HPV 13 was not determined in case no. 13).

How could failure to detect HPV nucleic acids be explained?

First, in some cases staining was sparse and confined to only a part of the examined sections. Thus, positive cells are easily missed in small biopsies, when serial sections cannot be obtained for lack of material. Second, working on formalin-fixed, paraffin-embedded material probably lowers the percentage of positive reactions in comparison with freshly frozen tissue. Strong fixation with formalin can render nucleic acids unaccessible for viral probes (Unger et al. 1986). Third, the low amount of HPV DNA in some lesions could be out of the sensitivity range of the used detection system. Some hundred copies of HPV DNA are estimated to be necessary for a positive signal (Crum et al. 1986). Thus, in lesions which are productive to a lesser degree at the time of biopsy the copy number might be beneath the detection limit.

It is, of course, possible that in some of our negative cases other HPV types, as yet not included in our panel of HPV probes, are present and relevant. Our study, however, demonstrates the markedly high frequency of HPV 13 infection in FEH and thus confirms the association of HPV 13 with lesions of FEH. The question whether the role of this virus in the pathogenesis of FEH is a causal or a casual one is beyond the scope of this study and remains to be solved. Our experiments further underline the importance of type-specific viral probes and conditions of high stringency for sensitivity and specificity of in situ hybridization.

Further studies are needed to assess the prevalence of HPV13 infection in a larger series of normal appearing mucosa and in lesions other than FEH in order to confirm the specificity of HPV 13 for this disease.

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