Virchows Archiv A Pathol Anat (1992) 420:295–300

Virchows Archiv A
Pathological Anatomy and Histopathology
© Springer-Verlag 1992

In situ hybridization with digoxigenin-labelled DNA probes for the detection of human papillomavirus-induced focal epithelial hyperplasia among Venezuelans

Gloria Premoli-de-Percoco [1], Ivan Galindo [2], and José Luis Ramirez [2]

[1] Instituto de Investigaciones "Raúl Vincentelli", Facultad de Odontologia, [2] Centro de Biologia Celular, Universidad Central de Venezuela, Caracas, Venezuela

Received June 17, 1991 / Received after revision August 30, 1991 / Accepted September 6, 1991

Summary. An in situ hybridization assay with digoxigenin-labelled probes was used to detect the presence of human papillomavirus (HPV) sequences in ten related Venezuelan patients with the diagnosis of focal epithelial hyperplasia. The samples displayed HPV sequences in all cases. Further restriction analysis in four of the patients suggested the presence of HPV-13 in oral lesions.

Key words: Human papillomavirus – Focal epithelial hyperplasia – In situ hybridization

Introduction

Focal epithelial hyperplasia (FEH) is one of the oral squamous cell lesions caused by human papillomavirus (HPV). The lesions appear as painless multiple nodular soft tissue masses distributed over the mucosal surfaces, especially the buccal and labial mucosa, and the tongue. These lesions have the same colour as the adjacent mucosa. The patients often do not notice their presence until they become aesthetically disturbing or are traumatized. The lesion occurs mainly in children and young adults, with frequent manifestations within the same family. The lesions may persist for several years but do not become malignant and finally tend to remit spontaneously, with occasional recurrence.

The condition, previously described by other groups (March 1881; Estrada 1956; Reyes 1962; Soneira and Fonseca 1964), was defined as a distinct entity by Archard et al. in 1965. FEH has been found with variable incidence in particular ethnic groups, mainly in Eskimos and American Indians (Beaudenon et al. 1987; Van Wyk et al. 1977; Henke et al. 1987) where familial occurrences have been observed (Gomez et al. 1969). Nevertheless, the role of genetic factors in susceptibility to infection by HPV remains obscure.

Offprint requests to: G. Premoli-de-Percoco, Apartado postal 51513, Sabana Grande, Caracas 1050, Venezuela

The association of HPV with FEH was not appreciated until 1970, when Praetorius-Clausen and Willis (1971) described viral particles in these lesions. The observation was confirmed independently by several researchers (Hanks et al. 1972; Kuffer and Perol 1976; Sattgast and Limmer 1976; Van Wyk et al. 1977; Goodfellow and Calvert 1979; Petzoldt and Dennin 1980; Kuhlwein et al. 1982). The relationship of FEH to a specific HPV type (HPV-1) was originally reported in a single case using DNA-DNA hybridization (Petzoldt and Pfister 1980). Lang et al. (1984) isolated DNA from lesions of FEH which reacted both with HPV-1 and HPV-18. HPV-6-related sequences have been detected in two patients (de Villiers et al. 1986). In addition, HPV-13 (which has weak cross-hybridization with HPV-6 and HPV-11) and HPV-32 have been isolated in Danish and Greenlandic patients (Pfister et al. 1983; Syrjanen et al. 1984; Beaudenon et al. 1987; Henke et al. 1987). Since HPV-13 and HPV-32 have only been reported infrequently in other oral lesions, they appear to be specific to FEH.

In the present work, we have used in situ hybridization with digoxigenin-labelled DNA probes (D-L HPV probe) in paraffin-embedded tissue specimens, to search for HPV retrospectively. Ten Venezuelan patients were admitted to the hospital with different clinical diagnoses and their oral lesions were identified as FEH by light microscopy. The sample, carefully collected through the years, reveals a tight correlation between FEH and the presence of HPV genomes associated with the cell nuclei, as well as the involvement of genetic factors.

Materials and methods

The biopsies were taken from patients with FEH, as assessed by the criteria outlined below (Pilgard 1984; Praetorius et al. 1985). All specimens were fixed in neutral formalin and embedded in paraffin. Seven cases (each corresponding to a different family group) was undertaken for study. In one case, four relatives (patients 7–10; Table 1) were available for DNA extraction. The familial aggregation, patients 7–10, was detected in a direct genealogy study and was traced to six generations of the kindred. In the

Table 1. Clinical characteristics of patients and results of the in situ hybridization

Patient	Age (years)	Sex	In situ hybridization	Familial aggregation	Race
1	14	F	+	Yes	Me
2	6	F	+	Yes	Me
3	6	M	+	Yes	Me
4	7	F	+	Yes	Me
5	7	M	+	Yes	Me
6	24	F	+	Yes	Me
7	8	M	+	Yes	Me
8	15	F	+	Yes	Me
9	14	F	+	Yes	Me
10	15	F	+	Yes	Me

Me, Mestizo

remaining cases an evident familial aggregation was obtained from clinical records (patients 1–6). The samples were provided by the Department of Oral Pathology, Universidad Central de Venezuela.

The following diagnostic characteristics of FEH were required. The lesions appear as nodular elevations, where epithelial acanthosis, prominent clubbing and anastomosis of epithelial ridges with basal orientation is observed. Mild hyperparakeratosis is usually present. Enlarged ballooning cells with abnormal nuclear chromatin patterns and multinucleated cells are also common. Cells immediately beneath the surface often display large cytoplasmic vacuolization with deeply basophilic nuclei.

The detection of viral sequences in the biopsy samples was achieved through in situ hybridization on paraffin-embedded sections with cloned genomes from HPV types 6, 11, 16 and 18. The cloned DNAs were generously provided by Drs. H. zur Hausen and L. Gissmann, Heidelberg, Federal Republic of Germany. The recombinant plasmids were amplified and purified following standard techniques (Maniatis et al. 1982). The DNA probes were labelled by random priming with digoxigenin-11-dUTP according to the manufacturers' recommendations (Genius, DNA labelling and detection kit; Boehringer, Mannheim, FRG).

Histological sections (4–6 μm) were cut and mounted on poly-L-lysine-coated slides. After deparaffinization, the tissue sections were hydrated (through xylene and graded ethanols), pretreated with 0.2 M HCl for 10 min, then with phosphate buffered saline (PBS, pH 7.4) containing 0.2% glycine (w/v) for 6 min, and followed by 1 h of postfixation in 4% paraformaldehyde in PBS. After washing in PBS containing 0.2% glycine (w/v), slides were dehydrated (graded ethanols), and air dried.

Two genital condyloma and two oral condyloma were used as positive controls (P). Negative controls (N) consisted of normal oral tissues. Sections were examined by routine light microscopy. In situ DNA hybridization positivity was identified as a deep blue nuclear reaction product in transmitted light microscopy.

For in situ hybridization the tissue section specimens were hybridized with D-L HPV probe. After 2 h (37° C) of prehybridization in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.0015 M sodium citrate pH 7.0), 20% formamide, 5 × Denhardt mixture (0.2% bovine serum albumin, 0.2% Ficoll, 0.2% polyvinyl pyrrolidone) (Denhardt 1966), and 100 μg/ml heat-denatured salmon sperm DNA, the HPV DNA probe was adjusted to a final concentration of 40 ng/ml to the hybridization mixture (6 × SSC, 20% formamide, 10% dextran sulphate, 5 × Denhardt mixture). The target and probe DNAs were simultaneously denatured in a 92° C waterbath (boiling water) for 10 min in a humidified chamber. The hybridization was carried out under an airtight cover glass sealed with rubber cement. The specimens were hybridized with HPV-6 and HPV-11, overnight at 37° C in the same humidified chamber under low stringency conditions (−42° C) to detect the presence of HPV genomes.

After hybridization, the cover glasses were removed, submerged in 6 × SSC at room temperature (RT). After washing in 6 × SSC, 2 × SSC and 0.5 × SSC (42° C), the slides were blocked for 30 min in 2% blocking reagent solution (Boehringer). Then they were washed for 1 min in buffer I (0.1 M TRIS-HCl, pH 7.5, 0.15 M NaCl), and incubated with Dig-AP conjugate (polyclonal sheep anti-digoxigenin alkaline phosphatase conjugate) in buffer I for 2 h, washed with buffer I three times for 15 min each, and finally equilibrated in buffer III (0.1 M TRIS-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$).

The colour reaction was developed overnight under a nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate solution (NBT/BCIP) at RT in complete darkness. The colour reaction was stopped with TE [10 mM TRIS HCl, 1 mM ethylene diamine tetraacetic acid (EDTA), pH 8.0].

The slides were dehydrated through graded ethanols, counterstained with haematoxylin, cleared in xylene, coverslipped, and mounted with Permaunt.

DNA was extracted from biopsies from patients with FEH (nos. 7–10), with normal tissue (N), and with oral condyloma (P) as follows: The solid material (approx. 10–100 μg) was frozen at −70° C and ground. The treated sample was resuspended in 3 volumes of 10 mM TRIS-HCl, pH 7.4, 100 mM NaCl, 10 mM EDTA. Lysis was achieved by the addition of sarcosyl up to 1%, proteinase K 20 ng/μl. Most proteins were eliminated at 56° C during 3 h of incubation. The samples were then treated three times with a chloroform:phenol (1:1) mixture. The DNA was precipitated overnight by the addition of 2.5 volumes of absolute ethanol at −20° C. The precipitated DNA was resuspended in TE and treated with ribonuclease A (20 μg/ml) for 1 h at 56° C. The DNA was repeatedly phenol/chloroform extracted, until all traces of cellular protein were removed. The final supernatant was concentrated by addition of 0.1 volume 3 M sodium acetate and 2.5 volumes of ethanol. The DNA precipitate was ethanol washed, dried and redissolved in TE to a concentration of 0.5 μg/μl.

For dot blot hybridization aliquots containing 40 μg DNA were placed into the manifold apparatus (Schleicher and Schuell, Dassel, FRG) and absorbed to Hybond-N filters (Amersham, Buckinghamshire, UK) by suction. The DNA was heat denatured (95° C, 10 min) and chilled on ice. Then, the air-dried nylon membrane was baked for 60 min at 80° C. Prehybridization was carried out for 90 min at 42° C in the following solution: 6 × SSC, 40% formamide, 5 × Denhardt's solution and 100 μg/ml denatured salmon sperm DNA. The hybridization was conducted overnight in the same solution containing 40 ng/ml of HPV-DNA 6 and 11 probes with digoxigenin and previously denatured at 95° C for 10 min. After hybridization, the filter was washed in several changes of 2 × 15 min with 2 × SSC, 0.1% sodium dodecyl sulphate (SDS) at RT, 2 × 15 min with 1 × SSC, 0.1% SDS at 42° C.

Immunological detection of hybridized D-L HPV probe was performed according to the instructions in the Genius Kit.

Southern blotting was performed in order to typify the HPV DNA sequences present in the biopsies (patients 7–10); 10 μg of total DNA was cleaved with *Bam*HI restriction endonuclease (Bethesda Research Laboratory md., USA), and run in 0.8% agarose gel electrophoresis (Sigma, MO, USA). The gels were stained with ethidium bromide and photographed under ultraviolet light. Finally, the bands were denatured and neutralized, and transferred to Hybond-N filters (Amersham). The membrane was prehybridized, and hybridized, and washed at intermediate stringency as above.

Results

Ten biopsies of FEH conforming with the histological criteria previously described were included in this study. Adequate data on familial aggregation were found in all cases (Table 1). Age was specified with a mean of 11.6 years (range 6–24). The first and second decades

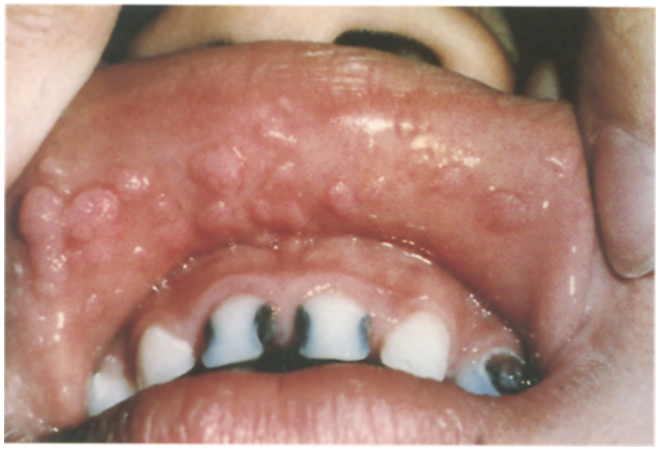

Fig. 1. Focal epithelial hyperplasia (FEH) on the upper lip mucosa (patient 3). The lesions were multiple, with scattered growth over the oral mucosa, particularly in the lip and tongue. No lesions were seen in the floor or the mouth

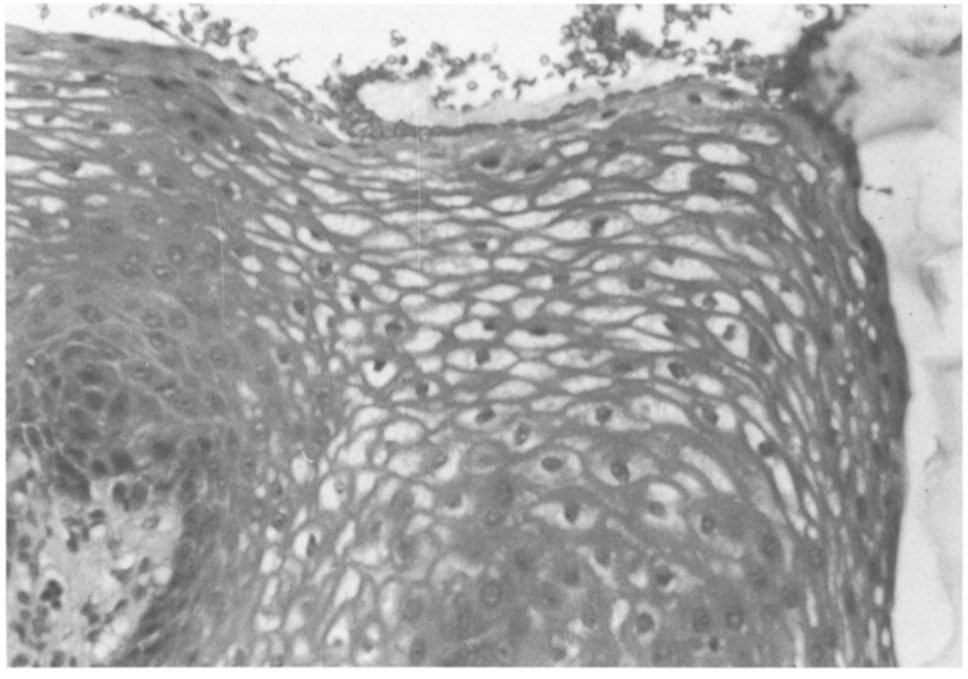

Fig. 2. Human papillomavirus (HPV) positive (patient 3) showing flattened surface with minimal or no keratosis and marked acanthosis. Koiliocytosis is unusually prominent. H & E, ×200

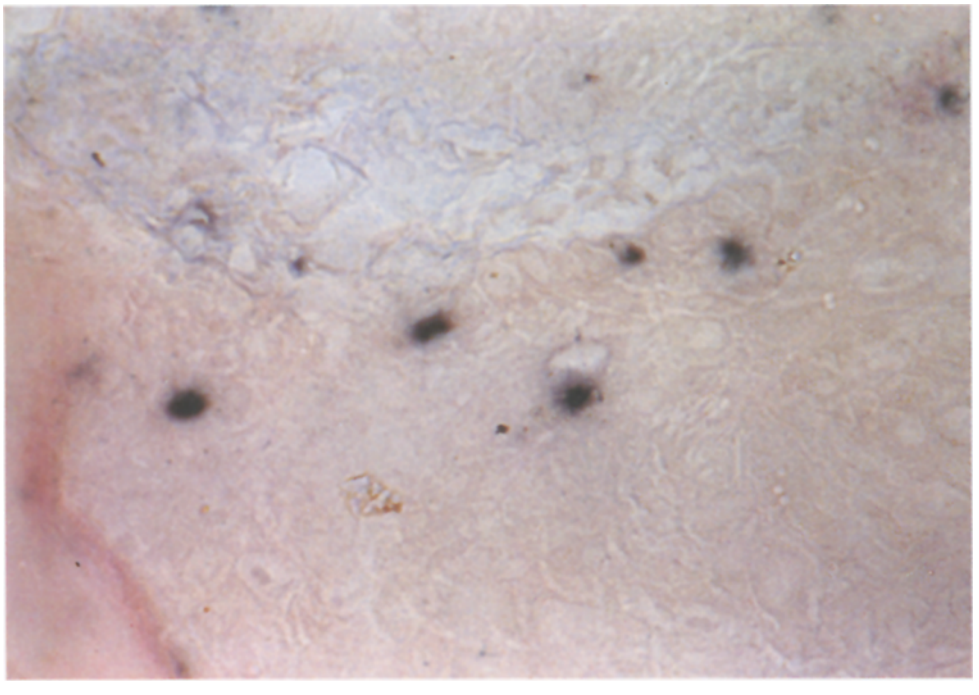

Fig. 3. In situ hybridization with digoxigenin HPV-6 and HPV-11 probes under low stringency conditions. Note hybridization strictly confined to nuclei. ×400

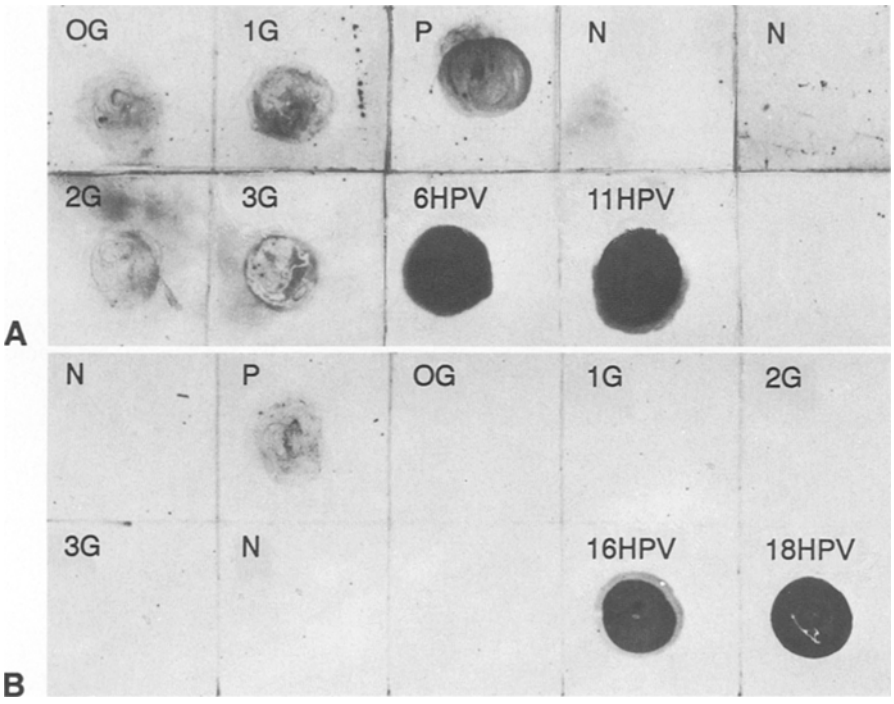

Fig. 4A, B. Confirmation of the positivity of the in situ hybridization results. Biopsies were DNA-extracted [patients 7–10 with FEH; patient with normal tissue (*N*); patient with oral condyloma (*P*)] and blotted on to a nitrocellulose membrane and hybridized with probes HPV-6 and -11 and HPV-16 and -18 labelled with digoxigenin under medium stringency conditions. Negative for HPV-16 and -18

accounted for 90% of the cases. All were Mestizo (the offspring of a Spaniard and an American Indian); 70% were females and 30% males. In two cases, spontaneous regression and recurrence were reported. The lesions were multiple, with scattered growth over the oral mucosa, particularly in the lip and tongue. No lesions were observed on the floor of the mouth (Fig. 1). Examination of the haematoxylin and eosin-stained sections fulfilled the morphological criteria for FEH to a high degree (Fig. 2). Parakeratosis, acanthosis, pseudomitotic figures and koilocitotic cells were found in all specimens, although to a variable degree. Koilocytotic changes were very marked and present already in suprabasal cell layers in some of the cases.

In situ hybridization under low stringency conditions with a mixture of D-L HPV probes revealed a positive reaction in all cases. Detection of HPV DNA was most prominent in the nuclei of superficial cell layers, although staining of nuclei in deeper cells layers of the stratum spinosum was observed in some specimens (Fig. 3). 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. In order to obtain an approximation to the HPV types involved, hybridization procedures were carried out under medium stringent conditions with HPV-6 and 11 and HPV-16 and 18. The hybridization signal was only observed with the HPV-6 and 11. As a guide to the specificity of the process, sections of virus-positive oral and genital condylomata reacted positively with D-L HPV probe (P). In order to ascertain the specificity controls (oral and genital condylomata) were performed by omitting D-L HPV probe from the hybridization solutions (N). For further control, hybridization solution with D-L HPV probe was used on normal oral sections (N).

To confirm the positivity of the in situ hybridization results, biopsies were DNA-extracted and blotted on to a nitrocellulose membrane and hybridized with probes of HPV genomes (types 6, 11 and types 16, 18) labelled with digoxigenin under medium stringency conditions (Fig. 4). Next, using the viral probes available, we decided to get closer to the identity of the viral types involved through the analysis with *Bam*HI. Total biopsy DNA was digested with *Bam*HI endonuclease, electrophoresed, and transferred to membrane filter (Southern 1975). After hybridization with digoxigenin-labelled HPV-6 and 11 under medium stringency, the characteristic HPV-13 *Bam*HI pattern was observed (Pfister 1983), i.e. 5.3, 1.9, and 0.66 kbp respectively, thus indicating the involvement of this viral type in the lesions under study (Fig. 5). The total DNA extraction from the samples was only possible in four cases and prolonged colour development was necessary to achieve the result. This explains the heavy background in Fig. 5.

Discussion

Although FEH is a rare condition, a differential incidence in ethnic populations and familial occurrence have been observed. Our results demonstrate an evident familial aggregation. Indeed, the fact that four patients presented a strong American Indian genetic component, coupled with occurrence among cousins and siblings, suggests a possible genetic factor influencing the susceptibility to oral infection by HPV. This hereditary aspect

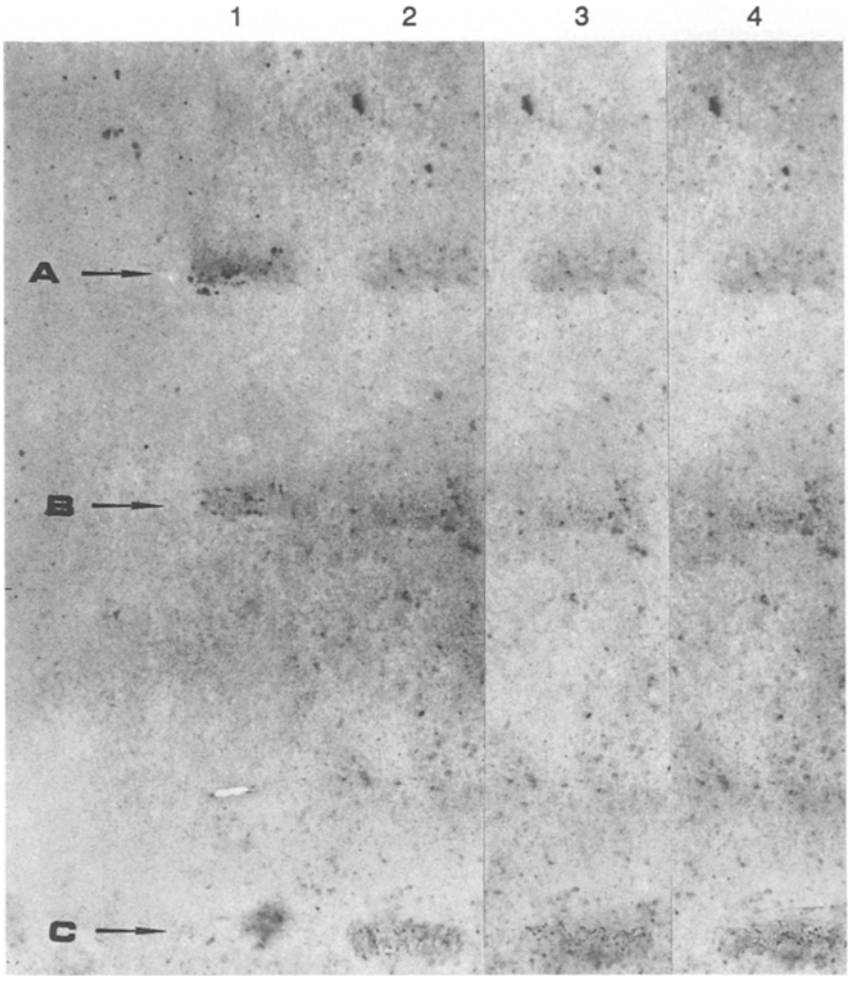

Fig. 5. Southern blots of total biopsies DNA digested with *Bam*HI endonuclease. Lesions from patients 7–10 were electrophoresed and transferred to membrane filter. After hybridization with digoxigenin-labelled HPV-6 and -11 under medium stringency, the characteristic HPV-13 *Bam*HI pattern was observed, i.e. 5.3(*A*), 1.9(*B*), and 0.66(*C*) kbp respectively, thus indicating the involvement of this viral type in the lesions under study

is currently under study in order to clarify the elements involved.

The cases presented here are clearly compatible with the clinical picture for FEH; there are multiple, discrete and confluent papules on the labial, buccal and gingival mucosa, as well as on the tongue and palate. All the cases studied by light microscopy satisfied our criteria.

The presence of HPV-6 and HPV-11-related sequences in all cases confirms previous reports regarding the involvement of HPV in FEH. Pfister et al. (1983) found that FEH-HPV shows 4% cross-hybridization with HPV-6 and 3% with HPV-11. This could explain the observed cross-reaction between these two viruses under medium stringency conditions. Although the sequence of HPV-13 has not been published, hybridization experiments reveal some homology with viral types 6 and 11, and very little with types 16 and 18. The different hybridization conditions used by other authors may explain the association of FEH to other viral types (Syrjanen et al. 1984; de Villiers et al. 1986; Beaudenon et al. 1987). Although it has been impossible for us to obtain a specific probe for HPV-13 and HPV-32, the typical *Bam*HI restriction pattern observed in our results, supports the involvement of HPV-13 in the sample examined. Not enough samples were available to perform a Southern analysis of undigested viral DNA; nevertheless, since we obtained the same restriction pattern for the whole viral genome and the fact that nobody has been able to observe integrated HPV-13 leads us to conclude that the virus is present in its episomal form (Hernandez-Jauregui et al. 1987).

Recently, in situ DNA hybridization techniques have been introduced and successfully applied in HPV typing with radioactive and biotinylated DNA probes. The non-radioactive digoxigenin detection system for in situ hybridization was specific, simple and sensitive enough to be used for retrospective and routine screening of HPV-suspect oral lesions, without radioactive hazard or the assistance of highly trained personnel. In addition, the test allows the study of the tissue morphology, along with the precise location of the viral particle, without background staining.

Acknowledgements. We gratefully acknowledge Mariela Luna and all those who have helped us during this study. This study was supported by grant 11.30.2166.89. from Concejo Desarrollo Cientifico y Humanistico – Universidad central de Venezuela, from Concejo Nacional de Investigaciones Cientificas y Tecnologicas, and from Fundacion Polar, Venezuela.

References

Archard HO, Heck JW, Stanley HR (1965) Focal epithelial hyperplasia: an unusual oral mucosal lesion found in Indian children. Oral Surg 20:201–212

Beaudenon S, Praetorius F, Kremsdorf D, Lutzner M, Worsaae N, Pehaw-Arnaudet G, Orth G (1987) A new type of human papillomavirus associated with oral focal epithelial hyperplasia. Invest Dermatol 88:130–135

Denhardt DT (1966) A membrane filter technique for the detection of complementary DNA. Biochem Biophys Res Commun 23:641

De Villiers EM, Neumann C, Le JY, Weiduer H, zur Hausen H (1986) Infection of the oral mucosa with defined types of human papillomaviruses. Med Microbiol Immunol (Berl) 174:287–294

Estrada L (1956) Aporte al estudio odontologico de los Indios Katios. Heraldo Dental 2:5

Gomez A, Calle C, Arala G, Pindborg JT (1969) Focal epithelial hyperplasia in a half-breed familial of Colombians. J Am Dent Assoc 79:663–667

Goodfellow A, Calvert H (1979) Focal epithelial hyperplasia of the oral mucosa. Br J Dermatol 101:341–344

Hanks CT, Fischman SL, Guzman MN de (1972) Focal epithelial hyperplasia. A light and electron microscopic study of one case. Oral Surg 33:934–943

Henke RP, Milde-Langosch K, Loning T, Koppang HS (1987) Human papillomavirus type 13 and focal epithelial hyperplasia of the oral mucosa: DNA hybridization on paraffin embedded specimens. Virchows Arch [A] 411:193–198

Hernandez-Jauregui P, Eriksson A, Tamayo Perez R, Pettersson U, Moreno-Lopez J (1987) Human papillomavirus type 13 DNA in focal epithelial hyperplasia among Mexicans. Arch Virol 93:131–137

Kuffer R, Perol Y (1976) Hyperplasie epitheliale focale. Premier cas français. Mise en évidence d'un virus papova en microscopie électronique. Rev Stomatol 77:318–321

Kuhlwein A, Nasemann T, Janner M, Schaeg G, Reinel D (1982) Nachweis von Papillomaviren bei fokaler epithelialer Hyperplasie Heck und die Differentialdiagnose zum weiben Schleimhautnävus. Hautarzt 32:617–621

Lang E, Zabel M, Ikemberg H (1984) Fokale epitheliale Hyperplasie (Morbus Heck), Dtsch Med Wochenschr 109:1763–1766

Maniatis T, Fritsh EF, Sambrook J (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

March CJ (1881) Multiple papillary tumors of labial, buccal and glossal mucous membrane. Dent Cosmos 23:165

Petzoldt D, Dennin R (1980) Isolierung virusartiger Partikel bei fokaler epithelialer Hyperplasie Heck. Hautarzt 31:35–36

Petzoldt D, Pfister H (1980) HPV1 DNA in lesions of focal epithelial hyperplasia Heck. Arch Dermatol Res 268:313–314

Pfister H, Hettich I, Runne U, Gissmann L, Chilf GN (1983) Characterization of human papillomavirus type 13 from focal epithelial hyperplasia Heck lesions. J Virol 47:363–366

Pilgard G (1984) Focal epithelial hyperplasia. Oral Surg 57:540–543

Praetorius-Clausen F, Willis JM (1971) Papova virus-like particles in focal epithelial hyperplasia. Scand J Dent Res 79:362–365

Praetorius F, Praetorius-Clausen P, Mogeltoft M (1985) Immunohistochemical evidence of papillomavirus antigen in focal epithelial hyperplasia. Tandlaegeblaadet 89:585–589

Reyes DG (1962) Verruga de la cavidad oral. Riv Colegic Med (Guatemala) 13:223

Sattgast JE, Limmer BL (1976) Focal epithelial hyperplasia: a case report with light and electron microscopic findings. J Assoc Mil Dermatol 2:41–44

Soneira A, Fonseca N (1964) Sobre una lesion de la mucosa oral en los niños de la Mision los Angeles del Tokuko. Venezuela Odont 29:109

Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98:503–517

Syrjanen S, Syrjanen K, Ikenberg H, Gissmann L, Lamberg M (1984) A human papillomavirus closely related to HPV 13 found in a focal epithelial hyperplasia lesion (Heck disease). Arch Dermatol Res 276:199–200

Van Wyk CW, Staz J, Farman AG (1977) Focal epithelial hyperplasia in a group of South Africans: its ultrastructural features. J Oral Pathol 6:14–24