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Human Papillomavirus in Oral Premalignant Lesions

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The aetiology of oral premalignant lesions is generally accepted to be multifactorial. Tobacco and alcohol are established as important cofactors in malignant development in the oral cavity, but in addition microorganisms, such as human papillomavirus (HPV), have gained much interest over the past decade. For many years, HPV has been accepted as an important cofactor in the development of cervical cancer, originating from a mucous membrane with similarities to the oral mucosa. 49 patients with oral premalignant lesions and 20 control patients with normal oral mucosa and no history of HPV infection were examined for the presence of HPV by immune histochemical staining using the peroxidase anti-peroxidase technique (PAP), DNA–DNA *in situ* hybridisation (ISH), and polymerase chain reaction (PCR) analysed by Southern blot hybridisation with an HPV 16 specific probe. The investigations revealed that HPV was found in 62.5% of the verrucous leucoplakias, 50.0% of the erythroplakias, 45.5% of the homogeneous leucoplakias, 33.3% of erythroleucoplakias and in 12.5% of the nodular leucoplakias. An overall HPV detection rate in the examined premalignant lesions was 40.8% and no patients in the control sample were positive. Concerning oral cancer development, it seems likely that HPV may be a cofactor, as 100% of patients who developed oral cancers within 4–12 years were all positive for HPV, one being positive for HPV 16. Copyright © 1996 Elsevier Science Ltd

Keywords: human papillomavirus, *Candida albicans*, oral premalignant lesions, leucoplakia, erythroplakia, normal oral tissue, malignant transformation

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INTRODUCTION

Oral premalignant lesions are defined as a morphologically altered tissue in which cancer is more likely to occur than in its apparently normal counterpart [1]. The malignant transformation rates range from 1 to 19% [2–6]. Examples of premalignant lesions are leucoplakia and erythroplakia. The prevalence of oral leucoplakia in an unselected adult Swedish population was 3.6%, the most frequently affected being the middle-aged and elderly [4–6]. Oral leucoplakia has been subtyped into homogeneous and non-homogeneous lesions and particularly the non-homogeneous type seems to possess a high potential for malignant transformation [1, 7–11]. The aetiology of oral premalignant lesions is generally accepted to be multifactorial [2, 12], tobacco and alcohol being important cofactors in the transition from premalignancy to malignancy [12–14]. However, these factors were not involved in several well-documented cases [7, 15–17], so speculations on the possible involvement of a virus evolved [18]. In particular, human papillomavirus (HPV) type 16 and type 18 have gained much interest because these viruses for more than a decade have been accepted as important correlates of cervical cancer [19–21]. In the oral cavity HPV 16 has been shown in both homogeneous leucoplakia, verrucous leucoplakia, and in normal oral mucosa [22–26]. Oral squamous cell carcinomas have also been shown to contain HPV 16 [23, 25–27] and HPV type 2 and type 11 have been found [25, 28–32]. However, the role of HPV in the aetiology of oral premalignant lesions and in the transition into malignancy is still uncertain. The purpose of the present study was to reveal the possible presence of HPV genus specific antigen and, HPV DNA type 16 in the initial lesion of a sample of oral premalignant lesions with a long follow-up period. Furthermore, it was the purpose to correlate the presence of HPV genus specific antigen, and HPV type 16 DNA to clinical and

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histological manifestations and finally to correlate oral HPV infection to later malignant transformation and well-known co-factors of malignant transformation.

MATERIALS AND METHODS

Study population

The study population included excision material from 49 patients (25 women, 24 men; median age: 67 years (range: 34–94 years)) referred to the Department of Oral Surgery and Oral Medicine, University Hospital (Rigshospitalet), and Department of Oral Surgery, School of Dentistry, Faculty of Health Sciences, University of Copenhagen for treatment of oral premalignant lesions [33]. The patients were included in the study if a pre-operative biopsy had shown epithelial dysplasia or the lesion was situated on the lateral borders of the tongue or in the floor of the mouth. The distribution of the clinical diagnoses is seen in Table 2. All the included patients had their initial lesions excised and received antimycotic treatment for 4–6 weeks before the operation if the pre-operative biopsy showed signs of Candidal infection as revealed by the presence of hyphae-like structures in PAS-stained sections. The patients were followed for a median period of 6.3 years (range: 3–14 years) after surgery.

From archive material which has served as normal controls in former investigations in the Department of Oral Pathology, School of Dentistry, Faculty of Health Sciences, University of Copenhagen, a control group of 20 patients, 12 females and 8 males with clinically and histologically normal oral mucosa was selected. The control sample had the following topographical distribution: 14 from the buccal mucosa/lip, 3 from regio sublingualis, and 3 from the tongue. The controls were selected to match the age and gender of the patients. The median age of the control group was 60.0 years (range: 39–79 years).

All surgical specimens were fixed in 10% neutral buffered formalin and embedded in paraffin. Sections (5 μm thick) were cut for routine light microscopy, immunohistochemistry staining using peroxidase anti-peroxidase technique (PAP), and DNA–DNA *in situ* hybridisation. The histological examination was carried out on haematoxylin–eosin (HE) stained sections and, if adequate biopsy material allowed it, two areas from each lesion were included for further analysis, one with severe dysplasia, the other one with no or slight epithelial dysplasia. The evaluation of the degree of dysplasia was based on criteria, as previously described [34]. The biopsy from each patient in the control group had a similar histological examination. The study was approved by the Danish Committee of Ethics (No. KF 03-008/94).

Detection of HPV genus specific antigen

An immunohistochemical method (peroxidase anti-peroxidase technique (PAP)) was used to detect HPV genus specific antigen. The sections were dewaxed in xylene and endogenous peroxidase activity was blocked with methanol and H_2O_2. After that the sections were rehydrated through alcohol to water. To block unspecific background staining the sections were incubated for 1.5 h with normal swine serum (normal swine serum, DAKO X 901). The sections were then incubated overnight at 4°C with a primary antibody against common papillomavirus antigen (rabbit anti-bovine BPV-1 antiserum, DAKO B 580). The sections were then washed in a PBS standard buffer and incubated for 30 min with a secondary antibody (swine anti-rabbit immunoglobulin, DAKO Z 196), followed by a new wash with standard PBS buffer and incubation for 30 min with peroxidase anti-peroxidase complex (PAP) (peroxidase-rabbit anti-peroxidase, DAKO Z 113). Finally, the sections were incubated with DAB (diaminobenzidine) for 10 min, dehydrated and mounted with Eukitt. A staining reaction was registered positive if a dark brown colour of the cell nucleus was found. Staining procedure included three sections from each biopsy. Two sections were incubated with primary antibody and one of them was counterstained with Mayer's haematoxylin to ensure the orientation of the specimen. In one section, the primary antibody was substituted by a control rabbit serum (normal rabbit immunoglobulin, DAKO X 903). The PAP technique was further controlled by staining a specimen with known HPV content (oral condyloma) and by staining a specimen without any history of HPV infection (a radicular odontogenic cyst). All specimens were read by the first author (H.N.) and independently by a trained pathologist (F.P.) without information on the origin of the specimen.

DNA–DNA in situ *hybridisation*

In situ hybridisation (ISH) was performed on paraffin embedded tissue sections placed on organosilane pretreated slides. Sections were baked overnight at 60°C on the pre-treated slides and after that dewaxed in xylene and passed through alcohol and air dried. The sections were incubated for 15 min in a moist chamber at 37°C with proteinase-K (0.5 mg/ml, Boehringer-Mannheim Biochemicals). The sections were then rinsed in PBS, passed through alcohol and air dried. A DNA probe consisting of a mixture of the following biotin-labelled HPV types 6, 11, 16, 18, 31 and 33 (BIOHIT OY, Helsinki) were slightly modified in order to reduce the costs of the ISH technique. One millilitre of probe mixture consisted of 100 μl biotin-labelled HPV DNA (0.5–1.0 μg, BIOHIT OY, Helsinki), 300 μl (30%) deionised formamide, 200 μl 50% dextran sulphate, 100 μl 2 × SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.0), 40 μl (0.4 mg/ml) sonicated, denatured Herring sperm DNA (Herring sperm 10 mg/ml, Promega) and 260 μl aqua purissima. This was 10% of the amount of DNA recommended by the manufacturer, but because of the addition of dextran sulphate, the reduced probe concentration gave optimal hybridisation. A 10-fold increase in the concentration has been shown to give the same results [35]. The probe solution was added to the sections and then probe DNA and cellular DNA was denatured in an oven at 110°C for 5–6 min. Hybridisation was carried out for 18 h at 37°C in a moist chamber with 0.2 SSC. These conditions approximated those of low stringency. Furthermore, all lesions that were positive with the ISH method under low stringency were examined under high stringency with a specific probe for HPV type 16. This was accomplished by using 50% deionised formamide and a hybridisation temperature of 60°C. Detection of hybridised DNA was accomplished by sequential incubations with streptavidin alkaline phosphatase 1:200 for 30 min at 37°C, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) for 2 h at 37°C in a moist chamber. Finally, the sections were rinsed with distilled water and counter-stained with Carbo-fucin, dehydrated and mounted with Depex mounting medium. From each biopsy three sections were used, two with probe mixture and

one without DNA in the probe mixture. The latter section thus served as a negative control. For positive control of the hybridisation reaction, sections from an oral condyloma with known HPV content were used and sections from a radicular odontogenic cyst served as a further negative control. A positive reaction was seen as a bluish staining over the nucleus. All specimens were read by the first author (H.N.) and independently by a trained pathologist (F.P.) without information on the origin of the specimen.

PCR and Southern blot analysis of the amplification products

PCR (polymerase chain reaction). DNA was extracted from 30 μm thick paraffin embedded tissue sections in 100 μl 10 mM Tris and 1.6 μl proteinase K (12.5 mg/ml Boehringer-Mannheim Biochemicals) in a waterbath at 55°C for 3 h. To inactivate proteinase-K the sample was heated to 95°C for 10 min, put into an icebath and then centrifuged. The supernatant was then ready for PCR. A mastermix was made fresh for each PCR, 1 ml consisted of 100 μl $MgCl_2$, 100 μl 10 × buffer, 640 μl H_2O, 160 μl dNTP-mix (12.5 μl dATP-100 mM starch, 12.5 μl dTTP, 12.5 μl dGTP, 12.5 μl dCTP and 950 μl H_2O) together with 100 pmol primer (50 pmol of each) specific for HPV 16 (described in [36]), 1.5 U Ampli Taq DNA polymerase (Promega), 10 μl sample supernatant and one drop of oil. The sample was subjected to 40 cycles of amplification in a DNA thermal cycler. A cycle represents primer extension for 2 min at 72°C, denaturation for 1 min at 95°C, and reannealing for 1 min at 55°C followed by primer extension. To control the PCR reactions, one positive control (consisting of an HPV fragment of the HPV type in question) and one negative control (DNA from a guinea pig muscle) was amplified. Furthermore, the reactions contained a negative control from the DNA extraction (buffer solution without sample tissue). To ensure that the samples contained DNA, an internal control (here β-globulin [37]) reaction was amplified before amplifying with specific primers.

Southern blot analysis of the amplification product. The PCR reaction product (15–20 μl sample supernant) was electrophoresed in a 2.5% agarose gel for band separation. The final oligonucleotide product was visualised by ethidium bromide stain and ultraviolet illumination and 0.5 μg pBR/HinfI served as a marker. After photography, the gels were rinsed in 0.25 N HCl for 15 min, denaturation buffer (50 ml 2.5 N NaOH, 80 ml 3 M NaCl and 170 ml H_2O) for 30 min and neutralisation (500 ml 3 M NaCl and 500 ml 1 M Tris) buffer for another 30 min. The gels were transferred to Nytran filters (Schleicher & Schuell) overnight using standard procedures and hybridised with an alpha ^{32}P-dATP (Amersham, PB10235)-end-labelled oligonucleotide probe specific for HPV 16 (Amersham 3'-end-labelling kit N4020). The hybridisation could be visualised on an X-ray film (Kodak T-MAT G5500) after 1 week of exposure.

Statistical analysis

The statistical analysis included Fisher's exact test (two-tailed) and binomial confidence limits, (Medstat, Version 2.1, The Astra Group, Copenhagen), P values ≤ 0.05 were considered significant.

RESULTS

An almost equal distribution of patients and control patients according to age and gender at operation are seen in Table 1. Table 2 shows the number of clinical diagnoses between males and females. The distribution is almost equal except from erythroplakia and nodular leucoplakia where females, respectively males, are preponderant. All 20 patients in the control group had a diagnosis of normal oral mucosa. Table 3 shows the histological diagnoses of the HE stained specimens from the patients. No dysplasia was seen in almost half of the patients (24 out of 49) and in the 20 control patients.

The results of the different techniques to demonstrate HPV revealed that only 20 out of 49 patients were HPV positive (40.8%). The distribution of the HPV positive patients is shown in Table 4. All control patients and control specimens were HPV-negative despite the technique employed. It is interesting to note from Table 4 that only 3 patients were positive with more than one technique and the majority of positive lesions were identified by *in situ* hybridisation. In addition, all lesions were negative with the ISH method using

Table 1. Distribution of patients and controls according to age and gender at operation

	Male		Female	
Age group	Patients	Controls	Patients	Controls
30–39	2	0	0	1
40–49	2	3	0	2
50–59	6	1	4	2
60–69	6	3	8	5
70–79	7	1	10	2
80–89	1	0	2	0
90–100	0	0	1	0
n	24	8	25	12

Table 2. Distribution of patients by gender and clinical diagnosis

	Male	Female	*n*
Erythroplakia	1	9	10
Homogeneous leucoplakia	5	6	11
Non-homogeneous leucoplakia			
Erythroleucoplakia	6	6	12
Nodular leucoplakia	7	1	8
Verrucous leucoplakia	5	3	8
n	24	25	49

Table 3. Histological diagnoses of patients with oral precancerous lesions and control patients

Epithelial dysplasia	Patients	Controls
No dysplasia	24	20
Slight	11	
Moderate	9	
Severe	4	
Carcinoma *in situ*	1	
n	49	20

Table 4. Results of HPV demonstration in 20 biopsies

Patient number	Gender	PAP	ISH	PCR	BLOT
1	M	0	0	1	0
7	F	0	1	0	0
8	F	0	1	0	0
9	F	0	1	0	0
14	**M**	**0**	**1**	**0**	**1**
15	F	0	0	0	1
20	F	0	0	0	1
22	M	0	1	0	0
25	**M**	**1**	**1**	**0**	**1**
26	M	0	1	0	0
27	F	0	1	0	0
31	F	0	1	0	0
33	M	0	1	0	0
34	**M**	**1**	**1**	**0**	**0**
36	F	0	1	0	0
44	F	0	1	0	0
48	M	0	1	0	0
56	F	0	1	0	0
57	M	0	1	0	0
60	F	0	1	0	0
n	11F, 9M	2	17	1	4

0 = HPV negative with present technique; 1 = HPV positive with present technique; F = female; M = male; bold type illustrates patients positive with more than one technique.

a specific probe for HPV type 16. The reduction in the amount of DNA in the probe is expected to be without influence on the result [35]. Table 4 also shows that there was no gender predilection for HPV positivity as 9 males and 11 females were positive. However, the number of HPV positive patients was significantly increased as compared with the control sample ($P = 0.0005$) in which no patients were HPV positive.

Tables 5 and 6 show an interesting feature of the patient sample, namely the topographical distribution of the HPV positive and negative lesions. The majority of HPV positive lesions were found in the buccal mucosa, predominantly as verrucous leucoplakia with no or slight dysplasia, or as erythroplakia with moderate dysplasia. The majority of HPV negative lesions were found in the sublingual region predominantly as erythroleucoplakia and nodular leucoplakia with slight to moderate dysplasia.

In Table 7, the clinical and histological characteristics of the patients who developed cancer are listed. 3 patients developed oral cancer and all 3 were HPV positive (100%). The three HPV positive lesions in which oral cancer developed were two erythroleucoplakias and one verrucous leucoplakia. It is interesting to note that all 3 were smokers and that the length of the follow-up period before development of cancer was 4–12 years.

DISCUSSION

Four different techniques were used to demonstrate HPV in oral premalignant lesions with varying degrees of dysplasia (Fig. 1). All techniques proved to be reliable as all control specimens gave consistent results irrespective of the technique employed. The study revealed that only 3 patients were positive with more than one technique and that the majority of the positive lesions were found with the ISH technique (Figs 2, 3 and 5). Only 2 patients were PAP positive which is

Table 5. Distribution of HPV +/− lesions according to clinical diagnosis, degree of dysplasia and localisation

	No dysplasia		Slight dysplasia		Moderate dysplasia		Severe dysplasia/ carcinoma *in situ*		HPV positive patients in % with 95% confidence limits
	HPV+	HPV−	HPV+	HPV−	HPV+	HPV−	HPV+	HPV−	
Homogeneous leucoplakia	1 lip 2 sublingual 1 buccal mucosa 1 tongue	4 sublingual 2 buccal mucosa							45.5 (16.7–76.6)
Erythroplakia		2 tongue 1 sublingual		1 buccal mucosa	3 buccal mucosa		1 sublingual	1 buccal mucosa	50.0 (18.7–81.3)
Erythroleucoplakia		2 sublingual	2 buccal mucosa	1 sulcus 1 palate	1 palate	2 sublingual 1 lip	1 sublingual	1 sublingual	33.3 (9.9–65.1)
Nodular leucoplakia	1 buccal mucosa	1 buccal mucosa 1 palate		3 sublingual		1 sublingual		1 sublingual	12.5 (0.3–52.6)
Verrucous leucoplakia	3 buccal mucosa	1 tongue 1 sublingual	1 buccal mucosa 1 sublingual	1 palate					62.5 (24.5–91.5)

Table 6. Distribution of HPV +/− lesions according to localisation

Localisation	Number	HPV+	HPV−
Buccal mucosa/lip	18	12	6
Sulcus	1	0	1
Sublingual region	21	5	16
Tongue	4	1	3
Palate	5	2	3
n	49	20	29

Fig. 1. A patient demonstrating severe dysplasia in a sublingual erythroleucoplakia (HE stain).

Fig. 2. A positive lesion with the *in situ* hybridisation screening probe.

Fig. 3. An oral condyloma serving as a positive control for the *in situ* hybridisation with the screening probe.

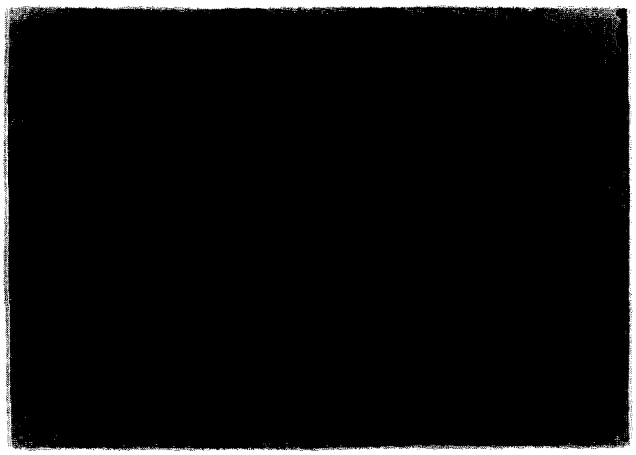

Fig. 4. The two dark nuclei illustrate a positive lesion with peroxidase anti-peroxidase technique (PAP).

probably due to the technique developed to show viral structural proteins produced late in the infectious cycle (Fig. 4). The negative PAP findings demonstrate that viral particles are not made. PCR was carried out on all samples with a specific primer set for HPV 16. Interestingly, the PCR revealed only one positive sample but four more were identified after Southern blot analysis, which approximately increases the sensitivity by 10-fold [38]. Two of them were also ISH positive. The high number of positive lesions with the ISH technique compared with PCR and Southern blot may be a consequence of the criterion used for positivity. In our study, a blue stain over just one nucleus was regarded as evidence of HPV being present. However, weak signals complicated the registration (Fig. 2), probably due to a low copy number of

Table 7. Clinical and histological characteristics of patients developing oral cancer

Patient number	Sex	Age	Clinical diagnosis	Histological diagnosis	Localisation	Year of excision	Tobacco	Year of cancer development	Transmission period in years	HPV+
8	F	57	Verrucous leucoplakia	No dysplasia	Buccal mucosa	1975	Yes	1987	12	+
25	M	64	Erythroleucoplakia	Severe dysplasia	Sublingual	1982	Yes	1986	4	+
57	M	59	Erythroleucoplakia	Slight dysplasia	Buccal mucosa	1982	Yes	1988	6	+

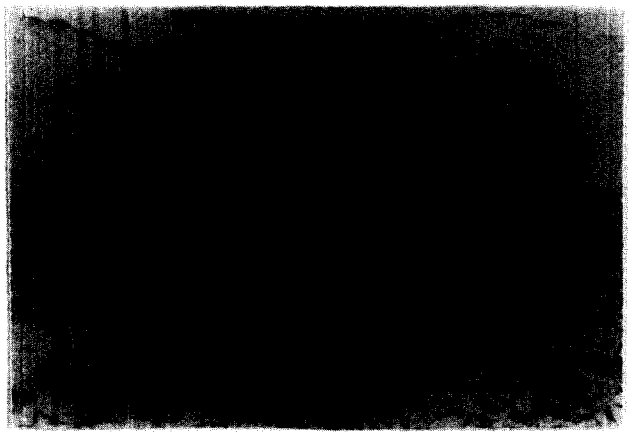

Fig. 5. A section from a lesion without probe mixture serving as negative control for the *in situ* hybridisation.

HPV or due to uneven distribution of HPV positive cells in the lesions, as previously suggested [32].

The registration of the specimens was further complicated by a supposed unspecific staining that occurred in the upper layer of the epithelium, most likely due to the proteinase-K digestion that caused hollow cells. These hollow cells might retain staining granules occasionally and thereby resemble a positive staining reaction of the cell, although the specimens were vigorously rinsed. A similar phenomenon has been experienced in a comparative study of two commercial ISH kits for detection of HPV [39]. However, it is important to emphasise that the high number of ISH positive lesions which were negative with PCR and Southern blot technique may be explained by the fact that the ISH probe contained DNA specific for several HPV types whereas PCR and Southern blot were only carried out for HPV 16.

The result of the investigations was an overall rate of HPV positive lesions of 40.8%. However, only five of them were HPV 16 positive. The remaining were positive for one or more of HPV types 6, 11, 18, 31 and 33. Our findings show higher HPV prevalence than studies reported by Syrjänen *et al.* who found an overall rate of 28.6% using the same HPV probes [25] or by Shroyer *et al.* who found an overall rate of 14.6% using the similar HPV probes [40]. Their results are in line with a study by Gassenmaier and Hornstein who found HPV DNA in 18% of the precancerous lesions using probes specific for HPV type 2, 6, 11, and 16 [41]. However, high prevalences in oral leucoplakias have been found by Scully *et al.* who found an overall rate of 42% using probes specific for HPV 16 [42] and by Maitland *et al.* who reported HPV detection in 82% of oral leucoplakias using specific probes for HPV 16 [43]. These differences in detection of HPV may be accounted for by different sensitivities of methods used.

None of our patients from the control sample with normal oral mucosa were HPV positive, which disagrees with other investigations reporting high percentages of HPV infection, even in clinically normal oral mucosa [26, 44]. The discrepancy may be explained by different sensitivities of the techniques used or by the fact that some investigators have included control and pathological specimens from the same patient [26].

In the present study, HPV seems to possess a predilection of lesions in the buccal mucosa as Table 6 shows that 12 out of 18 lesions in that region were HPV positive. On the contrary, the HPV negative lesions were most frequent in the sublingual region with 16 out of 21 lesions being negative. In a study by Syrjänen *et al.*, the most prevalent HPV infected dysplasias were those of the tongue, followed by the floor of the mouth and the palate [25]. In our study, as demonstrated in Table 5, the verrucous leucoplakia is the type of lesion most often harbouring HPV, this occurring in 62.5% of the cases, primarily in the buccal mucosa. Erythroplakias also harbour HPV occurring in 50% of the cases, primarily in those from the buccal mucosa. This is interesting as these two types of lesions are believed to possess a high potential for malignant transformation. Furthermore, erythroleucoplakia and nodular leucoplakia are harbouring HPV in 33.3% and 12.5%, respectively. This observation implies that other aetiological factors, besides tobacco, alcohol, and HPV, are involved in these lesions. One such factor may be yeast infection by *Candida albicans*. Studies by Krogh *et al.* and Hogewind *et al.* support this as they have shown that *Candida albicans* is often present in erythroleucoplakias and nodular leucoplakias. Infections with *Candida albicans* in these lesions are believed to represent an increased risk for malignant development [45, 46].

Finally, the hypothesis that the HPV infection might be latent for several years from initial infection to cancer development [47] seems also to be valid in our study as 3 patients developed oral cancer within a period of 4–12 years. Furthermore, they were all smokers, suggesting that infection with HPV alone is not sufficient for cancer development, but cofactors such as tobacco are very important.

In conclusion, HPV is present in oral mucosa affected by homogeneous leucoplakia, erythroplakia and verrucous leucoplakia and to a lesser extent in erythroleucoplakias and nodular leucoplakias. Concerning oral cancer development, it seems likely that HPV may be a cofactor as 3 out of 3 patients that developed oral cancer were all infected by HPV, 1 being positive for HPV 16.

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