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Human Papilloma Viruses in Oral Lesions

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Abstract. *Oral mucosa biopsies from 53 patients with different oral diseases, and from 12 healthy control patients, were examined by the PCR-technique using the L1 consensus primers and type-specific primers for HPV 6/11, 16 and 18. Three out of 24 (12.5%) oral cancer biopsies were HPV positive, 1 for HPV 18, 1 for HPV 16 and 6/11 and 1 for none of the specific primers. Six out of 22 (27.3%) lichen planus were HPV positive, 5 for HPV 18 and 1 for none of the specific primers. Seven leukoplakias were included in the study and 2 (29.6%) were positive for both HPV 16 and 6/11. None of the 12 control patients was HPV positive. No statistical difference between the use of tobacco and alcohol and HPV prevalence was found. An association between HPV infection and oral lesions was demonstrated in the studied population but the pathogenic influence of HPV infection remains unclear.*

The association between human papilloma virus (HPV) and development of benign and malignant genital tumours has been well established and previously reviewed by several authors (1, 2). Some reports suggest that HPV may be an etiological factor in oral squamous cell papillomas, focal epithelial hyperplasia, condyloma accuminatum and epithelial dysplasia, *i.e* these lesions may be caused by one or more of the recognised types of HPV (3, 4). The presence of HPV has been correlated to the degree of dysplasia of premalignant disorders such as oral leukoplakia (5). HPV has also been identified in oral squamous cell carcinomas and a large number of studies have examined the relationship between HPV and squamous cell carcinoma in the head and neck region (6-11).

The association between HPV and oral lichen planus has been studied by Jontell *et al* (1990), who found a clear correlation, identifying HPV in 65% of erosive lichen planus lesions by means of PCR techniques (12). It is intriguing, in the light of these reports, that the prevalence of HPV in normal oral mucosa varies from 0 to 60%, which makes the role of HPV as a pathogen dubious in oral lesions (13-15).

At least 70 different types of HPV are currently known. Of these, HPV 13 and 32 primarily infect the oral cavity but several others have been associated with a number of oral benign, premalignant and malignant lesions. HPV 13 and 32 are strongly associated with focal epithelial hyperplasia (16). HPV 6 and 11 have been associated with other benign oral lesions and HPV 16 and 18 have been associated with oral cancer (3, 4, 7, 11).

The aim of the present study was to examine the possible etiological role of HPV in the development of premalignant and malignant oral mucosal lesions and to study the effect of tobacco and alcohol exposure on the prevalence of HPV in oral lesions.

Materials and Methods

Patients. The study population consisted of 53 patients (33 men and 20 women, mean age 62.0±15.2 years) who were diagnosed or treated at the Department of Oral & Maxillofacial Surgery, Faculty of Odontology, Gothenburg, Uppsala University, Uppsala, and the Division of Oto-Rhino-Laryngology, Sahlgrenska Hospital, Gothenburg, Sweden. Of these 53 patients, 24 had squamous cell carcinoma (18 men and 6 women, mean age 66.8±16.8 years), seven patients had leukoplakia, one of which was dysplastic (5 men and 2 women, mean age 60.4±14.3 years), and 22 had lichen planus (10 men and 12 women, mean age 57.3±12.7 years). Fourteen of these were reticular, 4 erosive, 2 atrophic, 1 plaque-like and 1 unspecified. The control group consisted of 12 patients (6 men and 6 women, mean age 56.8±14.6 years) attending the same clinics with clinically healthy oral mucosa and with no or only moderate alcohol consumption and no tobacco habits. The rationale for chosing control patients with no previous history of tobacco consumption was to exclude patients with minimal, clinically undetectable, lesions of the oral mucosa. The biopsies from the control patients were taken after informed consent and the study was approved by the ethics committee of Göteborg University.

Questionnaire. All patients were asked to complete a questionnaire that focussed on their tobacco and alcohol habits during the last five years. The questionnaire data did not permit definitive conclusions regarding earlier consumption. The response rate to the questionnaires was 96%, since two of the cancer patients were too ill to answer. The amount of tobacco and alcohol used was classified as none (0), low (1), moderate

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(2) or high (3), as shown in Table I. The classification of smoking and alcohol habits was a slight modification of the Rothman and Keller (1972) classification (17).

Tissue processing and morphological examination. Biopsies were obtained under local anaesthesia (Lidocaine 20 mg/ml + 12.5 µg Adrenaline, Astra, Södertälje, Sweden) or general anaesthesia. The specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned (5 µm) and stained with hematoxyline and eosin according to standard procedures. Histologically identified lesions were used for identification of HPV-DNA.

DNA-extraction and deparaffinisation. A 50 µm section was prepared with a microtome from the blocks of fixed tissue and put into a 1.5-ml microfuge tube containing 300 µl of xylene. The tubes were sealed and put into a 60°C water-bath for 30 seconds. Two minutes of mixing followed by a 1 min equilibration was repeated three times. The tissue was then pelleted by centrifugation at 12800 g and the xylene removed with a clean Pasteur pipette. 300 µl of xylene was again added and mixed for 2 min followed by a 1 min equilibration. This procedure was repeated 3 times. The tissue was further pelleted and 600 µl of 99.5% ethanol was added and mixed slowly for 10 min. The tissue was again pelleted by centrifugation at 12800 g and the supernatant removed with a clean Pasteur pipette. This step was repeated once, after which 50 µl of acetone was added and the open tube was put in a water-bath at 60°C to increase the evaporation of the acetone. 100 µl of digestion buffer (50 mM Tris pH 8.5, 1 mM EDTA and 0.5% Tween 20) and 1.0 µl (20 mg/ml) proteinase K was added. The tubes were incubated at 37°C overnight, followed by incubation at 45°C for 10 min and then at 95°C for 5 min to inactivate the protease. Residual paraffin or tissue was pelleted by centrifugation at 12800 g for 30 sec and the DNA transferred to a new tube.

Polymerase chain reaction. Each PCR reaction mixture contained 2.5 µl 10 times PCR-buffer (500 mM KCl, 100 mM Tris-pH 8.5), 0.6 µl 10 times dNTP, 3.5 µl $MgCl_2$ (25 mM), 0.3 µl Primer 1 (100 µM), 0.3 µl Primer 2 (100 µM) and 14.2 µl H_2O, which makes the total amount of each reaction 21.4 µl. 3.5 µl of the sample and 0.15 µl of Taq-polymerase (0.75 U) was added to the reaction mixture. The tubes were covered with 1 drop of mineral oil to prevent evaporation. DNA was denatured for 5 min at 95°C followed by 40 cycles of amplification in a Perkin Elmer Thermal Cycler. Each cycle consisted of 1 min at 95°C, 1 min at 55°C and 2 min at 72°C. After the amplification was completed, the mixture was heated at 72°C for 5 min. The HPV-DNA consensus primers used were as follows:

Positive Strand Primer (MY 11): GCMCAGGGWCATAAYAATGG
Negative Strand Primer (MY 09): CGTCCMARRGGAWACTGATC
M=A+C, R=A+G, W=A+T, Y=C+T

The consensus primers MY 11 and MY 09 promote the amplification of an approximately 450-base-pair product from at least 25 distinct genital HPVs in the L1 open reading frame (18). For typing of the positive samples, the following specific primers against HPV 6/11 and 16 as described by Ostwald *et al* (1994) (19) and specific primers against HPV 18 as described by Kellokoski *et al* (1992) (20) were used:

6/11 primers:	5'ATGCCTCCACGTCTGCAAC	115-133
(113 bp)	5'CTCTGCCGGTGGTCAGTGCAT	227-208
16 primers:	5'TGCTAGTGCTTATGCAGCAA	110-129
(398 bp)	5'ATTTACTGCAACATTGGTAT	507-488
18 primers:	5'AAGGATGCTGCACCGGCTGA	L1 6903-6922
(216 bp)	5'CACGCACACGCTTGGCAGGT	L1 7100-7119

As a positive control in the PCR reaction, paraffin-embedded anal condylomas, SiHa cell lines (ATCC nr HTB 35) and HeLa cell lines (ATCC nr CCL 2) were used. As a negative control, reaction mixture with water was used. Each HPV-reaction was controlled with a parallel reaction of human ß-actin (18). Every specimen was tested twice and judged by two independent people.

Table I. *Classification of tobacco and alcohol consumption modified from Rothman and Keller (1972) (17).*

Alcohol (100%)	
None (0):	non users
Low (1):	<1.1 cl/day
Moderate (2):	1.1-4.2 cl/day
High (3):	>4.2 cl/day
Cigarettes	
None (0):	non users
Low (1):	<10 cig./day
Moderate (2):	10-20 cig./day
High (3):	>20 cig./day
Snuff	
None (0):	non users
Low (1):	<3 cans/week
Moderate (2):	3-5 cans/week
High (3):	>5 cans/week

Gel electrophoresis. The PCR product was analysed by gel electrophoresis in a 3% agarose gel. The amplified product was visualised by staining the gel with ethidium bromide (1 µg/ml in Tris borate buffer) and inspected under ultraviolet illumination. The size of the amplified product was determined by comparison with a 50-base-pair ladder size marker (Pharmacia, Uppsala, Sweden) in the gel.

Southern blot hybridisation. Southern blot hybridization was used to confirm the PCR results with the consensus primers. The DNA was transfered to nylon membranes (Boehringer Mannheim, FRG) by Southern blotting with 20 x SCC. Prehybridization was carried out for 1 hour at 50°C in a solution of 5 x SCC, 5 x Denhardt' solution, 0.1% SDS and 100 µg/ml denatured salmon sperm DNA. The hybridization was performed using oligonucleotide probes specific to internal target DNA sequences which did not overlap primer sequences. The products of PCR with consensus primers were hybridized on three parallell gels to consensus probes GP1/GP2. The consensus-probes used were (19) :

5'CTGTGGTAGATACCACWCGCAGTA
5'CCTGTTGTTGATACTACACACGCAGTAC
W= A or T

Oligonucleotides probes were 3' labeled with digoxigenin-dUTP by terminal transferase (Boehringer, Mannheim, FRG). Hybrids were visualized by alkalinephosphatase-immunoassay as blue precipitates

using nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolylphosphatyl toluidine salt (X-phosphate) or by chemiluminescence using AMPPD (Serva, Heidelberg, FRG).

Statistics. Difference in HPV prevalence was analysed with the Chi-square test and a repeated measures ANOVA was used for analysis of tobacco and alcohol habits. $P<0.05$ was considered as statistically significant.

Results

HPV-prevalence. Of the 53 patients in this study, 11 (20.8%) were found HPV positive (7 men and 4 women, mean age 65.7 years). Fortytwo patients were HPV negative (26 men and 16 women, mean age 66.9 years). Diagnoses and tobacco and alcohol habits in the HPV-positive patients are presented in Table II. Two snuff users were HPV positive. In the control group, all 12 specimens were HPV negative. Six out of 22 (27.3%) lichen planus were HPV positive and of seven leukoplakias included in the study, 2 (29.6%) were HPV positive. Three out of 24 (12.5%) oral cancers were HPV positive (Figure 1). The difference in HPV prevalence between the subgroups of oral cancers, lichen planus and leukoplakias was not statistically significant.

HPV-typing. Six samples were positive for HPV 18, three for HPV 16 and three for HPV 6/11 (Table IV). The three samples positive for HPV 16 were also positive for HPV 6/11. Two of the samples positive with the concensus primers were not positive with any of the specific primers against HPV 18, 16, or 6/11. Of the three HPV-positive cancers, one was positive for HPV 18, one was positive for both HPV 16 and 6/11 and one was not positive for any of the specific primers. Of the six HPV-positive lichen planus, five were positive for HPV 18 while one was HPV-positive but not for any of the specifically tested HPV types. The two HPV-positive leukoplakias were positive for both HPV 16 and 6/11 (Figure 2).

Tobacco and alcohol data. Fiftyone of the 53 (96%) patients with oral lesions and all (100%) of the control patients answered the questionnaires. The 2 patients that did not answer were cancer patients and too ill to answer. All of the 11 HPV-positive patients and 40 of 42 HPV-negative patients answered the questionnaire (Table III). Ten of the eleven (91%) HPV-positive patients were tobacco and/or alcohol users and only one of the eleven HPV-positive patients did not use any tobacco or alcohol at all (Table II). One of the 40 HPV-negative patients that answered the questionnaire did not use tobacco or alcohol at all. In two of the three HPV-positive cancer patients both alcohol and tobacco consumption was high (Table II) and the total tobacco and alcohol consumption was higher in the cancer group (Table III). No correlation between the grade of dysplasia in the leukoplakias or the type of lichen planus and HPV positivity was found.

Table II. *Demographics, diagnosis and classification of tobacco and alcohol consumption in the eleven HPV-positive patients.*

Sex	Age	Smoke	Snuff	Alcohol	Diagnosis
F	82	0	0	0	Lichen
F	74	0	0	1	Lichen
M	79	1	0	1	Leukoplakia
M	47	2	0	1	Leukoplakia
F	49	2	0	1	Lichen
F	84	0	0	1	Cancer
M	56	3	3	3	Cancer
M	57	2	0	2	Cancer
M	66	0	1	0	Lichen
M	69	2	0	1	Lichen
M	60	1	0	0	Lichen

Discussion

In this study, we found a statistically significant difference in HPV prevalence between patients with oral mucosal lesions and the controls. It is unlikely that this difference could be explained by different tobacco and alcohol habits or age. The current literature provides evidence of an association between HPV and oral cancer but the prevalence in previous reports varies widely from 0% to 78% in oral lesions (7-9). Van Rensburg *et al* (1996, 1995) found by PCR analysis only 2 HPV-positive samples among 146 oral squamous cell carcinomas in a black African population, (10) whilst in another study only 1 HPV-positive sample was found among 66 oral squamous cell carcinomas using the *in situ* hybridisation technique (9). In our study, one oral cancer was HPV-18 positive and one positive for both HPV 16 and 6/11. This result corresponds to earlier studies suggesting HPV 16 and 18 may be oral oncogenic HPV types (2). However, the majority of oral cancers were negative for HPV but those answering the questionnaires revealed a history of tobacco and alcohol consumption.

In this study, the mean age among HPV-positive patients (65.7 years) was similar to that in the HPV-negative patient group (66.9 years). The mean age of the control group was 56.8 years but this difference was not statistically significant and does not explain the difference in HPV prevalence. Miller *et al* (1994) (21) and Brandwein *et al* (1994) (22) did not find any age difference between HPV positive and negative patients but Cruz *et al* (1996) (23) suggested an

Table III. *Classification of tobacco and alcohol consumption with regard to diagnosis and HPV status.*

Diagnosis	HPV	n	Tobacco				Alcohol			
			0	1	2	3	0	1	2	3
Cancer	neg	21*	4	1	11	3	1	13	3	2
	pos	3	1	0	1	1	0	1	1	1
Lichen	neg	16	9	1	4	2	2	8	6	0
	pos	6	3	2	1	0	3	3	0	0
Leukoplakia	neg	5	1	2	0	2	0	3	1	1
	pos	2	0	1	1	0	0	2	0	0
Control	neg	12	12	0	0	0	2	7	3	0
	pos	0	-	-	-	-	-	-	-	-

* data unavailable in 2 patients

association between HPV and oral squamous cell carcinoma in patients under 60 years of age. Although two of three patients with HPV-positive oral squamous cell carcinoma were less than 60 years old, the number was too small to allow statistical analysis.

The prevalence of HPV in the control group, 0%, is low compared to what has previously been reported (13, 14). Our results, however, correspond well to those of Eike *et al* (1995) and Ostwald *et al* (1994), who found 0% and 1% HPV positivity in normal oral mucosa respectively (15, 19).

Several published studies have concerned the prevalence of HPV in specific lesions, and not in the general population. Kellikoski *et al* (1992) reported a high prevalence of oral HPV in females with genital HPV infection (23.1%), which suggests that there is a cross infection between genital and oral HPV (20). Scwartz *et al* (1998) reported an increased risk of oral cancer among men with a history of genital warts (11). The control group in our study consisted of patients with clinically normal mucosa admitted for tooth extraction, and was chosen for comparison of HPV prevalence in the patients with oral lesions. However, no information is provided on HPV prevalence in the oral mucosa of the general population.

The highest prevalences of HPV was found in patients with leukoplakia (28.6%). Leukoplakia is considered to be premalignant but it remains unclear to what extent premalignant leukoplakias develop into squamous cell carcinomas (24). Earlier studies show a variation between 0 and 82% of HPV prevalence in oral leukoplakias, hyperkeratoses and epithelial dysplasias (4, 6, 25, 26). HPV 6, 11 and 16 seem to be the most frequent types associated with oral leukoplakias and dysplasias. In our study, the two HPV-positive leukoplakias were positive for both HPV 16 and 6/11. Syrjänen *et al* (1986) also found double infection in a leukoplakia with HPV 6 and 11 (27). In our study, one of the leukoplakias had mild dysplasia but this patient was HPV negative, although previous studies have reported a higher prevalence of HPV in dysplastic lesions (5).

Table IV. *Summary of HPV status and HPV type in the different oral lesions.*

Diagnosis	Total number of biopsies	HPV positive	HPV type		
	n	n (%)	6/11	16	18
Lichen planus	22	6 (27.3)	0	0	5**
Leukoplakia	7	2 (28.6)	2*	2*	0
Cancer	24	*3 (12.5)*	1*	1*	1**
	53	11 (20.8)			

*=double infection

**=one HPV-positive oral cancer specimen and one lichen specimen was not positive for any of the tested type-specific primers.

Oral lichen planus has been associated with malignancies of the oral cavity (28, 29). Jontell *et al* (1990) analysed biopsies from oral erosive lichen planus samples and found 65% HPV-positive samples, Syrjänen *et al* (1986) found 2 HPV-positive out of 2 samples (100%), Maitland *et al* (1987) found 7 positive out of 8 (87.5%) and Cox *et al* (1993) found 2 HPV-positive out of 4 (50%) (12, 26, 27, 30). In the present study, we found 22.3% HPV-positive biopsies, which does not confirm such a high prevalence of HPV in connection with lichen planus. Furthermore, another study, Young *et al* (1991) did not find any HPV-positive samples at all among six lichen planus patients (6). In our study, five of the six positive

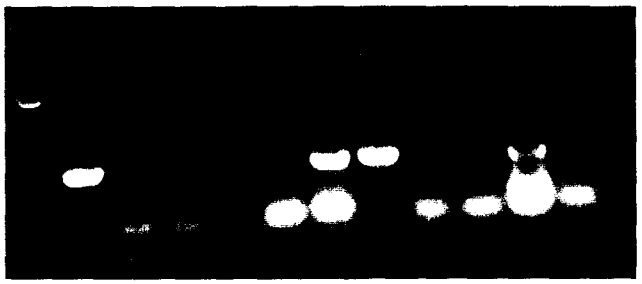

Figure 1 *(a). Detection of HPV with L1 consensus primer-mediated PCR (450 bp) in oral squamous cell carcinomas. The products are visualised on a 3% agarose gel stained with ethidium bromide. Lanes 7, 8 and 11 show positive cases and Lanes 4, 5, 6, 9, 10 and 12 negative cases. Lane 1 shows a molecular size marker (1 Kb). Lane 2 shows the positive control and Lane 3 the negative control.*

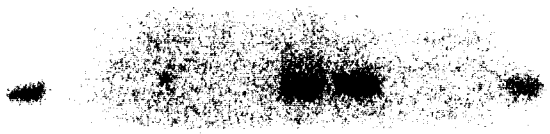

Figure 1 *(b). Subsequent Southern blot hybridization with HPV consensus probes GP1 / GP2 of the cases in Figure 1 (a).*

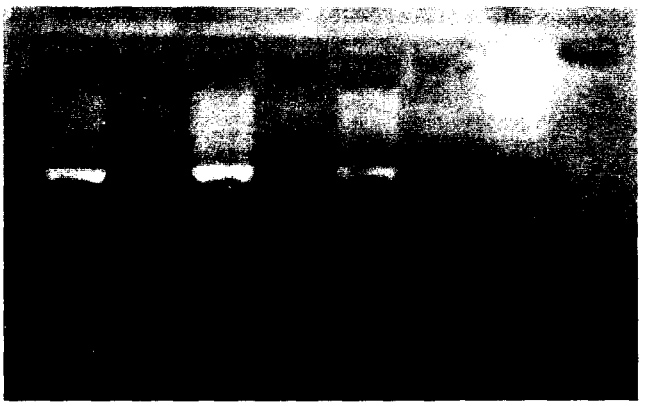

Figure 2. *Visualisation of PCR-products with HPV 16-specific primers (398 bp) in leukoplakias. The products are visualised on a 3% agarose gel stained with ethidium bromide. Lanes 1 and 5 show positive cases and Lanes 4 and 6 negative cases. Lane 7 shows a molecular size marker (123 bp). Lane 3 shows the positive control and Lane 2 shows the negative control.*

samples were positive for HPV 18. In the earlier studies, the most common HPV types are HPV 6, 11 and 16. Four of the HPV- positive samples in our study were of the common reticular type, one was erosive/atrophic and one was unspecified. There was thus no correlation between the type of lichen planus and HPV infection.

In the present material, no statistical evidence was found regarding alcohol and tobacco use and prevalence of HPV but it was evident that the cancer patients had a history of extensive tobacco use. Our results are in accordance with Paz *et al* (1997), who did not find any significant difference in HPV prevalence between tobacco users and non tobacco users (8). However, in two of the three HPV- positive oral cancer patients the alcohol and tobacco consumption was high (group 2-3 in Rothman's classification). No firm conclusions can be drawn from these two patients, even though it is an interesting note since Scwartz *et al* (1998) concluded that HPV 16 in connection with cigarette smoking may contribute to the development of oral squamous cell carcinoma (11).

Neither the patients' socioeconomic status nor sexual history was evaluated in this study. However, we have no reason to suspect any difference between the study group and the control group with regard to these variable. In a study among American citizens, Schwartz *et al* (1998) concluded that low income increased the risk of oral cancer, as did decreasing age of first intercourse and increasing number of sex partners (11).

Patients with oral mucosal lesions have a higher prevalence of HPV infection but a major etiological role of HPV is not supported by the present data. The prevalence of HPV infection in oral lichen planus and oral leukoplakia exceeds the HPV-prevalence in oral cancer. The significance of this will be followed up in a prospective study.

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