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Age-dependence of Human Papillomavirus DNA Presence in Oral Squamous Cell Carcinomas

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The aetiology of oral cancer is thought to be multifactorial. Apart from the two known major risk factors (tobacco and alcohol), a viral aetiology has been proposed, with special reference to human papillomavirus (HPV).

35 cases of oral squamous cell carcinoma (OSCC), seen at the Departments of Oral & Maxillofacial Surgery and Oral Pathology and Otolaryngology of the Free University of Amsterdam, were analysed as well as 12 biopsies of clinically and histologically normal gingival mucosa collected from healthy individuals after tooth extractions, using the polymerase chain reaction (PCR) and two different sets of primers that are able to detect a broad spectrum of HPV types.

An overall HPV positivity of 54.3% in OSCC was found, the majority of positive cases (78.9%) harbouring HPV type 16. In contrast, no positivity for HPV was detected in the clinically normal oral mucosal samples analysed. Furthermore, a significant association between HPV presence and age was found: patients older than 60 years showed a lower prevalence of the virus (29.4%) compared with patients below this age (77.8%) ($P<0.05$). The results from the present study suggest an association between HPV and OSCC, particularly in patients under the seventh decade.

Keywords: DNA, human papillomavirus, human papillomavirus type 16, polymerase chain reaction, age

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INTRODUCTION

Squamous cell carcinoma is the most common cancer of the oral cavity (OSCC) and accounts for approximately 3% of all malignancies of the Western world. In the developed countries, occurrence of OSCC rises with age, with a peak incidence in the seventh decade. Alcohol and tobacco are two known risk factors, but there is evidence that other factors, such as the infection with certain human papillomavirus (HPV) types and genetic susceptibility, could also be involved in the carcinogenic process.

The HPV group is a large group of viruses in which more than 70 types have already been designated [1, 2]. There is epidemiological and molecular biological evidence for involvement of certain HPV types in anogenital cancer, particularly carcinoma of the uterine cervix, in which HPV DNA has been detected in more than 90% of cases [3]. The oral cavity shows several similarities with the region of the cervix uterine, with regard to histological and embryological features. Also, similar risk factors (i.e. smoking and life style) have been implicated in the development of malignancies in both regions. Moreover, a parallel can be established between benign, premalignant and malignant lesions found in these regions [4]. In addition, HPV has been implicated in a subset of carcinomas of the aerodigestive tract, including squamous cell carcinomas of the oral cavity [5–8].

In vitro studies have shown the ability of the high risk types HPV 16 and 18 to immortalise oral keratinocytes [9, 10], and the subsequent exposure of HPV immortalised oral epithelial cells to tobacco-related chemicals resulted in the progression towards a fully malignant phenotype [10, 11]. These data suggest a role for HPV infection in concert with exposure to carcinogens in the multifactorial aetiology of oral cancer.

The presence of common genital HPV genotypes in the oral cavity has already been demonstrated by several studies involving both benign and malignant lesions of this region. Oral warts, condyloma acuminatum and oral papillomas have been found to harbour mainly low risk HPV types, HPV 6 and 11 [6], whereas high risk HPV types, predominantly HPV 16,

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have been demonstrated in OSCC by many groups ([5–8, 12, 13] and references therein). However, different HPV prevalence rates have been reported for OSCC, which may reflect either different technical approaches or differences in study populations. On the other hand, HPVs such as HPV 13 and 32 have almost exclusively been demonstrated in focal epithelial hyperplasia [6], pointing to the possibility of the existence of specific HPV types showing a particular tropism for the oral cavity.

The present study aims to determine the prevalence of HPV in OSCC and to investigate whether there is any association between the presence of HPV and certain patients' features, such as age and gender, tobacco and alcohol habits, and histological features, in particular grade of differentiation. In an attempt to avoid missing positivity, two general-consensus primer PCR assays were used, allowing the amplification of different regions within the HPV genome. Together, these two PCR techniques allow the detection of the most common genital and cutaneous HPV types.

PATIENTS AND METHODS

Clinical specimens and sample preparation

35 patients presenting at the Department of Oral & Maxillofacial Surgery and Otolaryngology of the Free University of Amsterdam, The Netherlands, with squamous cell carcinomas of the oral cavity (OSCC), were evaluated in this study. The medical records of these patients were reviewed in order to obtain information concerning age and gender of the patients, their tobacco and alcohol habits, and location and grade of the tumours. Twelve biopsies of clinically and histologically normal gingiva of the third molar region were obtained from patients who underwent extraction of impacted third molars. Of these patients, who were between 18 and 60 years of age, 7 were males and 5 were females.

OSCC patient data were divided into broad categories for Chi-square analysis. The parameter age was divided into three categories (<40 years, 40–60 years and >60 years), as well as into two categories (≤ 60 years and >60 years) for statistical analysis. Concerning tobacco and alcohol consumption, patients were considered to be non-smokers, non-drinkers, incidental smokers (<10 cigarettes/day), incidental drinkers (<2 U alcohol/day), moderate smokers (10–20 cigarettes/day), moderate drinkers (2–4 U alcohol/day), heavy smokers (>20 cigarettes/day) and heavy drinkers (>4 U alcohol/day). A close association existed between tobacco and alcohol consumption. About 80% of heavy drinkers were also heavy smokers, and the remaining 20% of heavy drinkers were moderate smokers. All incidental or non-drinkers were non-smokers ($P<0.001$). Tumour grades included well differentiated, moderately differentiated, poorly differentiated, as well as the intermediate categories (well to moderately differentiated and moderately to poorly differentiated).

Frozen blocks from the pathological specimens were serially sectioned. The first and last sections (5 μm) were stained with haematoxylin and eosin, and histologically examined for confirmation of diagnosis and estimation of the percentage of normal, dysplastic and neoplastic cells. Ten to 20 in-between sections (10–20 μm) were used for DNA extraction. Sections were first digested with proteinase K (10 μg/ml) in a 3 ml volume containing 10 mM Tris–HCl, 400 mM NaCl, 2 mM EDTA, pH 8.2, 1% SDS. Digestion was performed overnight at 37°C. Afterwards, 1 ml of saturated 6 M NaCl was added, and tubes were centrifuged at 4000 rpm at room temperature for 25 min. The supernatant containing the nucleic acids was collected and two volumes of absolute ethanol were added for precipitation. Finally, the pellet was dissolved in 100–400 μl 10 mM Tris–HCl, pH 8.0, 1 mM EDTA and incubated at 65°C for 15 min. DNA was stored at 4°C until use. The HPV status for each patient, as determined with the methodology described in the next paragraph, was categorised as HPV positive or HPV negative for statistical analysis.

HPV detection by PCR

To analyse the quality of the target DNA, the samples were first screened by PCR using β-globin specific primers, which direct the amplification of a 209 bp fragment [14]. All samples showed successful amplification of β-globin sequences and were subjected to general-consensus primer PCR.

Two different general-consensus primer pairs were used: GP5+–GP6+, which span a region of 150 bp from the L1 open reading frame (ORF) of a broad spectrum of mucosotropic HPV genotypes [15], and CPI–CPII which direct the amplification of a 188 bp fragment from the E1 ORF of a broad spectrum of cutaneous and mucosotropic HPV genotypes [16].

The PCR reaction mixtures of 50 μl contained 1 μl (100–500 ng) of extracted DNA, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 3.5 mM $MgCl_2$ (for GP5+–GP6+) or 1.5 mM $MgCl_2$ (for CPI–CPII), 200 μl of each dNTP, 1 unit of a thermostable DNA polymerase (AmpliTaq DNA polymerase; Perkin-Elmer Cetus, U.S.A.) and 50 pmol of each primer. The mixtures were overlaid with several drops of paraffin oil and incubated for 5 min at 96°C for DNA denaturation, followed by 40 cycles of amplification using a PCR processor (Bio-med, Theres, Germany).

Each cycle included a denaturation step to 96°C for 1 min, an annealing step to 40°C for 2 min and a chain elongation step to 72°C for 1.5 min. To ensure a complete extension of the amplified DNA, the final elongation step was prolonged by another 4 min.

A total of 10 μl of PCR mixture was finally analysed by electrophoresis on 1.5% agarose gels, stained with ethidium bromide and visualised under u.v. light.

Positive controls consisted of dilution series of SiHa and C4-1 cell line DNA in human placental DNA. These cell lines contain one copy of HPV 16 and HPV 18 per cell, respectively.

Type-specific PCR (TS-PCR) for the common mucosotropic HPVs 6, 11, 16, 18, 31 and 33 was performed on general-consensus primer PCR positive samples, using HPV 16 primers and combinations of HPV 6/33 and HPV 11/18/31 in a multiplex PCR assay, as described by Van den Brule *et al.* [17]. PCR conditions were the same as described for PCR with the CPI–CPII primer pair, except that 25 pmol of each primer and an annealing temperature of 55°C were used.

Special care was taken to control contamination problems. Negative controls consisted of: (a) frozen embedding compound cut between samples; (b) a mixture of all reagents used in the DNA extraction procedure, except DNA; (c) all reagents used in the PCR mixture preparation, adding 1 μl of distilled water instead of the DNA; and (d) liver DNA (HPV-negative sample). These samples were submitted to PCR, to exclude the detection of contamination which could have occured during all steps from sample preparation to PCR. Disposable tubes and filter tips were used. Furthermore, the different PCR steps, such as sample preparation, electro-

phoresis and PCR solution preparation were carried out in spatially separated rooms.

Southern-blot analysis of PCR products

The electrophoretically separated PCR products were blotted overnight, on to nylon membranes (GeneScreen Plus; Du Pont) in 0.4 N NaOH, 0.6 M NaCl. The membranes were saturated with 2 × SSC (1 × SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) and dried at room temperature between filter paper.

Then, incubation in a prehybridisation solution (0.5 M sodium phosphate, pH 7.4, 7% SDS, 1 mM EDTA) was performed for 1 h, at 55°C, followed by hybridisation in the same solution supplemented with the probe, for 16 h at 55°C. The cocktail probes consisted of a mixture of the respective general-consensus primer PCR products, specific for HPV 6, 11, 16, 18, 31 and 33 (GP5+–GP6+) or for HPV 2, 4, 5, 10, 11, 13, 16, 18, 25, 31, 33, 46, 51 and 52 (CPI–CPII), generated from cloned HPVs or characterised clinical samples, which were electrophoretically separated in NA agarose (Pharmacia, Uppsala, Sweden), excised from the gel and purified after freeze-squeezing.

A total of 2.5 ng of a mixture of these purified DNAs was used to generate α-^{32}P-dCTP random primed labelled cocktail probes. After hybridisation, the filters were washed three times for 30 min in 3 × SSC, 0.5% SDS at 55°C (for GP5–GP6 cocktail probe) and twice for 30 min in 3 × SSC, 0.5% SDS plus twice for 30 min in 0.5 × SSC, 0.5% SDS, at 55°C (for CPI–CPII cocktail probe).

Autoradiography was performed overnight, at −70°C, with Kodak Royal X-Omat film and intensifying screens. Samples showing a 150 bp product after hybridisation with the GP5–GP6 cocktail probe, or a 188 bp product after hybridisation with the CPI–CPII cocktail probe, were considered to be HPV positive.

For analysis of HPV TS-PCR products, hybridisation was performed with HPV 6, 11, 16, 18, 31 and 33 specific

Table 1. Information concerning patients, tumours and HPV PCR data

Tn	Sex	Age	Tob*	Alc*	Locat†	Grade‡	HPV Type	GP5+–GP6+ PCR	CPI–CPII PCR
1	M	60	0	0	OL	W–M	16	+	+
2	M	37	4	3	T	W	X	+	+
3	M	70	3	4	FM	M	16	+	+
4	F	49	4	4	FM	W–M	16	+	+
5	M	59	4	4	FM	M	16	–	+
6	M	48	4	4	OL	—	—	–	–
7	M	77	4	3	T	W	16	+	+
8	M	71	3	3	FM/T	W–M	—	–	–
9	M	39	4	3	T	W	X	–	+
10	M	58	0	0	OL	M–P	16	+	+
11	M	27	1	3	FM/T	W–M	16	+	+
12	F	79	1	2	T	W	—	–	–
13	F	55	4	4	T	W–M	16	+	–
14	M	59	1	2	T	W–M	16/6	+	+
15	F	63	4	4	T	W	—	–	–
16	M	69	3	3	T	W–M	—	–	–
17	M	56	1	2	OL	W	16	+	+
18	M	62	4	3	T	M	—	–	–
19	M	67	3	3	T	M	—	–	–
20	F	73	1	1	OL	W–M	—	–	–
21	F	50	0	0	OL	M–P	—	–	–
22	M	72	0	4	T	M	—	–	–
23	F	66	1	1	OL	W–M	—	–	–
24	F	63	4	4	T	W–M	—	–	–
25	F	55	4	4	FM	W–M	16	–	+
26	M	52	4	0	OL	W	16	+	+
27	M	77	0	0	OL	W	16	+	+
28	M	51	3	4	FM	W–M	16	+	+
29	M	59	4	4	OL	W	16	+	+
30	F	81	1	2	OL	W	X	+	+
31	F	84	3	3	FM	M	X	–	+
32	M	56	3	3	T	W–M	—	–	–
33	F	87	0	0	FM	M–P	—	–	–
34	F	75	1	1	T	M	—	–	–
35	F	56	0	0	OL	M	—	–	–

Tn = tumour sample number; Tob = tobacco use; Alc = alcohol consumption; Locat = location.

*Tobacco/alcohol habits: 0 = not known; 1 = no smoking/alcohol; 2 = < 10 cigarettes/< 2 U alcohol/day; 3 = 10–20 cigarettes/2–4 U alcohol/day; 4 = > 20 cigarettes/> 4 U alcohol/day.

†T = tongue; FM = floor of mouth; OL = other location.

‡W = well differentiated SCC; W–M = well to moderately differentiated SCC; M = moderately differentiated SCC; M–P = moderately to poorly differentiated SCC.

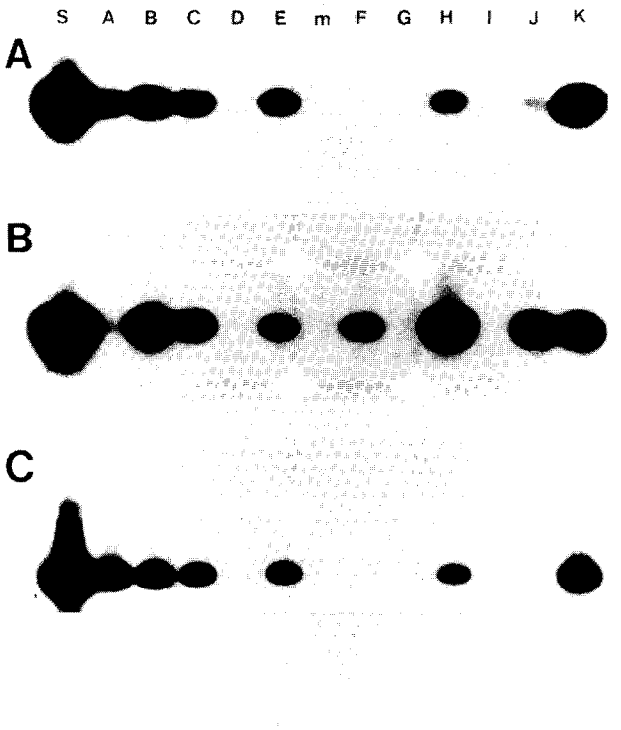

Fig. 1. Southern blot analysis of PCR products derived from DNA extracted from OSCC. Panel A—hybridisation of GP5+–GP6+ PCR products with the GP5–GP6 cocktail probe. Panel B—hybridisation of CPI–CPII PCR products with the CPI–CPII cocktail probe. Panel C—hybridisation of TS-PCR products (HPV 16) with HPV 16 oligonucleotide probe. Lanes A–E and F–K, OSCC samples. S, SiHa; m, size marker for agarose gel electrophoresis.

γ-^{32}P-dATP end-labelled oligonucleotides [17]. Hybridisation and washing conditions, as well as exposure times, were as described for filters hybridised with the GP5–GP6 cocktail probe.

The general-consensus primer PCR positive samples that were negative by TS-PCR, were designated HPV X, indicating the presence of an HPV type different from HPV 6, 11, 16, 18, 31 and 33.

General-consensus primer PCR negative samples were interpreted as HPV-negative samples.

Statistical analysis

Statistical analysis was performed using the Chi-square test after the Yates correction. The values were considered significantly different when P was less than 0.05.

RESULTS

The data concerning gender and age of the patients with OSCC, tobacco and alcohol consumption, location and histological grade of the lesions, as well as HPV PCR results, are presented in Table 1.

Out of 35 patients with OSCC, 19 (54.3%) showed positivity for HPV in their tumours, whereas in 16 (45.7%) cases no HPV DNA could be detected. Representative Southern blots of PCR products are shown in Fig. 1. No HPV positivity could be detected in clinically normal oral mucosas of the control group.

Amongst the 19 HPV-positive tumours, 14 cases were detected with both GP5+–GP6+ and CPI–CPII primers. For some samples, the PCR signal intensities between GP5+–GP6+ and CPI–CPII fragments differed markedly (Fig. 1, panels A and B, lanes A, H and J), pointing to differences in efficiency of both methods to detect certain HPV genotypes or HPVs showing alterations affecting either the E1 (CPI–CPII) or L1 (GP5+–GP6+) region. Moreover, 1 case was only positive in the GP5+–GP6+ PCR, whereas the remaining 4 cases were detected exclusively with the CPI–CPII primer pair (Fig. 1, panels A and B, lane F).

Further typing by type specific PCR revealed that 15 samples (78.9%) contained DNA of HPV type 16 (Fig. 1, panel C, lanes A, B, C, E, H and K), whereas in 4 cases a type different to HPV 6, 11, 16, 18, 31 and 33 (HPV X) was present (Fig. 1, lanes F and J). From these, only 2 cases could be detected by CPI–CPII PCR (Fig. 1, lane F), whereas the other 2 cases could be detected with both general-consensus primers (Fig. 1, lane J). The HPV type 16 PCR positive cases included one sample which was only positive by GP5+–GP6+ PCR and two samples which appeared positive only by CPI–CPII PCR. In addition, 1 HPV 16 positive case revealed evidence for co-infection with HPV 6.

After relating HPV DNA presence with clinical and histological data (Table 2), a tendency for a higher HPV prevalence was found in male patients (66.7 versus 35.7% in female), in lesions located in the floor of the mouth (85.7% of the tumours at this location were HPV positive), and in the highest grades of tumours (well-differentiated tumours displaying 80% of positivity). However, none of these differences were of statistically significant value. Statistical significant differences were found when HPV prevalence was related to the age of the patients. Patients belonging to younger age groups displayed a higher percentage of positive cases (Table 2). Similar results were obtained when age was dichotomised as above 60 years, and 60 years and younger: 17 patients above 60 years of age displayed 29.4% positive cases, whereas in 18 younger patients an HPV positivity of 77.8% was found. These differences were statistically significant ($P<0.05$). A graphical representation of HPV status in different age groups is depicted in Fig. 2.

The two age groups were dichotomised with regard to tobacco and alcohol habits into a lower risk group including no or incidental smokers/drinkers, and a higher risk group of moderate or heavy smokers/drinkers. The HPV prevalence in these groups, in the two sexes, is depicted in Table 3. In the younger age group the clear predominance of HPV-positive cases appeared to be independent of alcohol and tobacco habits in both sexes. In contrast, in the older patient group the predominance of HPV-negative cases was found most notably in the lower risk group of female patients. Owing to the small number of patients in these groups, no statistical analysis was attempted on these data.

DISCUSSION

In this study, special care was taken to prevent false positivity, which is a well-known drawback of the sensitive

Table 2. Statistical analysis of HPV prevalence according to sex and age of the patients, location and grade of the tumours

		HPV		
		Positive	Negative	*P* value
Sex	Male ($n=21$)	14 (66.7%)	7 (33.3%)	$P=0.146$
	Female ($n=14$)	5 (35.7%)	9 (64.3%)	
Age	<40 years ($n=3$)	3 (100%)	0 (0%)	$P=0.011$
	40–60 years ($n=15$)	11 (73.3%)	4 (26.7%)	
	>60 years ($n=17$)	5 (29.4%)	12 (70.6%)	
Location*	T ($n=14$)	5 (35.7%)	9 (64.3%)	$P=0.185$
	FM ($n=7$)	6 (85.7%)	1 (14.3%)	
	T/FM ($n=2$)	1 (50%)	1 (50%)	
	OL ($n=12$)	7 (58.3%)	5 (41.7%)	
Grade†	W DIF ($n=10$)	8 (80%)	2 (20%)	$P=0.251$
	W–M DIF ($n=13$)	7 (53.8%)	6 (46.2%)	
	M DIF ($n=8$)	3 (37.5%)	5 (62.5%)	
	M–P DIF ($n=3$)	1 (33.3%)	2 (66.7%)	

*T = tongue; FM = floor of mouth; OL = other location. †W DIF = well differentiated; W–M DIF = well to moderately differentiated; M DIF = moderately differentiated; M–P DIF = moderately to poorly differentiated.

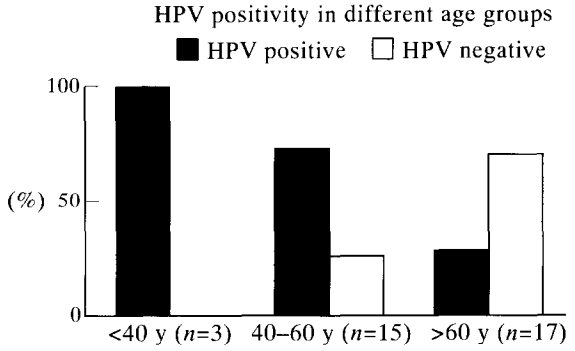

Fig. 2. HPV DNA status in different age groups.

PCR technique. This was accomplished by the special measures on handling the material as well as by the inclusion of a large number of negative controls for different steps of the sample processing. Furthermore, in an attempt to decrease the rate of false negative results, two sets of general-consensus primers were used. These primers target different regions of the HPV genome of a large number of HPV types, which minimises the possibility of missing cases exhibiting viral integration and disruption of a certain primer matched region. However, it should be noted that although the combination of both assays ensures the detection of a broad spectrum of common genital and cutaneous HPVs, putatively distantly related HPVs which might be specific for the oral cavity could have been missed.

HPV 16 appeared the predominant type found, which is in general agreement with other studies [5, 7, 12, 13]. One HPV 16 positive case was only detected with the GP5+–GP6+ primers (localised within the L1 region) and two other HPV 16 containing cases were only detected with the CPI–CPII primers (localised within the E1 region). This may reflect viral integration events or alterations within the viral genome disrupting parts of the L1 or E1 region. Intragenomic alterations affecting the HPV 16 L1 region have been reported before [13, 18]. The 2 HPV X cases which could only be detected with the CPI–CPII PCR may represent a cutaneous HPV type or its relative. In contrast to the GP5+–GP6+ PCR assay, which is specific for mucosotropic HPVs [15], the CPI–CPII PCR system also allows the detection of cutaneous HPVs [16]. It is worthwhile to note that so-called cutaneous HPV genotypes, such as HPV 2 and HPV 4, have occasionally been detected in oral cavity lesions, including carcinomas [13, 19, 20]. Therefore, the possibility of cutaneous types being present in these carcinomas would not be surprising. Further characterisation of these HPV X containing samples was hampered since these displayed very weak signals, only visible after Southern blot hybridisation of the PCR products.

In this study we found a higher prevalence of HPV in males

Table 3. HPV status versus tobacco and alcohol habits in different sex and age groups

Sex	Age	Smoking/drinking habits	HPV	
			Positive	Negative
Male* ($n=17$)	≤60 years	No or incidental ($n=20$)	2 (100%)	0 (0%)
		Moderate or heavy ($n=8$)	6 (75%)	2 (25%)
	>60 years	No or incidental ($n=0$)	—	—
		Moderate or heavy ($n=7$)	2 (28.6%)	5 (71.4%)
Female ($n=11$)	≤60 years	No or incidental ($n=0$)	—	—
		Moderate or heavy ($n=3$)	3 (100%)	0 (0%)
	>60 years	No or incidental ($n=5$)	1 (20%)	4 (80%)
		Moderate or heavy ($n=3$)	1 (33.3%)	2 (66.7%)

*One patient from the younger group of males (27 years old) harboured HPV 16 in the respective OSCC, but was not included in the table owing to the absence of correlation between tobacco and alcohol habits (non-smoker, moderate drinker).

and younger patients, in lesions located in the floor of the mouth, and in well-differentiated tumours. It is interesting to note that the higher HPV prevalence in younger ages was statistically significant. A same tendency was found for vulvar intraepithelial neoplasia [21], although such a relation was not found in previous studies of Brandwein *et al.* [22] and Miller *et al.* [23], involving OSCC. Technical aspects or differences in study populations could be responsible for these discrepancies. In the studies performed by Brandwein *et al.* [22], the material analysed was formalin-fixed and paraffin-embedded, which may result in missing positivity in OSCC [12], particularly when primers are used that direct the amplification of a DNA fragment longer than 200 bp (data not shown). In addition, the population studied by Miller *et al.* [23] was older than the one analysed in our study and belongs to the American continent, a different geographical area. However, in spite of these discrepancies, Miller *et al.* [23] also found that carcinomas associated with dual HPV infections occurred at a lower mean age, reflecting a relationship between HPV presence and age.

Several studies have reported the presence of OSCCs in young patients, in which the two known major risk factors (tobacco and alcohol consumption) were not present [24–26]. In these cases an agent, such as an oncogenic virus, would be a suitable candidate as an aetiologic agent. Our results partially support this hypothesis, since all non-smokers and all incidental drinkers under 60 years of age were HPV positive. However, all patients who were much younger than the common age of OSCC development (the 3 patients under 40 years of age: T2, T9 T11) were moderate alcohol consumers, and 2 of them were also heavy smokers. The results obtained in this particular group, although it includes a small number of cases, could reflect the importance of the combined effect of HPV infection and other risk factors, such as alcohol and tobacco, in OSCC development at an early age. This is in accordance with *in vitro* data showing that the combined effect of oncogenic HPV types with tobacco-related chemicals may result in the transformation of normal oral epithelial cells [10, 11].

The clear predominance of HPV-negative cases in patients over 60 years of age, suggests a less important role of HPV in the older age group in our study population. The presence of moderate or heavy tobacco/alcohol intake in all the male patients over 60 years of age could reflect the importance of these habits, over a long period, in the development of these tumours. In contrast, the majority of females over 60 years of age were either non-smokers or only incidental smokers, and HPV negative. This suggests a putative new pathway for cancer development other than the one related to alcohol and tobacco usage, without any HPV involvement. Although these results have to be interpreted with caution because of the small number of patients analysed, it is interesting to note that Wey *et al.* [27] also reported a subset of oral cancer cases occurring in elderly women (mean age: 71 years), who were non-users of either tobacco or alcohol. Unfortunately, the HPV status of these patients was not reported. Iron deficiency is a condition that has been associated with the development of cancer in elderly women [28], but this information was not available in the present study.

Given the fact that the three HPV types commonly found in the genital tract (HPV 6, 11 and 16) are also found frequently in lesions of the oral cavity [6], it has been suggested that the genital region could be an important route for oral HPV infections. However, in a study involving women from Finland, harbouring genital HPV infections, Kellokoski *et al.* [29] were not able to find a clear correlation between the HPV types present in the oral region and in the anogenital region of these women or their male partners. Moreover, we do not have information about the sexual behaviour of our study population. Therefore, no data exist to support the hypothesis that the different prevalence rates of HPV in the two age groups analysed might be a result of different sexual behaviour of these groups.

The relevance of the higher prevalence of HPV found in well-differentiated OSCC remains to be determined. Although it is tempting to speculate that well-differentiated lesions would be better able to support the viral life cycle, in carcinomas the viral genome can be integrated into the host-cell genome [30]. Hence, viral persistence would not necessarily be dependent on conditions supporting viral replication.

In spite of the limited number of patients analysed, it seems justified to draw attention to the possible importance of age in HPV prevalence in OSCC. However, the data obtained from this study deal with PCR and do not necessarily imply the presence of HPV DNA in carcinoma cells. Therefore, morphological techniques have to be applied to determine HPV presence at the cellular level and to study clonality, before consistent conclusions can be drawn regarding the role of HPV in oral carcinogenesis.

None of the clinically normal gingival samples showed

positivity for HPV even though these were from people in the same age group as the OSCC patients in which a higher HPV positivity was found. Similarly, Ostwald *et al.* [12] were only able to detect HPV DNA in 1 case out of 97 (1%) normal buccal mucosas of a group of healthy volunteers, using PCR techniques. However, it should be considered that subclinical HPV infections may be difficult to detect by analysing biopsies from a limited area alone. This has been supported by a study described by Lawton *et al.* [31] showing a significantly higher HPV positivity rate using mouthwashes instead of scrapes or biopsies of any particular site in healthy volunteers. Still, the failure to detect HPV DNA in the control subjects analysed in this study, together with the recent demonstration of transcriptionally active integrated HPV 16 DNA in a cell line derived from an OSCC [30], suggests a role for HPV in oral carcinogenesis.

Based on the information currently available it is likely that different pathways, with and without HPV involvement, exist for OSCC development. To what extent HPV plays a role might also depend on different geographical, cultural, ethnical and socio-economic factors. Ostwald *et al.* [12] found a tendency for decreasing HPV PCR positivity of normal mucosa at increasing distance from OSCCs. Although this suggests a role for HPV in oral carcinogenesis, HPV infection can only be considered a risk factor for OSCC development if epidemiological evidence is obtained after extensive case-control and prospective studies. The outcome of such studies might be relevant for the future preventive, therapeutic (vaccines) and prognostic approaches of OSCC.

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