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Human papillomavirus infection in oral squamous cell carcinomas from Chilean patients

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ABSTRACT

Human papillomavirus (HPV) is the causal agent of cervical, anogenital and a subset of oropharyngeal carcinomas. In addition, the role of HPV in oral carcinogenesis has been suggested, although the findings are inconclusive. In this study, using conventional polymerase chain reaction (PCR) and genotyping by specific PCR and DNA sequencing, we analyzed the HPV presence in 80 oral squamous cell carcinomas (OSCCs) from Chilean subjects. In addition, we determined the expression of p16, p53, pRb and Ki-67 using immunohistochemistry (IHC). The CDKN2A (p16) promoter methylation was evaluated using methylation-specific PCR (MSP). HPV sequences were found in 9/80 (11%) OSCCs. Non-statistically significant association with p53, pRb, Ki-67 and p16 levels were found ($p = 0.77$; 0.29; 0.83; 0.21, respectively). HPV-16 and 18 were the most prevalent HPV genotypes in 8/9 (89%) OSCCs. In addition, CDKN2A (p16) was methylated in 39% of OSCCs. No association with HPV presence ($p = 0.917$) was found. These results suggest that HPV positive OSCCs are entities that do not resemble the molecular alterations of HPV-associated tumors in a Chilean population. More studies are warranted to determine the role of HPV in OSCCs.

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1. Introduction

Human papillomavirus (HPV) is a double-stranded DNA virus that is the etiological agent of cervix-uterine, anogenital and oropharyngeal carcinomas (Zur Hausen, 2009). In addition, HPV has been detected in oral squamous cell carcinomas (OSCCs), although an etiological role of this virus in this neoplasia has not been established. Oral and oropharyngeal malignancies represent approximately 3% of all malignant tumors in males and 2% in females in the United States (Neville and Day, 2002). In Chile, oral cancer corresponds to 1.6% of total cancer cases. In addition, deaths due to oral cancer represent 1% of total deaths in Chile with a mortality rate of 1.33/100,000 inhabitants in 2002 due to this cause (Riera and Martinez, 2005). The SQC histological type is the most frequent oral tumor among all malignancies in the oral cavity (Rojas-Alcayaga et al., 2010). The most important risk factors for OSCC development are tobacco smoking and alcohol drinking. However, these tumors can also develop in subjects who have never smoked or drank alcohol. Previous studies in OSCCs showed that HPV infection varies between 9.8% (Lingen et al., 2013) and 42% (Nemes et al., 2006). A meta-analysis published in 2001 reported that HPV was present in 46.5% (95% CI, 37.6%–55.5%) OSCCs (Miller and Johnstone, 2001). However HPV frequency in oral carcinomas is relatively low when compared with oropharyngeal carcinomas (Gillison et al., 2008; Pannone et al., 2011). In Chile, there is no published information about HPV prevalence in oral cancer. Studies in cervix uterine cancer and oropharyngeal carcinomas show a characteristic pattern of gene expression. In general, p16 overexpression and downregulation of p53 and pRb are molecular alterations caused by high-risk HPV (HR-HPV) (Rautava and Syrjanen, 2012). High-risk E6 and E7 oncoproteins are directly related to the loss of p53 and pRb, respectively (Boulet et al., 2007). It has been previously suggested that nuclear and cytoplasmic p16 overexpression and pRb loss are surrogate biomarkers of HPV infection in cervix-uterine and oropharyngeal cancers (Chen et al., 2012; Gillespie et al., 2009; Goon et al., 2009; Lewis et al., 2010; Weinberger et al., 2004). However, it seems that in OSCC, the expressions of these genes are different because the p16 expression is not always detected in HPV positive cases and a small proportion of the cases is positive in

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HPV negative cases (Nemes et al., 2006; Harris, 2011). However, in oropharyngeal tumors, where a subset of tumors is related to HPV infection, there is a significant proportion of cases that are HPV negative and p16 positive (Thomas and Primeaux, 2012). The aim of this study was to determine the presence of HPV in OSCCs in the Chilean population and to establish if there are associations between HPV infection and expression of molecular biomarkers related to cell cycle control and apoptosis.

2. Materials and methods

2.1. Sample collection

3Eighty OSCCs were collected considering a database from the Register of the Pathological anatomy service from the Faculty of Dentistry, University of Chile (SAP-FOUCH), between the years 2000 and 2014 and from the National Cancer Institute, between the years 2013 and 2014. The demographical and clinical data were directly obtained from the clinical record. The study was approved by the Ethical Committee Board from the Faculty of Medicine and Faculty of Dentistry, University of Chile.

2.2. DNA extraction and polymerase chain reaction (PCR) for HPV presence

Paraffin-embedded samples were cut into 10 μm slices and prepared according to a previously described method (Greer and Alexander, 1995), followed by digestion with proteinase K (200 mg/mL) at 55 °C overnight. The integrity status of DNA was confirmed by PCR for a 110 base pairs (bp) beta-globin fragment using primers PCO3: 50-ACACAA CTGTGTTCACTAGC-30 and PCO4: 50-CAACTTCATCCACGTTCACC-30. The presence of HPV DNA was evaluated by PCR using GP5+/GP6+ primers (150 bp) for the HPV L1 gene (De Roda Husman et al., 1995). The PCR reaction was performed in a total volume of 25 μL containing 1 U of HotStar Taq (Qiagen, Hilden, Germany), 50 mM of each primer, 0.2 mM of each dNTP and HotStar Taq PCR buffer as supplied by the enzyme manufacturer (Qiagen) (contains 1.5 mM $MgCl_2$, Tris–Cl, KCl, $(NH_4)_2SO_4$ pH 8.7). The amplification was carried out with initial enzyme activation at 95 °C for 5 min, followed by 45 cycles that included a 1 min denaturation step at 94 °C, a 2 min annealing step at 40 °C and a 1.5 min chain elongation step at 72 °C; and a final elongation at 72 °C for 5 min. The amplification of HPV-16 and -18 was carried out through specific PCR in generic HPV positive samples. The primers used for HPV-16 amplification were: Forward: 5′-GGTCGGTGGACCGG TCGATG-3′ and Reverse: 5′-GCAATGTAGGTGTATCTCCA-3′ and for HPV-18 amplification were: Forward: 5′-CCTTGGACGTAAATTTTTGG-3′ and Reverse: 5′-CACGCACACGCTTGGCAGGT-3′. The PCR conditions were: initial denaturation at 95 °C for 5 min followed by 45 cycles consisting of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s and a final extension at 72 °C for 5 min. PCR products were characterized by 2% agarose gel electrophoresis and visualized under UV transillumination after ethidium bromide staining. As positive controls for amplification, full genomes of HPV16 and 18 (kindly donated by Prof. Michelle de Villiers, German Cancer Research Centre, Heidelberg, Germany) were used, and nuclease-free water was used as negative control.

2.3. RNA extraction and HPV-16 E6/E7 transcript detection

RNA purification was carried out using the High Pure RNA paraffin kit (Roche), following the manufacturer's instructions. The RNA obtained was suspended in 50 μL of TE buffer (10 mM Tris–Cl, 1 mM EDTA) and stored at −80 °C until use. The cDNA preparation was made as follows: 100 ng of purified RNA was transformed to cDNA using RNAsin 1 U/μL (Promega, USA); 1 × buffer TR (Promega, USA); 10 μg/μL random primers (Promega, USA); 20 U/μL MMLV (Promega, USA) and 2 mM dNTPs in a final volume of 20 μL. MMLV negative controls were included. The reaction mixture was incubated at 37 °C for 1 h, 70 °C for 15 min and stored at −20 °C. We used GAPDH amplification as an inner constitutive expression control. The E6 and E7 transcription detection was carried out using transcriptase-reverse PCR according to a previously described method (Aguayo et al., 2011).

2.4. CDKN2A promoter methylation

The CDKN2A promoter methylation was evaluated in oral carcinomas using methylation-specific PCR (MSP) according to previously reported methods (Guzman et al., 2007). MYOD1 amplification was used as an inner positive control. Hela cells (harboring HPV-18 genomes) treated with SssI methylase were used as positive controls and nuclease-free water was used as a negative control.

2.5. Immunohistochemistry

Sections of 4 μm thickness from paraffin blocks of each case were obtained and collected in positively charged slides (Lab Cellpath, England). Later they were deparaffinized in xylene and rehydrated in descending alcohols to distilled water. Immunohistochemical staining was performed using the streptavidin–avidin–biotin complex (ABC) and the reaction was visualized with diaminobenzidine tetrahydrochloride (DAB). Sections were placed in sodium citrate buffer (pH 6) for 45 min in a pressure cooker for antigen retrieval, and then were washed with PBS for 5 min. Endogenous peroxidase activity was blocked by incubating the sections in 3% hydrogen peroxide in methanol at room temperature for 30 min. The sections were pre-incubated with horse serum for 20 min at room temperature and then incubated for 30 min with primary antibodies; p53 (Cell Marque Laboratory, USA. R.T.U. dilution); p16 (Biogenex Laboratory, USA. R.T.U. dilution), pRb (Thermo laboratory, USA. 1:50 dilution) and Ki-67 (Cell Marque Laboratory, USA. R.T.U. dilution), in a moist chamber at 37 °C. Sections were then washed with PBS for 5 min and incubated with biotinylated secondary antibody for 30 min at 37 °C and then with peroxidase-conjugated streptavidin (Universal Detection System Vectastain Elite Kit wide spectrum ABC-HRP, RTU, Vector-USA, EE.UU) for 20 min at 37 °C. The reaction was finally visualized with diaminobenzidine (DAB) and staining with Harris hematoxylin. Negative controls were obtained by substitution of the primary antibody with PBS. The immunostaining was evaluated and interpreted by an experienced oral pathologist. The immunopositive cells were evaluated using an Olympus BX41 microscope using 40× and five areas were selected in a non-random manner in neoplastic epithelia areas with a higher positive labeling. For mutated TP53, Ki67 and pRb the signal was considered positive when the nucleus was stained without considering signal intensity. For p16, the signal was considered positive when the nucleus and cytoplasm were stained without considering intensity. The fields were photographed using an Olympus BX41 microscope using the software Micrometrics SE Premium program. One thousand neoplastic epithelial cells were counted using the Image J software, determining the number of positive epithelial cells for each of the used antibodies.

2.6. Statistical analysis

Shapiro–Wilk test was used for an exploratory normality analysis of the data. To determine differences in gender, age and differentiation grade between HPV positive and negative OSCCs the Fischer's exact test was used. To determine the association among HPV16/18 presence and p16, pRb, p53 and Ki-67 positive OSCCs the Mann–Whitney test was used. The results were expressed as median and interquartile range. A p-value less than 0.05 was considered statistically significant. All of the tests were determined using the Stata 11.0 software.

3. Results

3.1. Clinical–pathological features

Eighty OSCCs were selected for this study. The cases were confirmed histologically. Thirty-six specimens were from women and 44 specimens were from men. In addition, non-statistically significant differences were observed in age, differentiation grade and invasion pattern between men and women (Chi 2, p = 0.35, 1.000 and 0.19, respectively) (Table 1).

3.2. HPV, p53, pRb, p16 and Ki-67 detection in OSCCs

Using conventional generic PCR, 9 cases (11%) were positive for generic HPV infection. There was not a statistically significant association between HPV presence and age, gender, differentiation grade and invasion pattern (Chi 2, p = 1.00, 0.75, 0.12 and 0.44, respectively) (Table 2). In addition, we determined the expression of p53, pRb and p16 using IHC. 10 positive cases were observed for p16 expression, where 2 cases were HPV positive and 8 cases were HPV negative. The expression of pRb showed 44 positive cases, 4 HPV positive and 40 HPV negative. Seventy cases were p53 positive, of which 8 cases were HPV positive and 62 were HPV negative. All of the cases were positive for Ki-67.

There was not a statistically significant association between the number of cells with the expression of p53, pRb, Ki-67 and p16 and HPV presence in OSCCs (Mann–Whitney test, p = 0.77, 0.29, 0.83 and 0.21, respectively).

3.3. HPV genotyping and E6/E7 expression

Using specific PCR for HPV-16 and -18 and confirmation through direct sequencing of the amplified DNA fragments, we determined that 5/9 (56%) HPV positive cases were genotyped as HPV-16, 3/9 (33%) were HPV-18 and 1 case was HPV-10. Table 3 shows the clinical pathological features of the HPV-16/18 positive cases. HPV-16 E6/E7 transcripts were detected in 4/5 (80%) of HPV-16 positive specimens and in 1/3 HPV-18 positive cases.

3.4. CDKN2A promoter methylation

Because the collected tissue was insufficient in a significant number of cases, we determined the CDKN2A promoter methylation in 46 OSCCs. All of the cases were positive for MYOD1 amplification post-treatment with sodium bisulfite. CDKN2A was methylated in 18/46 (39%) OSCCS. There was not a statistically significant association between HPV presence and CDKN2A promoter methylation (Chi 2, p = 1.00). In addition, CDKN2A methylation was not associated with p16 detection using IHC.

Table 1
Clinicopathological features of OSCC patients.

		Number of subjects (%)		
		Women	Men	p-Value
Age	<60	17 (47)	26 (59)	0.35
	≥60	19 (53)	18 (41)	
	Total	36 (100)	44 (100)	
Differentiation grade	Poor	1 (3)	2 (5)	1.00
	Moderate	13 (36)	14 (33)	
	Well	22 (61)	28 (62)	
	Total	36 (100)	44 (100)	
Invasion pattern	Expansive	6 (17)	14 (32)	0.19
	Infiltrative	30 (83)	30 (68)	
	Total	36 (100)	44 (100)	

Table 2
Comparison between HPV status and clinicopathological and molecular features of OSCC patients.

		Number of subjects (%)		
		HPV−	HPV+	p-Value
Age	<60	27 (53)	3 (60)	1.00
	≥60	44 (47)	6 (40)	
	Total	71 (100)	9 (100)	
Gender	Female	31 (45)	5 (55)	0.75
	Male	40 (55)	4 (45)	
	Total	71 (100)	9 (100)	
Differentiation grade	Poor	2 (3)	1 (9)	0.12
	Moderate	22 (30)	5 (55)	
	Well	47 (67)	3 (36)	
	Total	71 (100)	9 (100)	
Invasion pattern	Expansive	19 (18)	1 (20)	0.44
	Infiltrative	52 (82)	8 (80)	
	Total	71 (100)	9 (100)	
Protein expression	p53−	9 (13)	1 (9)	0.73
	p53+	62 (87)	8 (91)	
	Total	71 (100)	9 (100)	
	pRb−	31 (43)	5 (55)	0.53
	pRb+	40 (57)	4 (45)	
	Total	71 (100)	9 (100)	
	p16−	63 (88)	7 (82)	0.26
	p16+	8 (12)	2 (18)	
	Total	71 (100)	9 (100)	
	Ki-67+	71 (100)	9 (100)	1.00
	Ki-67−	0	0	
	Total	71 (100)	9 (100)	
P16 methylation	Negative	24 (62)	4 (57)	1.00
	Positive	15 (38)	3 (43)	
	Total	39 (100)	7 (100)	

4. Discussion

In this study, HPV infection was detected at low prevalence in OSCCs from Chilean patients. Using conventional PCR and sequencing we determined that HPV was present in 11% of OSCCs. This HPV frequency is in agreement with reports that show that HPV presence worldwide ranges between 0 and 45% (Kruger et al., 2014; Lingen et al., 2013; Nemes et al., 2006; St Guily et al., 2011; Zhang et al., 2004) being HPV-16 the most prevalent genotype found in oral carcinomas with a higher prevalence in women (Kreimer et al., 2005; Syrjanen et al., 2011). Our results are in agreement with these previous reports although we did not find some statistically significant association between HPV presence and gender. In the HPV positive cases analyzed, one specimen was genotyped as HPV-10. This genotype is associated with flat warts and it has not been reported in OSCCs. Probably this genotype was not directly related to the development of oral carcinomas and it was a bystander infection into the tissue. However, some studies reported the presence of low-risk HPV such as HPV-6 and -11 in oral carcinomas (Kreimer et al., 2005). In this respect, it has been shown that low-risk HPV presence in OSCC patients is related to a poor prognosis (Lee et al., 2013; Rautava et al., 2012). Thus, some authors suggested that low-risk HPV genotype could be related to OSCC development in some patients (Kreimer et al., 2005; Syrjanen, 2010).

It is known that the mere DNA presence of HPV in clinical specimens is not a sufficient condition for causality. Thus, additional markers need to be analyzed in order to determine if the role of HPV in OSCCs resembles the role of this virus in HPV-related tumors such as cervical or oropharyngeal carcinomas. In this respect, a very important event in HPV-associated malignancies is the E6 and E7 overexpression, caused by the loss of E2 protein by viral integration into the host genome. For this reason, we were interested in knowing the expression of E6 and E7 transcripts in HPV-16/18 positive specimens. Our results showed that HPV-16/18 E6 and E7 transcripts were detected in 5/8 (63%) HPV positive specimens suggesting viral functionality in these patients.

Table 3
Clinicopathological and molecular features of HPV 16/18 cases.

Patient	Age	Gender	Location	Differentiation	HPV genotype	E6/E7 expression	CDKN2A methylation
1	75	F	Tongue	Moderate	16	Yes	N/D
2	68	M	Tongue	Well	16	Yes	Positive
3	51	M	N/D	Well	16	Yes	N/D
4	60	F	Reborde	Moderate	16	Yes	Positive
5	61	F	N/D	Moderate	16	No	N/D
6	48	M	Tongue	Poor	18	Yes	Negative
7	70	M	Reborde	Moderate	18	No	Negative
8	80	F	Reborde	Moderate	18	No	Negative

It has been suggested that HPV positive tumors have a different biological profile when compared with those HPV negative. The first are associated with p16 overexpression and pRb p53 loss (Rautava and Syrjanen, 2012).

The high-risk E6 oncoprotein is able to induce the p53 degradation into the proteasome. On the other hand, E7 oncoprotein induces the pRb degradation releasing the E2F for S-phase induction (Boulet et al., 2007). This event is associated with p16 overexpression in HPV-associated tumors.

Klaes et al. (2002) were the first to show that p16 detection using immunohistochemistry was a specific and reliable biomarker for the identification of HPV-infected neoplastic cervical cells (Klaes et al, 2002). In addition, it has been reported that nucleus/cytoplasmic p16 detection and pRb loss may be used as a surrogate marker of HPV presence (Chen et al., 2012; Gillespie et al., 2009; Goon et al., 2009; Lewis et al., 2010; Weinberger et al., 2004).

In this study we found only 4/9 (44%) HPV-16/18 cases that were pRb negative and only 2/9 (22%) cases were positive for nuclear and cytoplasmic p16 staining. Thus, our results suggest that p16 and pRb alterations are not necessarily associated with HPV presence in OSCCs from Chilean patients. This finding is in agreement with some reports that found that p16 expression is not always positive in HPV-related OSCCs and a small proportion is positive in HPV negative cases (Harris et al., 2011; Nemes et al., 2006). This situation has been described in HNC where a proportion of about 15 to 20% are p16 positive and HPV negative (Smeets et al., 2007) suggesting that p16 expression is detectable in the absence of HPV and is not necessarily a consequence of HPV infection (Compton et al., 2011). The results allow us to speculate that non-viral mediated mechanisms are involved in p16 overexpression. However, we cannot deny the possibility that other viral infections be related to this finding. To this respect, more studies are warranted. Taken together, our results suggest that p16 is not a surrogate marker of HPV infection on OSCCs.

The absence of p16 expression in HPV negative or positive cases may be explained by the frequent inactivation of this gene by deletion, mutations or gene hypermethylation (Ruesga et al., 2007). It has been previously reported that tobacco smoking is associated with frequent CDKN2A (p16) hypermethylation (Scesnaite et al., 2012). Unfortunately, in our study it was not possible to obtain the tobacco smoking information in some patients, so we did not find any association between smoking and p16 promoter methylation.

It has been reported that in HNCs with p16 overexpression, an improved prognosis and lower recurrency are shown when compared with cases without p16 overexpression (Vairaktaris et al., 2007; Weinberger et al., 2004). In addition, the HNC cases HPV negative/p16 negative/p53 positive have a poor survival when compared with HPV positive/p16 positive /p53 negative cases (Smith et al., 2010).

The p53 detection by immunohistochemistry has been used as a surrogate biomarker of p53 mutations (Braakhuis et al., 2014).

In our study, it was found that p53 immunostaining was positive in a high percentage of the analyzed OSCCs, however the expression of this protein was not related to the presence of HPV. This finding is in agreement with studies that show that HPV infection in OSCCs is not related to an increased p53 expression (Nemes et al., 2006; Shuyama et al., 2007).

On the other hand, Ki-67 is considered a biomarker for cell proliferation and was used in order to compare the proliferation in the presence or absence of the virus (Freudlsperger et al., 2012; Mimica et al., 2010). However, no association between Ki-67 and HPV was found, which is in agreement with data previously reported.

A possible limitation of this study is the use of not other additional methods of HPV detection, e.g. in situ hybridization studies. However, the method for generic HPV detection that was used in this study (using GP5/6+ primers) is highly sensitive and specific for HPV detection (Aguayo et al., 2007, 2010). In order to confirm the HPV positivity, all of the specimens were analyzed at least two times using adequate controls. In situ hybridization (ISH), although is a good alternative to confirm the positivity in the context of the tissue, is less sensitive than PCR.

Taken in consideration the data obtained in this study we conclude that the molecular profile in HPV positive OSCCs is different when compared with tumors currently caused by HPV presence. However, we cannot deny the possibility that other factors are related to this different molecular profile. As previously mentioned, as the oral mucosa is exposed to components such as tobacco smoke or alcohol, molecular alterations caused by these components are plausible.

In conclusion, in this study the prevalence of HPV in oral carcinomas from Chilean patients was 11%. No association with molecular changes related to a causal role of HPV was observed. More studies are needed to determine the role of HPV infection in a subset of oral carcinomas.

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