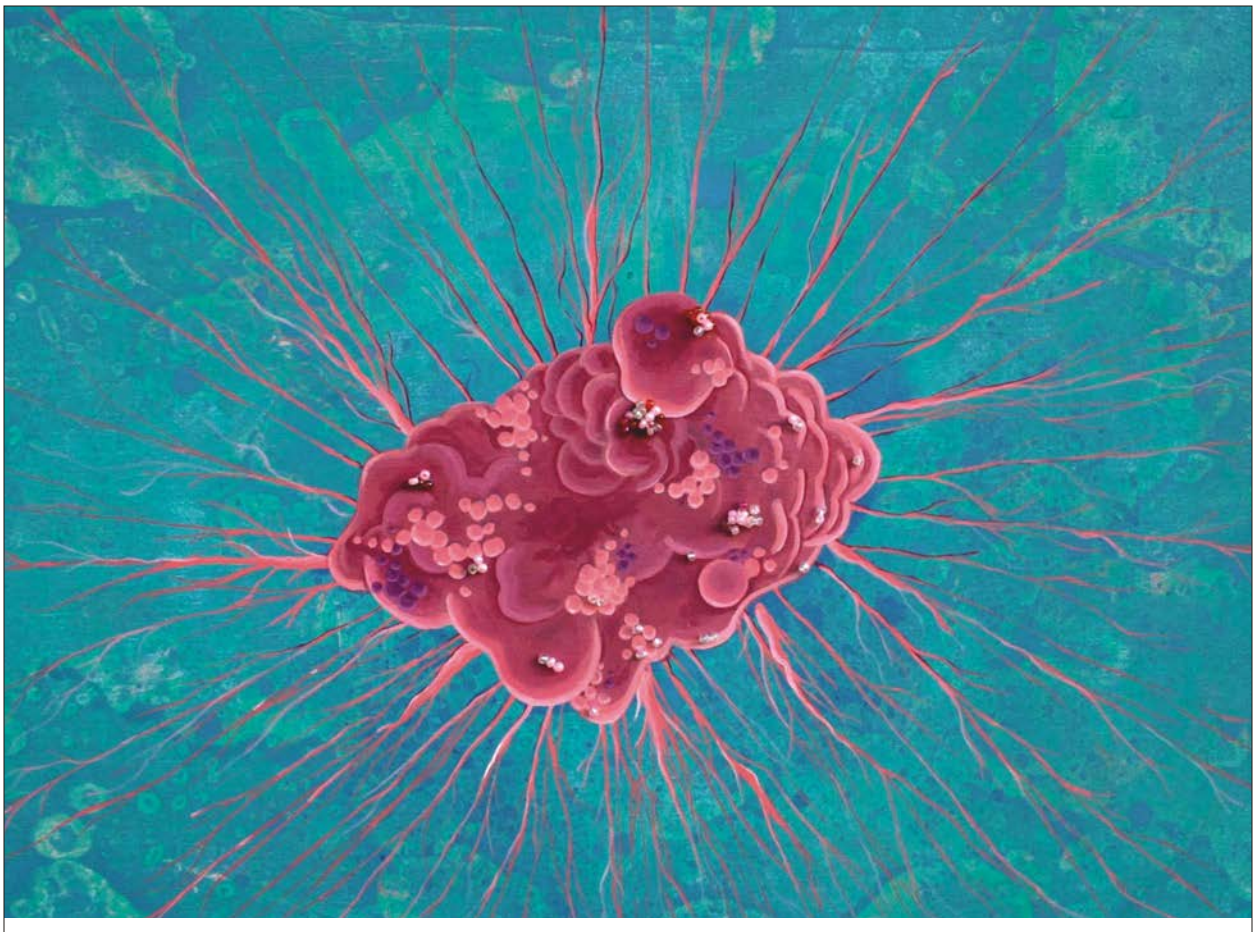

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Role of Human Papillomavirus Type 16 in Squamous Cell Carcinoma of Upper Aerodigestive Tracts in Colombian Patients

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ABSTRACT

Infections with mucosal High-Risk (HR) Human Papillomavirus (HPV) types are associated with a subset of Squamous Cell Carcinoma (SCC) of Upper Aerodigestive Tract (UAT). However, the prevalence of HR HPV-positive UAT cancers appears to vary in different geographical areas. To evaluate the role of HR HPV infection in Colombian population, we have characterized the prevalence of HPV16 infection and viral transcriptional activity in UAT cancers from 72 Colombian patients including tongue (n = 22), palate (n = 2) and gum (n = 2); larynx (n = 40) and esophagus (n = 6). HPV16 presence was determined by different molecular assays that allowed the evaluation of HPV16 DNA positivity, E6^E7 expression as well as the physical status of the viral genome i.e., integrated versus episomal. HPV16 DNA was detected in 35 and 30.8% of laryngeal and oral cavity cancers, respectively. In contrast, none of the esophageal cancers analyzed were HPV16-positive. Forty one percent of the UAT cancers HPV16 positives harboured the integrated form of HPV16 genome while 18.1% of the cases appeared to have both episomal and integrated viral DNA forms. E6 and E7 expression was detected in 41% of the UAT cancers independently of the HPV16 status. HPV16 DNA integration and E6 and E7 expression did not show any correlation with gender, age and type of cancer. These data provides evidence for the involvement of HPV16 in development of UAT cancers in the Colombian population.

Key words: Upper aerodigestive cancer, HPV16 infection, E6 and E7 expression, HPV integration

INTRODUCTION

Human Papillomaviruses (HPV) are Double-stranded DNA viruses that infect the cutaneous and mucosal epithelium. More than 100 HPV have been isolated and characterized so far and they have been classified in different genera (De Villiers *et al*., 2004). Epidemiological studies have demonstrated that approximately 15 mucosal HPV types, referred as High-Risk (HR) HPV types, are associated with intraepithelial neoplasia and invasive cervical carcinoma of the anogenital region, e.g., cervix, vagina, vulva, pines and anus (Munoz, 2000; Clifford *et al*., 2003; Bosch *et al*., 2002). The HVP 16 is the most frequent type detected in cervical Squamous Cell Carcinoma (SCC) being responsible for approximately 50% of cervical cancers worldwide (Munoz *et al*., 2003; Moosavi *et al*., 2008).

Two viral oncoproteins, E6 and E7, play a key role in cellular transformation altering fundamental cellular events and providing a selective growth advantage to the infected cells (Mantovani and Banks, 2001; Munger and Howley, 2002; Gallo *et al*., 2003; De Oliveira, 2007). In addition to the E6 and E7 oncoproteins, integration of viral DNA into host genome appears to favour carcinogenesis. Accordingly, episomal HPV genome is found in a high proportion of low grade HPV-positive pre-malignant lesions, e.g., cervical intraepithelial neoplasia grade I (CIN-I) while HPV DNA integration is frequently detected in CIN-III and cervical cancer lesions (Alazawi *et al*., 2002; Hopman *et al*., 2004; Hudelist *et al*., 2004; Saunier *et al*., 2008). The possible positive contribution of HPV DNA integration to cervical carcinogenesis is explained by the fact that this event results in the disruption of E2 gene and loss of E2 protein. Since E2 has the ability to negatively regulate the activity of the E6 and E7 promoter, its loss lead to an increased expression of the HPV oncoproteins, favouring cellular transformation (Schmidt *et al*., 2005; Sathish *et al*., 2004; Scheurer *et al*., 2005).

In addition to cervical cancers, HPV infection is associated with the carcinogenesis of the Upper Aerodigestive Tract (UAT). Several epidemiological studies provided evidence that HR-HPV are implicated in the development of a subset of SCC from oral cavity, esophagus, pharynx and larynx (Gillison *et al*., 2000; Gillison *et al*., 2008; Kreimer *et al*., 2005; Moradi and Talat, 2006). However, the prevalence of HR-HPV infection in the SCC of non-genital regions varies between 15-35% (Wong and Munger, 2000; Herrero *et al*., 2003). This phenomenon could be explained by genetic features of the examined population (Scheckenbach *et al*., 2004; Gatoo *et al*., 2011), presence of additional risk factor in a specific population/geographical region as well as the methodology used in the study e.g., HPV detection assay (Semnani *et al*., 2006; Marur *et al*., 2010; Kingma *et al*., 2010; Delavarian *et al*., 2010).

Additional studies are required to further corroborate the association of the HR HPV types with UAT in other populations, since they will have an important impact on preventing, screening and prophylactic strategies of these malignancies. Patients with HPV-positive oro-pharyngeal cancers appear to have higher response rates to radiation and chemotherapy and increased survival than the ones with HPV-negative tumours (D'Souza *et al*., 2010). Thus, determination of HPV positivity will influence therapeutic and follow-up strategies. Moreover the knowledge of HPV prevalence in non-genital tract cancers in specific geographical areas will contribute to estimate the impact of prophylactic HPV vaccination at the long term incidence of other type of cancers caused by the virus (Kim and Goldie, 2008).

Studies in non-genital cancer HPV-associated as the head and neck cancers, showed of between 86-95% of the cases are positive for HPV16 while the remaining HPV types appear to play only a marginal role (Ragin *et al*., 2004; Chaudhary *et al*., 2009). In this study, we have calculated the prevalence of HPV16 infection and characterized E6^E7 expression according the physical status of viral DNA in 72 UAT squamous cell carcinoma from Colombia, including oral cavity, larynx and esophagus, using different current molecular methods.

MATERIALS AND METHODS

Sample and data collection: This epidemiological descriptive study was performed from 2005 to 2007 years and the patients included were selected from the database and clinic archives of the pathology laboratory in the Hospital-University of Valle and the Clinic of Rafael Uribe of the health national services in Cali, Colombia. This study was approved by ethical committee of both institutions.

A total of 177 cases with SCC diagnostic localized in different regions of the upper digestive tract were selected from patients who were attended at the pathology laboratory since 1996 to 2005. Formalin Fixed Paraffin Embedded (FFPE) tissue cancer blocks were retrieved from the archive of the pathology section at the Colombian Hospitals (Cali). Tissue blocks included cancers from tongue (n = 45), gum (n = 1), palate (n = 2), lips (n = 3), floor of mouth (n = 5) and cheek (n = 1), oropharynx (n = 2), nasopharynx (n = 2), hypopharynx (n = 1), larynx (n = 100) and esophagus (n = 15). The histopathological diagnoses were confirmed by a new analysis of haemotoxilin and eosin stained sections performed by the pathologist of the Hospital-University of Valle. Five slides of 10 μm were obtained for each FFPE tissue cancer block, collected in one 1.5 mL Eppendorf tube and stored at room temperature for molecular biology analyzes.

DNA extraction: To remove the paraffin, two 10 μm slides were washed twice with xylene followed by one wash with absolute ethanol. The biopsies were then dried at 55°C for 20 min in a heating block to remove residual ethanol. An aliquot of 200-300 μL of dilution buffer (0.1 M Tris-HCL pH 7.4, 0, 5% Tween 20, 10 mM EDTA) with proteinase K (0.5 mg mL^{-1}) according to the amount of tissue available was added. Tissue digestion was performed by incubation at 56°C overnight with continuous shaking and Proteinase K was inactivated by a final step at 95°C for 10 min. DNA was stored at 4°C until their use. DNA quality was evaluated by amplification of a fragment of 268 Base Pair (bp) of β-goblin gene (De Roda Husman *et al*., 1995) using the primers GH20 and PC04 with the procedure reported by Saiki *et al*. (1985) (Table 1). PCR products were analysed by electrophoresis in a 2% agarose gel, stained with ethidium bromide and visualized under Ultraviolet Light (UV). The β-goblin-negative samples were excluded from further analyses.

HPV 16 DNA detection: The presence of HPV16 DNA was detected by PCR. A fragment of 82 bp and other one of 277 bp from E6 and E7 viral genes were amplified using specific primers (Table 1). Tumour samples with amplimers for both HPV genes were considered positive for HPV infection. The amplicons were detected by electrophoresis in a 2% agarose gel and the specificity evaluated by Southern blotting using a HPV16 genome probe labelled with ^{32}p. Briefly, 100 ng of the amplicons were blotted onto Hybond™ N^+ nylon membrane (Amersham™, UK) by capillary

Table 1: Sequences of forward (F) and reverse (R) primers and sizes of the PCR-amplified fragments

Gene	Name	Primer sequence (5'→3')	PCR fragment size (bp)	Reference
β-goblin	PC04	F. CAACTTCATCCACGTTCACC	268	Saiki *et al*. (1985)
	GH20	R. GAAGAGCCAAGGACAGCAGGTAC		
E6HPV16	16E6 F	F. GAGAACTGCAATGTTTCAGGACC	81	Peitsaro *et al*. (2002)
	16E6 R	R. TGTATAGTTGTTTGCAGCTCTGTGC		
E7HPV16	E7HPV16 F	F. CAT GGA GAT ACA CCT ACA TTG	277	
	E7HPV16 R	R. CAGATGGGGCACACAATTCC		
E2HPV16	16E2 F	F. AACGAAGTATCCTCTCCTGAAATTATTAG	82	Peitsaro *et al*. (2002)
	16E2 R	R. CCAAGGCGACGGCTTTG		
GAPDH	GAPDH F	F. GGTGAAGGTCGGAGTCAACGGA	240	McLaughlin *et al*. (1997)
	GAPDH R	R. GAGGGATCTCGCTCCTGGAAGA		
E6^E7	HPV16-768-24 D	F. ACAAAGCACACACGTAGACATTCG	800-1000	Thorland *et al*. (2000)
E7	RT-PCR	F. TCTGTTCTCAGAAACCATAATCTAC	800-1000	Thorland *et al*. (2000)
	E7 HPV16-839			

wetting and hybridized with a radiolabeled (α ^{32}p-dATP) full length HPV16 DNA probe. The hybridization was carried out at 50°C overnight in buffer containing 5X SSC-Denthard solution. The Nylon membrane was washed at room temperature in 2×SSC and 0,5% SDS twice for 30 min dried and submitted to a radiographic film for 24 h at -70°C.

E6 HPV16 sequencing analysis: In order to monitor the possible cross-contaminations between biopsies during their processing, the HPV16 E6 gene PCR product of 10 randomly selected SCC was sequenced. Direct sequencing reaction was performed by using the Big Dye Terminator Cycle Sequencing kit (Perkin Elmer) and an ABI PRISM Applied Biosystem model 310 A automated sequencer (Perkin Elmer Applied Biosystem, New Jersey, USA).

Physical status of HPV DNA: The integrated form of HPV16 DNA into the cell genome was determined by PCR assay of a fragment of 82 bp in the central region of E2 gene which is commonly disrupted during integration (Park *et al*., 1997; Kalantari *et al*., 1998). Positivity for E2 assay was used as criterion to differentiate the episomal from the integrated forms of viral genome (Peitsaro *et al*., 2002). DNA extracted from cervical carcinoma cell line CaSki which contain numerous integrated copies of HPV16 genome was used as control.

HPV16 E6^E7 expression: To determine the association of integrated form of HPV16 DNA with the expression of the oncogenes E6 and E7, APOT (amplification of papillomavirus oncogenes transcripts) assay was performed in the tumor cells HPV16 DNA positives. The APOT methodology allows the discrimination of HPV mRNAs derived from integrated and episomal viral genomes (Klaes *et al*., 1999). Total RNA was extracted from 3 or 4 slides of FFPE biopsies, using the Absolutely RNA miniprep Kit (Stratagene™). In order to remove any contaminating DNA, RNA solution was treated with DNAse and purified with affinity columns. cDNA synthesis was performed with a dT_{17}-p3 primer (5'-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3') using the Superscript First-Strand Synthesis System (Invitrogen™) according to the manufacturer's instructions. The cDNA quality was evaluated by the amplification of a Glyceraldehyde 3-Phosphate Dehydrogenase (GDPDH) gene fragment (McLaughlin *et al*., 1997). PCR assays were performed using the TagP3 sequence and HPV16 E7 primers (Table 1). A second round of PCR was performed with E7HPV16-839 primer containing E7 HPV16 internal sequences (Thorland *et al*., 2000).

Statistical analyses: HPV positive samples were stratified by anatomical cancer structures. HPV positive samples were analyzed according to the data of individual-level (e.g., gender, age, differentiation degree of the tumour). Smoking status was not considered because this data was not available in the clinical records. Viral variables including DNA presence, physical status and oncoprotein expression were determined and stratified by anatomic cancer structures and the frequencies of their variables were matched with the individual data.

The association of integrated HPV16 DNA presence with viral oncoprotein expression, tumour structure and individual data were calculated by Chi-square test. Fischer's exact test was applied to the analyses that include a low numbers of samples.

The possible association between HPV16 DNA integration into cell genome and E6^E7 genes expression were analyzed by a Fisher's exact test with statistically significant parameters ($\alpha = 0.05$ and $\beta = 80\%$). The statistical program SPSS 15.0 for Windows™ was used.

RESULTS

Prevalence of HPV16 infection in UAT cancers: We first evaluate the quality of DNA of 177 cases by PCR amplification of a fragment of the β-goblin gene. Seventy two samples were positive for β-goblin PCR assay and the remaining 105 cases were excluded from the study. The β-globin positive cases comprised SCC from the oral cavity (tongue (n = 22), palate (n = 2) and gum (n = 2)), larynx (n = 40) and esophagus (n = 6) (Table 2).

The population ages showed a normal distribution with a range between 25 and 91 years old and mean age of the population was 64.38±11.4 years old. Near to 57% of patients were older than 65 years at the time of the pathological report. More of 69% of tumors were from men and significant proportion of tumor had well differentiated grade (56.9%). All these characteristics are showed in the Table 2.

In total, 22 UAT of 72 cancers were HPV16 positives (30.6%). For 10 randomly selected HPV16-positive cases, a fragment of E6 gene was amplified and sequenced in order to evaluate whether different HPV16 variants were present. The sequencing analysis revealed the presence of three different E6 natural variants making sample contamination an unlikely explanation for our findings.

HPV16 DNA was detected in 35% (14/40) and 30.8% (8/26) of SCC from larynx and oral cavity, respectively (Table 3). Neither E6 nor E7 HPV16 DNA genes were detected in SCC from esophagus. The specificity of the amplification was confirmed by Southern blot assay using a full length HPV16 DNA probe labelled with ^{32}p. Statistically significance difference was not observed in the HPV16 frequency between the genders (p-value = 0.782 for Chi-square test, $\alpha = 0.05$) or the ages (p-value = 0.538 for Chi-square test). The HPV16-positive laryngeal cancers appeared to be more frequent in patients with age below 50 years old, although this association was not statistically significant (p-value = 0.06 for Chi-square test). HPV16 DNA positivity was slightly lower in poorly differentiated SCC (21.4%) in comparison to moderate and low differentiated SCC (31.7 and 35.3%, respectively). However, the association was not statistically significant (p-value = 0.68 for Chi-square test).

Physical status of HPV16 genome and E7^E6 oncogenes expression in UAT cancers: The HPV16 DNA was found integrated in 9 of 22 HPV16 DNA positive samples (40.9%) including

Table 2: Clinical and pathological characteristic of 72 β-globin positive SCC from upper digestive tract of Colombian cases

Characteristic	Esophagus (n = 6) (%)	Larynx (n = 40) (%)	Oral cavity (n = 26) (%)	Total (n = 72) (%)	p-value*
Age (years)	71.0±6.48	61.17±9.07	68.3±13.85	64.38±11.4	0.178
< 50	1 (16.7)	5 (12.5)	3 (11.5)	9 (12.5)	
50-65	1 (16.7)	16 (40.0)	5 (19.2)	22 (30.6)	
>65	4 (77.0)	19 (47.5)	18 (69.3)	41 (56.9)	
Sex					0.005
Female	4 (77.0)	5 (12.5)	13 (50.0)	22 (30.6)	
Male	2 (33.0)	35 (87.5)	13 (50.0)	50 (69.4)	
Differentiation grade of tumors					0.027
Well	4 (77.0)	24 (60.0)	13 (50.0)	41 (56.9)	
Moderate	1 (16.7)	10 (25.0)	9 (34.6)	17 (23.6)	
Poor	1 (16.7)	6 (15.0)	4 (15.4)	14 (19.5)	

Seventy-two SCC positive for β-globin PCR assay from 177 cases evaluated.. *The p-value of chi-squared between the different groups of age, sex and grade of differentiation were calculated with the total data

Table 3: HPV16 presence in 72 β-globin positive SCC from upper digestive tract of Colombian cases

Characteristic	n	HPV16 (%)	p-value	n	Larynx (%)	p-value	n	Oral cavity (%)	p-value
N	72*	22 (30.6)		40	14 (35)		26	8 (30.8)	
Age (years)			0.365			0.309			0.612
< 50	9	4 (44.4)		5	2 (40.0)		4	2 (50.0)	
50-65	22	7 (31.8)		21	8 (38.1)		5	1 (20.0)	
>65	41	11 (26.8)		14	4 (28.6)		17	5 (29.4)	
Sex			0.461			0.210			1.000
Female	22	7 (31.8)		5	3 (60.0)		13	4 (30.7)	
Male	50	15 (30.0)		35	11 (31.4)		13	4 (30.7)	
Differentiation grade of tumors			0.686			0.557			0.242
Well	41	13 (31.7)		25	10 (40.0)		15	3 (20.0)	
Moderate	17	6 (35.3)		9	3 (33.3)		5	3 (60.0)	
Poor	14	3 (21.4)		6	1 (16.7)		6	2 (33.3)	

*The total number of tumours evaluated was the 72 β-globin positive from 177 SCC: i.e., 40 samples from larynx, 26 from oral cavity and 6 SCC from esophagus. HPV16 DNA from neither E6 nor E7 genes did not detect in SCC from esophagus in then Colombian cases

Table 4: Physical state of DNA and oncogene expression of HPV16 in SCC of oral cavity and larynx from Colombian cases

		Physical status (%)				Oncoprotein expression	
	n	Integrated	Mixed	Episomal	p-value	(%)	p-value
Total	22	9 (40.9)	4 (18.2)	9 (40.9)		9 (40.9)	
Age (years)					0.941		0.594
< 50	4	1 (25.0)	2 (50)	1 (25.0)		2 (50.0)	
50-65	7	4 (57.1)	0 (0.0)	3 (42.9)		4 (57.1)	
>65	11	4 (36.4)	2 (50)	5 (45.5)		3 (27.3)	
Sex					0.899		0.083
Female	7	3 (42.9)	0 (0)	4 (57.1)		1(14.3)	
Male	15	6 (40.0)	4 (26.7)	5 (33.3)		8(53.3)	
Site of tumors					0.512		0.806
Larynx	14	5 (35.7)	2 (14.3)	7 (50.0)		5 (35.7)	
Oral Cavity	8	4 (50.0)	2 (25.0)	2 (25.0)		4 (50.0)	
Differentiation degree of tumors					0.137		0.203
Well	13	4 (30.8)	2 (14.3)	7 (57.1)		5 (38.5)	
Moderate	6	3 (50.0)	2 (33.3)	1 (16.7)		4 (66.7)	
Poor	3	2 (66.6)	0 (0)	1 (33.3)		0	

No statistically significant difference using chi-squared test, was found between the viral variables and individual data as age groups, site of the tumor and grade of differentiation of the tumorss

5 SCC of larynx (5/14; 35.7%) and 4 SCC from the oral cavity (4/8; 50%). The central fragment of E2 gene was not detected by PCR, even after a second round of PCR amplification. No statistically significant differences were found between the HPV16 genome integration status and tumour sites (Chi-square test, $p = 0.512$) (Table 4). Viral genome integration was confirmed in the same samples by the Transcriptional Papillomavirus Assay (APOT) which allows the discrimination between episomal and integrated HPV genome forms (Von Knebel Doeberitz, 1992).

In addition, four cancer specimens (18.1%) contained both episomal and integrated form of HPV16 genome including two SCC from the oral cavity and two from the larynx which resulted positive for both assays. No statistical association was found between HPV16 genome integration, gender and differentiation (Table 4).

The expression of HPV16 E7^E6 genes was detected in 9 out of 22 (40.9%) of the SCC (Table 4). E7^E6 gene expression was detected in 33.3% (3/9) of SCC with integrated HPV DNA and in 66.7% (6/9) of SCC with episomal form and was independent of gender and age of the patients and differentiation grade of the UAT cancers (Data not shown). A significant proportion of UAT cancers HPV16 DNA-positives was HPV16 RNA-negative (13/22; 59%).

DISCUSSION

The involvement of the HR HPV infection in the carcinogenesis of UAT has been proposed almost three decades ago (Syrjanen *et al*., 1982, 1983). Many studies in different geographical regions have shown that a subset of these cancers is consistently linked to HR HPV infection (Syrjanen, 2007; Ferlay *et al*., 2010). These studies also highlighted two important facts. Firstly, the majority of the HPV-positive UAT cancers appear to be almost exclusively associated with HPV16, in contrast to cervical cancer where HPV16 is responsible for approximately 50-75% of the cases worldwide. Secondly, the fraction of UAT linked to viral infection can substantially vary in different countries and over time (Marur *et al*., 2010; Ryerson *et al*., 2008). The data of this study contributed to the analyzed of HPV16 infection in UAT cancers in Colombian population. Our data showed that 30.6% of the head and neck cancers were HPV16 positives. A systematic review with 5046 cases of HNSCC from 60 studies worldwide demonstrated that the HPV16 prevalence in oropharyngeal, oral, laryngeal SCC was respectively 35.6, 23.5 and 24% (IARC, 2007). In present study, the majority of the specimens were laryngeal cancers and 35% of them (14/40) were HPV16-positives. Thus, the HPV16 prevalence in laryngeal cancers from Colombian patients is higher than the one determined in the worldwide meta-analysis (Kreimer *et al*., 2005). This higher prevalence of HPV16-positive laryngeal cancers may be determined by specific risk factors and/or lifestyle habits that are present in Colombia, however our study was not associated with a questionnaire; this issue cannot be further investigated in this current investigation. Our analysis showed that none of esophageal cancers examined resulted positive for HPV16, these data are in contrast with previous studies conducted in South-American populations that reported a HPV16-positivity of esophageal cancers in approximately 15% of the cases (Castillo *et al*., 2006). The esophageal analyses in this study are not conclusive due the very low number of esophageal cancers analyzed (n = 6) and we suggest more analyses in this country.

Another important issue of our study was the analyses of HPV16 DNA integration into host genome and the expression of E6 and E7 oncogenes. Our data clearly showed that both events were detected in a significant proportion of UAT cancer, further corroborating the role of HPV16 infection in these malignancies. However, we also observed that a significant proportion of UAT cancers were HPV16 RNA-negative, despite the presence of episomal or integrated HPV16 genome. These data are in agreement with previous studies that have also analyzed the expression of HPV oncogenes E6 and/or E7 in head and neck cancer lesions and showed that part of HPV DNA-positive cases were negative for oncogenes expression (Braakhuis *et al*., 2004; Jung *et al*., 2010). Together, these data suggest that viral oncogenes expression is lost at certain point during the multi-step process of carcinogenesis and it is not required for the maintenance of cancer phenotype. This scenario is clearly different from the one observed in cervical cancer. In fact, a large number

of studies have conclusively demonstrated in *ex-in vivo* and *in vitro* experimental models that HR HPV E6 and E7 expression is retained in all cervical cancers cells and its down-regulation lead the a reversion of the transformed phenotype. It is likely that others carcinogenic factors, known to play a role in the UAT carcinogenesis (e.g., alcohol and/or tobacco), induce additional cellular damages in HPV16 infected cells favouring cellular transformation and rendering cancer cells independent of E6 and E7 expression.

CONCLUSION

Although present results provide evidence for the role of HPV16 infection in cancer development in Colombian population, additional epidemiological studies are required to further characterization the relationship of HR HPV infection with other risk factors.

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