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Comprehensive Analysis of HPV Expression in Laryngeal Squamous Cell Carcinoma

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Role of human papillomavirus (HPV) in laryngeal carcinoma remains controversial. The aim of this study is to evaluate the role of HPV in laryngeal squamous cell carcinoma by determining presence of markers of viral infection. HPV DNA and E6*I mRNA status was determined by type-specific E7 PCR bead-based multiplex genotyping and RT-PCR assays in laryngeal squamous cell carcinoma biopsy samples. p16^{INK4a} and COX-2 expression was determined by immunohistochemistry. Four cases out of 32 (13%) were HPV DNA+: HPV 11 (n = 1), HPV 31 (n = 3), HPV 59 (n = 1). One double infection: HPV 11 and HPV 31. p16^{INK4a} was overexpressed in three cases (9%) and COX-2 in 17 cases (53%). Two of four HPV DNA+ samples had E6*I mRNA for HPV 31 and overexpressed p16^{INK4a} and COX-2. HPV appears to play an active role in a small subset of laryngeal squamous cell carcinoma. p16^{INK4a} can be used as a surrogate marker of transcriptionally active HPV infection; COX-2 expression had no correlation with HPV DNA and/or RNA positivity. ***J. Med. Virol. 86:642–646, 2014.***
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KEY WORDS: larynx cancer; human papillomavirus; COX-2; p16^{INK4a}

INTRODUCTION

Head and neck cancer accounts for 6.5% of all tumors in the USA [Cooper et al., 2009]. Although treatment options have improved, life expectancy for these patients has remained stable for the past 20 years. A subset of head and neck carcinoma, approximately 25% of cases worldwide, is associated with human papillomavirus (HPV) infection. HPV are small, double-stranded DNA viruses and are divided into mucosal and cutaneous types, according to their tissue tropism. Based on available epidemiological and biological data, 12 mucosal HPV types, that is, types HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59, have been classified as carcinogenic to humans (IARC Group 1) or high-risk (HR) HPV types [Bouvard et al., 2009]. HPV 16 and 18 are the most oncogenic types within the HR group and are responsible for approximately 55% and 16%, respectively, of cervical cancer cases worldwide [de Sanjosé et al., 2007]. In head and neck carcinoma, the majority of the HPV-associated cases (86–95%) are associated with HPV 16, and the remaining HPV types appear to play only a marginal role [Kreimer et al., 2005]. Among the different types of head and neck carcinoma, oropharyngeal carcinomas are the most frequently associated with HPV, whereas the role of HR HPV infection in other locations, for example, cancers of the larynx, has still not yet been fully evaluated. Pooled analyses of reports describe the presence of HR HPV DNA in 24% of cases of laryngeal squamous cell carcinoma [Kreimer et al., 2005; Isayeva et al., 2012]. In this study, the role of HR HPV in laryngeal squamous cell carcinoma was evaluated by determining the presence of viral DNA and RNA in cancer specimens. In addition, the validity of p16^{INK4a} and cyclooxygenase-2 (COX-2) immunohistochemical staining as a surrogate marker of HR HPV infection was assessed. Both cellular proteins are overexpressed in HR HPV-infected cells due to the activity of the viral oncoproteins E6, E7, and/or E5 [McKaig et al., 1998; Tsoumpou et al., 2009; Subbaramaiah and Dannenberg,

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2007; Kim et al., 2009]. In addition, COX-2 is rarely found in normal tissue, and it plays a crucial role in tumor progression since it stimulates cell proliferation and angiogenesis, and inhibits apoptosis [Greenhough et al., 2009].

MATERIALS AND METHODS

Patients and Samples

In this retrospective study, tumor samples that had been collected from 43 patients with head and neck carcinoma diagnosed between January 2007 and December 2011 at Hospital San Juan de Dios or Hospital Barros Luco Trudeau in Santiago, Chile, were included. All patients had signed informed consent, and the protocol was approved by the local ethics committees of both hospitals. Archived formalin-fixed, paraffin-embedded (FFPE) tumor samples were collected. The exclusion criteria were: histology other than squamous cell carcinoma, primary site other than larynx, recurrent tumors, and non-consenting patient.

Preparation of Paraffin Sections and DNA Extraction

All paraffin blocks were processed at the International Agency for Research on Cancer (IARC), Lyon. Three sections of 10 μm were cut from each paraffin block. To avoid cross-contamination, a new microtome blade was used each time a new case was sectioned, and the microtome was extensively washed with DNA Away (Dutscher, Brumath, France) to prevent the risk of cross-contamination between different specimens during the cutting. In addition, empty paraffin blocks were cut after every 10 cancer specimens and blindly analyzed to monitor possible cross-contamination. No contamination was detected throughout the study. DNA was prepared by incubating the paraffin tissue sections in digestion buffer (10 mM Tris/HCl pH 7.4, proteinase K 0.5 mg/ml, and Tween 20 0.4%) overnight.

RNA Extraction From Paraffin Sections

Following the same procedure described above, three sections of 10 μm were cut from each HPV DNA positive specimen. Two HPV DNA negative specimens were used as controls. Total RNA was purified from paraffin-embedded tissue sections using the PureLink FFPE Total RNA Isolation Kit (Invitrogen, Carlsbad, CA). Extracted RNA was eluted in 50 μl of RNase-free water and stored at –80°C until further use. A DNAse I treatment was carried out on the total RNA, following the manufacturer's instructions (Invitrogen).

cDNA Synthesis

For RT-PCR, the obtained RNA was reverse transcribed to cDNA with the RevertAid H Minus First Strand cDNA synthesis kit according to the manufacturer's instructions (Fermentas, Hanover, MD).

E6*I mRNA RT-PCR and Analysis

E6*I mRNA RT-PCR was performed using ultra-short primers allowing amplification of 65–75 bp sequences across the E6*I splice sites, as previously described (Halec et al., 2013). Primers for cellular ubiquitin C gene (ubC) were used to evaluate the quality of the recovered total RNA (Halec et al., 2013). The presence of E6*I spliced transcripts was verified by 2% agarose gel electrophoresis. To confirm the data, amplicons were excised and purified from the agarose gel using the MinElute kit (Qiagen, Hilden, Germany) and cloned into a PCR2.1 vector using the TA cloning kit (Invitrogen). Sequencing was performed using M13 primers.

HPV Type-Specific E7 PCR Bead-Based Multiplex Genotyping

The multiplex HPV type-specific E7 PCR uses HPV type-specific primers targeting the E7 region for the detection of 19 HR/pHR (probable high-risk) HPV types (HPV 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68a, 68b, 70, 73, and 82) and two low-risk (LR) HPV types (HPV 6 and 11), with detection limits ranging from 10 to 1,000 copies of the viral genome. Two primers for amplification of the beta-globin gene were also included to provide a positive control for the quality of the template DNA [Gheit et al., 2006; Schmitt et al., 2010]. After PCR amplification, 10 μl of each reaction mixture was analyzed by multiPlex HPV genotyping (MPG) using Luminex technology (Luminex Corporation, Austin, TX) as described previously [Schmitt et al., 2006, 2010].

Immunohistochemical Analysis

The expression of $p16^{INK4a}$ and COX-2 was evaluated by immunohistochemistry on FFPE sections. Samples included had a variable amount of tumoral cells ranging between 20% and 80%. Briefly, slides were de-paraffinized in xylene and rehydrated in graded alcohol. The antigens were retrieved for 10 minutes using a pH 9.0 epitope retrieval solution (95–99°C) followed by a 20-min cool-down period at room temperature for $p16^{INK4a}$, and microwaved in pre-heated Vector H-3300 unmasking solution for 15 minutes followed by a 30 minute cool-down period at room temperature for COX-2. This step was followed by incubation of primary antibodies: for $p16^{INK4a}$, mouse anti-human (Roche Laboratories, Heidelberg, Germany) for 30 min, and for COX-2, rabbit anti-human (Abcam), dilution 1:400, for 1 hr. The samples were then incubated with the secondary antibody goat anti-mouse for $p16^{INK4a}$ (Roche Laboratories, Heidelberg, Germany) and goat anti-rabbit for COX-2 (Vector Laboratories, Burlingame, CA), subsequently counterstained with haematoxylin, dehydrated, mounted

with permanent mounting medium, and coverslipped. Positivity of the inflammatory cells in the background of tumor tissue were considered as internal positive control for COX-2 expression, no positive control was included for p16^{INK4a} expression. For each staining, one slide was added to the run without implication of primary antibody as negative control. All steps, with the exception of epitope retrieval, were performed at room temperature. Immunoreactivity was visualized by light microscopy.

Immunohistochemical Scoring

p16^{INK4a} expression was scored based on both cytoplasmic and nuclear staining and by using a semi-quantitative scoring method [Koo et al., 2009]. Every case was given a score of intensity of staining (no: 0, weak: 1, moderate: 2, strong: 3) and a second score of extent (0% = 0, 1–10% = 1, 11–50% = 2, 51–80% = 3, 81–100% = 4). Then, a composite score was calculated by multiplying the two scores (ranging from 0 to 12). A composite score of ≥4 (moderate staining in >10% of cells) was considered as over-expression. All slides were scored by the same pathologist, who was blinded to the clinical and epidemiological data.

COX-2 expression was scored based on cytoplasmic staining using a semi-quantitative scoring method (0 = no staining, 1 = very weak diffuse cytoplasmic staining, 2 = moderate to strong granular cytoplasmic staining in 10–50% of tumor cells, 3 = strong intensity in >50% of tumor cells). A score of ≥2 was considered as overexpression [Milne et al., 2006].

Statistical analysis

Median differences were estimated by the Wilcoxon test with a significance level of 0.05. Contingency tables were analysed with Fischer exact test with a significance level of 0.05.

RESULTS

Of 43 cases, 11 were excluded for the following reasons: primary tumor not laryngeal (8 cases), no tumor in the selected paraffin block (1 case), and tumor recurrence (2 cases). Thus, 32 patients were included for the final analysis. Of these, 30 were male and 88% had tobacco consumption. The median age of the patients was 65 years (range, 38–83 years).

HPV DNA was identified in four cases (13%). Three of them were male, and three had tobacco consumption. The median age of this group was 48 years, significantly different from that of HPV negative cases (Wilcoxon test P: 0.005) (Table I). Three of them had a single infection, with genotypes 31 (n = 2) and 59 (n = 1), and one case had a double infection with genotypes 11 and 31.

The RT-PCR assay was performed to detect the most abundant splice variant within the HPV 31 open reading frame, namely E6*I. The analysis on agarose gel of ubC transcript showed valid RNA for all the HPV 31 DNA positive cases (Fig. 1), but not for the HPV 59 DNA positive sample (data not shown). E6*I mRNA expression was shown in two of the three HPV 31 DNA positive samples by RT-PCR (Fig. 1). We confirmed by sequencing that the detected band was indeed HPV31 E6*I mRNA with the identification of the junction between donor and acceptor sites (data not show). Both cases corresponded to young patients with tobacco consumption and different grades of disease.

TABLE I. Description of Cases According to HPV DNA Status

Characteristic	n	Positive HPV DNA	Negative HPV DNA	P
Median age (years)	32	48	66	0.005[b]
Gender				0.23[c]
Female	2	1	1	
Male	30	3	27	
Tobacco consumption	29	4	25	0.66[c]
Positive p16^{INK4a}	3	2	1	0.13[c]
COX-2[a]	17	2	15	0.65[c]

[a]Cyclooxygenase-2.
[b]Wilcoxon's test.
[c]Fischer's exact test.

p16^{INK4a} was overexpressed in 3 of 32 cases (9%). Two of these cases were HPV DNA and RNA positive, and one was negative for HPV DNA. There was no significant difference of p16^{INK4a} overexpression between HPV DNA positive and negative samples (Table I). High levels of COX-2 were found in 17 cases (53%), of which the majority were HR HPV DNA negative (Table I). No significant relation was found between COX-2 expression and HPV DNA positivity.

DISCUSSION

The link between HR HPV and a subset of oropharyngeal carcinomas is well established, but the role of these viruses in other locations of the head and neck remains controversial. The larynx is a known site of HPV infection, which can lead to benign lesions, namely airway papillomatosis, so a putative association with laryngeal carcinoma could be expected. Previous reports about the presence of

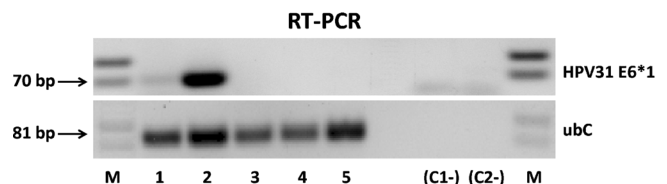

Fig. 1. Reverse transcriptase PCR (RT-PCR) for HPV31 E6*1 and ubiquitin (ubC) transcripts in HPV31 DNA positive specimens (Lanes 1–3) and HPV DNA negative specimens (Lanes 4 and 5). (C1-) and (C2-) correspond to negative controls for the RT-PCR and PCR, respectively.

HR HPV types in laryngeal squamous cell carcinoma vary considerably [Torrente et al., 2011]. Most HPV DNA detection methods used in research programmes are PCR-based assays and may differ in sensitivity and specificity according to the PCR primers used. In addition, many studies have used FFPE tissue blocks, which may present different levels of DNA fragmentation, affecting the performance of PCR-based assays [Karlsen et al., 1994]. Multiplex HPV type-specific E7 PCR has shown a higher sensitivity than other methods [Schmitt et al., 2010; Comar et al., 2012]. In addition, for those cases with positive HR HPV DNA, the presence of a viral transcript was assessed to further corroborate the potential role of the virus in the carcinogenic process. Four out of 32 cases were HPV DNA positive, and two of them showed E6*I mRNA expression, indicating that only a minority of laryngeal squamous cell carcinomas are associated with an active HR HPV infection.

The cases harboring viral DNA were from younger patients, both male and female, and 75% of them had tobacco consumption. HPV was initially explored as the cause why non smokers developed laryngeal carcinoma, but this has not been the case. Tobacco induced carcinogenesis might have a synergic relation with HPV carcinogenesis, that is, viral driven cellular replication could be the amplifier of mutations or epigenetic phenomena's induced by smoking.

p16^{INK4a} expression has been proposed as a surrogate marker of transcriptionally active HPV infection [El-Naggar and Westra, 2012]. This proposal has been challenged in head and neck carcinoma. Klingenberg [Klingenberg et al., 2010] described a series of 262 patients in which p16^{INK4a} overexpression was studied in tumor-free tonsillar tissue. 28% of the samples overexpressed p16^{INK4a}, and HPV 16 and 18 DNA were identified in only two cases by PCR analysis. Hoffman [Hoffmann et al., 2012] studied HPV DNA and RNA expression, coupled with p16^{INK4a} detection. Fourteen cases were positive for HPV DNA and RNA, whereas only 11 had high p16^{INK4a} levels. The authors concluded that using a surrogate marker for an initial selection of probable HPV-related tumors, as has been suggested [Smeets et al., 2007], could result in an underestimation of the real prevalence. p16^{INK4a} overexpression can be suppressed by mutations and hypermethylation [Dikshit et al., 2007; Kiwerska et al., 2010], maybe secondary to tobacco-related carcinogenesis, and this phenomenon could explain repressed p16^{INK4} expression in HPV DNA positive cases. Of the 32 cases analysed, p16^{INK4a} expression identified both cases harboring the E6*I transcript.

In cervical carcinoma, COX-2 expression also correlates with HR HPV infection [Kulkarni et al., 2001]. The viral oncoproteins E6 and E7 stimulate COX-2 transcription by the activator protein-1 pathway (Subbaramaiah and Dannenberg, 2007). This enzyme is rarely found in normal tissue but is frequently overexpressed in cancer and is associated with proliferation, angiogenesis, invasiveness, inhibited immune surveillance, and apoptosis [Kim et al., 2009]. COX-2 was overexpressed in more than 50% of the samples, as previously shown in head and neck carcinoma [Abrahao et al., 2010]. Most of the cases that overexpressed COX-2 were HPV DNA negative. Only two of the four HPV DNA positive samples overexpressed COX-2. These results showed a low correlation between COX-2 overexpression and HPV infection.

The HPV genotypes identified, HPV 31 and 59, do not reflect the scenario described in the literature, in which HPV 16 is the most prevalent HR HPV type detected in head and neck carcinoma [Dayyani et al., 2010]. It is likely that the prevalence of specific HR HPV types in head and neck carcinoma varies in different geographical areas. The analysis of a larger number of specimens is needed to confirm that in this particular population there is a different genotype relative distribution. An interesting observation was that the median age of the patients with biopsy samples identified as HPV DNA positive was significantly lower than that of the patients with HPV DNA negative samples. No association could be established with the stage of tumors since information was not available for all cases.

In conclusion, transcriptionally active HR HPV infection was identified in a small subgroup of laryngeal squamous cell carcinomas. p16^{INK4a} staining could be considered as a surrogate marker of transcriptionally active HR HPV infection, whereas high levels of COX-2 were detected also in many HR HPV negative tissues

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