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Does Transcriptionally Active Papillomavirus Associate with Glottic Laryngeal Cancer?

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Abstract

Purpose: To evaluate the association of human papillomavirus in its active form and invasive glottic laryngeal cancer.

Methods: A case-control study was conducted to evaluate the association of transcriptionally active human papillomavirus in patients diagnosed with glottic laryngeal squamous cell carcinoma and vocal cord polyps as cancer-free controls. p16INK4a immunohistochemistry and DNA in situ hybridization were used to identify positive cases for the virus.

Results: 132 subjects were included in the study, 66 patients diagnosed with laryngeal glottic squamous cell carcinoma, and the remaining 66 participants formed the control group. Among the individuals in the study group, only 8 were women with a mean age of 62.3 years. Smoking and tobacco exposure (pack years) were positively associated with laryngeal cancer. p16INK4a immunohistochemistry was positive in 5 out of the 66 (7.6%) patients with squamous cell carcinoma, whereas no case among the control group was positive. However, all the 5 cases presented negative in situ hybridization for human papillomavirus DNA and were therefore classified as negative human papillomavirus status. p16INK4a immunohistochemistry was not significantly associated with glottic laryngeal squamous cell carcinoma or human papillomavirus status, nor with any specific group. There was, however, a tendency to associate it with the laryngeal cancer group and patients who never smoked.

Conclusion: Transcriptionally active papillomavirus did not associate with glottic laryngeal cancer.

Keywords: Laryngeal Neoplasms; Glottis; Vocal Cords; Papillomavirus Infections; In Situ Hybridization

Introduction

The most important risk factors related to laryngeal cancer, as well as to other head and neck neoplasms, are smoking and alcoholism, which become even more important when associated [1]. However, during the last few decades, the studies involving the relationship among human papillomavirus (HPV) infection and the development of these neoplasms have increased [2]. Several studies have demonstrated a strong association among active HPV infection and oropharyngeal carcinoma [3,4]. On the other hand, the participation of HPV in the etiology of laryngeal squamous cell carcinoma (SCC) and other sites of the head and neck SCC has not been definitively established.

HPV-related SCC has an increased expression of $p16^{INK4a}$, whereas in non-HPV-related cases chronic exposure to tobacco and alcohol can generate mutational losses of $p16^{INK4a}$ and p53 protein genes, resulting in a low expression of this protein. Therefore, the immunohistochemical panel of $p16^{INK4a}$ has been described as a surrogate biomarker for active HPV infection in head and neck carcinomas [5], mainly in oropharynx cancer, but in laryngeal cancer as well [6].

However, detection of low levels of HPV-DNA and absence of viral transcription has a limited biological value and may indicate that HPV does not play a role in malignant transformation of laryngeal cancer [7,8]. HPV-DNA has also been found in normal tissues and in benign lesions of the vocal folds [9], which supports the idea that that the presence of HPV alone may not be related to a causal factor. Thus, detection methods that reflect active HPV

transcription, including $p16^{INK4a}$ immunohistochemistry (p16 IHC), reverse transcriptase polymerase chain reaction (RT-PCR) or in situ hybridization (ISH) for HPV-DNA or E6/E7 mRNA are required to demonstrate biologically significant HPV [10]. This study aims to evaluate the association of active HPV infection and the glottic laryngeal SCC.

Materials and Methods

Population and Research Design

We conducted a case-control study in which the association of transcriptionally active HPV was studied in tumors of patients previously diagnosed with invasive glottic laryngeal SCC. 66 consecutive adult patients (>18 years old) recently diagnosed with glottic laryngeal SCC through biopsy of the lesion under suspension laryngoscopy and anatomopathological analysis were recruited from July 2014 to June 2017. All patients previously treated with chemotherapy or radiotherapy were excluded.

The control group consisted of patients submitted to laryngeal microsurgery for vocal polyp, a known benign lesion, during the same period of the study. Patients under 38 years and women under 50 were excluded in order to make the sample more like the study group. It was also excluded those who presented dysplasia signs in anatomopathological analysis or who had progressed with head and neck cancer during follow-up. The work was done in accordance with the appropriate institutional review body and carried out with the ethical standards. Informed consent was obtained from all individual participants included in the study.

$p16^{INK4a}$ Immunohistochemistry (p16 IHC)

The immunohistochemical reactions for the $p16^{INK4a}$ antibody were carried out on paraffin-embedded tissue sections obtained from biopsies of tumors and polyps, using monoclonal primitive body GeneAB clone IHC 016 (GenomeMe, Canada).

The immunoblotting pattern of $p16^{INK4a}$ is cytoplasmic and nuclear and the evaluation was based on the extent of staining, related to the percentage of stained neoplastic cells. Staining was scored as positive if it showed nuclear and cytoplasmic staining in most tumor cells (> 70%), regardless of its intensity. It was scored as negative if it showed complete absence of $p16^{INK4a}$ or it appeared only on isolated tumor cells (<40%). Samples with intermediate staining pattern (between 40 and 70%) were classified as inconclusive [11].

HPV DNA *in situ* hybridization (ISH)

ISH was performed by manual technique using the Zytofast PLUS CISH Implementation kit (Zytovision, Germany) with the ability to detect high risk genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, and 82, and low-risk genotypes 6 and 11. The cases were classified as positive or negative. Negative results in ISH were considered as negative HPV status.

Statistical Analysis

The Statistical Package for Social Sciences (SPSS Inc., Chicago, IL, USA), version 17.0 for Windows, was used for database's elaboration and descriptive analysis. The results were presented as tables and graphs. Categorical variables were shown as frequencies and percentages -n (%). Continuous variables with normal distribution were expressed as mean and standard deviation, and those with non-normal distribution were described as median and interquartile range. The normality of the numerical variables was verified through descriptive statistics, graphical analysis and the Shapiro-wilk test.

Chi-square test for categorical variables was used to compare the clinical and demographic variables between the SCC and control groups and to compare positive and negative p16 IHC groups among individuals with laryngeal SCC. Fisher's exact test was used when the number of individuals was low. For the comparison of numerical variables between these groups independent t test was applied for those with normal distribution and Mann-Whitney test for those with non-parametric distribution. It was considered $p < 0.05$ for all analyzes.

Results

132 individuals were included in the study, 66 patients with glottic laryngeal SCC and 66 participants in the control group diagnosed with laryngeal polyp. The participants' clinical and demographic characteristics are detailed in Table 1. Regarding the study group, all patients were diagnosed with only one primary tumor and did not receive treatment prior to surgery. Only 8 were women and the mean age was of 62.3 years (± 9.2). Smoking history and tobacco exposure (pack years) were associated with laryngeal SCC (Figure 1). 5 out of the 66 (7.6%) patients with SCC presented positive p16 IHC, whereas no control cases were positive. Nevertheless, all 5 cases presented negative HPV DNA ISH and were therefore classified as negative HPV status. Cancer staging of patients with laryngeal SCC is shown in Figure 2. Among these patients, p16 IHC was positive in 3 T1A (60%), 1 T1B (20%) and 1 T3 (20%) cases. The p16 IHC distribution among the group with laryngeal SCC is shown in table 2.

Table 1: Clinical and demographic characteristics of patients with glottic laryngeal SCC and controls.

	Cases (n=66)	Controls (n=66)	p Value
Age	62,3 ±9,2	44,5 ±11,8	<0,001*
Gender			<0,001$^{\alpha}$
Female	8 (12,1)	28 (42,4)	
Male	58 (87,9)	38 (57,6)	
Smoking			0,005$^{\alpha}$

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Smoking history	51 (77,3)	31 (53,4)	
Never smoker	15 (22,7)	27 (46,6)	
Tobacco exposure (pack years)	44,5 (25-60)	20 (10-25)	<0,001$^{\lambda}$
Alcoholism			0,827$^{\alpha}$
Yes	9 (15,5)	6 (14,0)	
No	49 (84,5)	37 (86,0)	
p16 IHC			0,058$^{\beta}$
Positive	5 (7,6)	0	
Inconclusive	1 (1,5)	0	
Negative	60 (90,9)	66 (100,0)	
HPV status			
Negative	66 (100,0)	66 (100,0)	1,00$^{\alpha}$

*Independent T-Test; α= Chi-Square Test; β= Fischer's Exact Test; λ= Mann-whitney Test; HPV= Papilomavírus humano; p16 IHC= p16INK4a Immunohistochemistry.

Table 2: Distribution of patients with glottic laryngeal SCC according to demographic characteristics and p16^{INK4a} immunohistochemistry (p16 IHC).

	Positive p16 IHC (n=5)	Negative/ Inconclusive p16 IHC (n=61)	p Value
Age	56,0 ±6,2	62,7 ±9,3	0,115*
Gender			0,487$^{\beta}$
Female	1 (20,0)	7 (11,5)	
Male	4 (80,0)	54 (88,5)	
Smoking			0,073$^{\beta}$
Smoking history	2 (40,0)	49 (80,3)	
Never smokers	3 (60,0)	12 (19,7)	
Tobacco exposure	41,5 (34-41,5)	45 (25-60)	0,773$^{\lambda}$
Alcoholism			0,114$^{\alpha}$
Yes	2 (40,0)	7 (13,2)	
No	3 (60,0)	46 (86,2)	

*Independent T-Test; α=Chi-Square Test; β= Fischer's Exact Test; λ= Mann-whitney Test; p16 IHC= p16INK4a Immunohistochemistry.

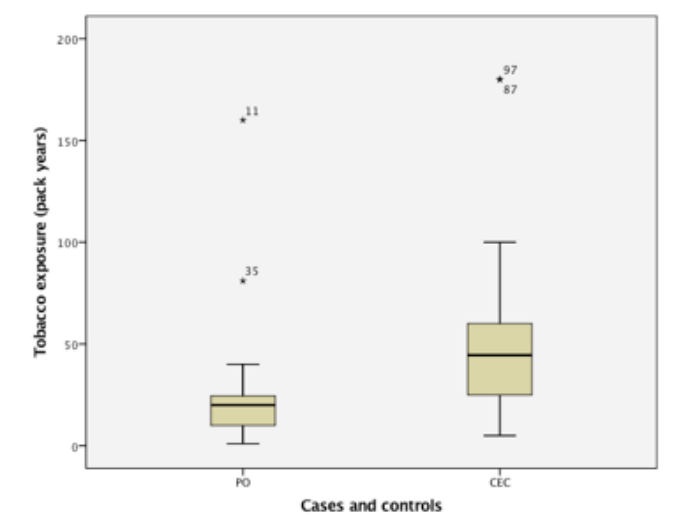

Figure 1: Box-plot association between cases and controls over tobacco exposure. SCC = squamous cell carcinoma; PO = polyp.

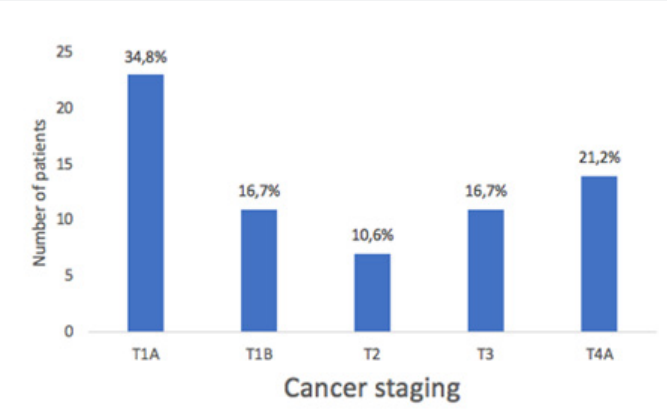

Figure 2: Cancer staging of patients with laryngeal SCC.

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Discussion

There is a large variation in the proportion of HPV-related laryngeal SCC in the literature. Besides the demographic and ethnic variation, the high variation in the diagnostic tests used may have influenced this result [9]. In the present study, no cases of glottic laryngeal SCC related to transcriptionally active HPV infection were identified. In order to confirm the results of p16 IHC, whilst avoiding false-positive cases, ISH reaction was performed in all p16 IHC positive cases. Thus, although p16 IHC was positive in 5 of 66 cases (7.6%), HPV DNA ISH was negative in all these samples, which demonstrated negative HPV status.

Rodrigo et al also did not find a significant role of HPV in laryngeal cancer in northern Spain. Only 1 patient out of 62 (1.6%) had positives HPV DNA 16 and E6 mRNA RT-PCR. However, this patient was 85 years old, with a smoking and heavy alcoholism history and presented positive p53 and negative HPV DNA ISH. Therefore, it was arguable HPV's participation in this patient's carcinogenesis. In this study, 11% of laryngeal tumors were positive for p16 IHC, which represented a high level of false-positive results and low correlation with HPV status [12]. Similarly, Lee et al investigated transcriptionally active HPV only in glottic laryngeal cancer and suggested that HPV does not appear to play a causal role in this type of tumor and the detection of this virus may only be incidental [13]. In the same way, Fakhyr et al reported that there was no HPV infection in a population of 34 patients with laryngeal cancer, investigated with PCR and ISH [4].

On the other hand, other studies investigated this relationship and demonstrated a small but positive association between active HPV and laryngeal SCC, ranging from 1.6% to 6.7% [9,14-16]. Chernock et al reported that only 4 out of 60 patients (6.7%) presented E6/E7 HPV mRNA [10] and Hernandez et al demonstrated only 2% p16 IHC and HPV DNA positivity in laryngeal SCC [17]. Both studies did not find a significant relationship of $p16^{INK4a}$ expression and HPV DNA status, unlike what is described in oropharyngeal SCC, which has a well-established etiologic and prognostic role [4].

Older studies or those which investigated only the presence of HPV, instead of its active form, tended to present a higher prevalence, reaching 16% [18], 27% [19], 35% [20] and up to 75% [21] of the cases. However, these studies used PCR to detect HPV DNA as diagnostic method, a method that does not confirm the integration of HPV DNA into the nucleus of the tumor cell and does not prove a causal role of the virus in laryngeal carcinogenesis [22]. The presence of HPV DNA has been demonstrated in a significant proportion of benign lesions of the larynx. Therefore, the presence of the virus in many cases of cancer appears to be only incidental [9,13].

Few studies, including ours, evaluated the participation of HPV only in glottic laryngeal SCC. Lee et al also found no relation between HPV infection and carcinogenesis in this population [23]. There are few studies comparing the prevalence of HPV infection in glottic and supraglottic cancer, but these are older studies that do not differentiate the active from inactive HPV forms, and have very small samples, making it difficult to reach reliable conclusions [24-27]. Therefore, it is possible that supraglottic laryngeal SCC behaves more like the pharynx SCC than to the glottic region SCC itself, which could justify these findings.

In this study, p16 IHC was not significantly associated with glottic laryngeal cancer or HPV status, nor with any of the specific groups, although there was a tendency to associate it with laryngeal SCC and patients who never smoked. HPV-associated SCCs have an increased expression of $p16^{INK4a}$, whereas those related to chronic exposure to tobacco and alcohol tend to generate mutational losses of $p16^{INK4a}$ and p53 protein genes, resulting in low expression of these proteins. Thus, p16 IHC has been used as an HPV-active infection biomarker in head and neck SCC, especially in oropharynx cancer [5].

Gheit et al have also suggested the use of this marker in HPV-related laryngeal SCC [6], however most studies published to date did not find a good correlation between $p16^{INK4a}$ overexpression and active infection in SCC of this region, suggesting that the hyperexpression of this protein in laryngeal carcinogenesis may reflect cellular changes not related to HPV. Some tumors may have positive $p16^{INK4a}$ due to mutation and inactivation of Rb protein or E2F amplification [17]. Thus, actual evidence suggests that p16 IHC alone is not a reliable marker for HPV participation in carcinogenesis of laryngeal SCC [9,10,17,28,29].

As expected, the group of patients with smoking history, as well as tobacco exposure (pack years) was associated with laryngeal cancer. The group of alcoholic patients, however, did not present the same association. The lack of information regarding the medical record, especially among patients with laryngeal polyp seems to have impaired this evaluation. These are the two main risk factors related to the development of head and neck SCC [1]. There was also a difference in gender and age between the study and control groups. Polyps are known benign lesions of the larynx which affect young patients (less than 40 years old). Although they are more related to males, they are also highly prevalent in females (55% x 45%, respectively) [30], whereas SCC affects men in a larger proportion and in older patients [31], which explains the difference found.

This study has some limitations. As the patients were retrospectively evaluated, the anatomopathological analysis were performed using paraffin-embedded tissue sections obtained

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from patients' surgeries. There was loss of data related to medical records, especially those concerning alcoholism and those of the control group. There was also a limited number of participants, which may have contributed to the identification of no HPV positive cases. Nevertheless, this work presents some other advantages, such as the use of tests that evaluate transcriptionally active HPV instead of only detecting the presence of the virus in the samples, which might not be related to tumor carcinogenesis. Besides it evaluated a single tumor site, the glottic region, avoiding generalization of other sites of the larynx's behavior.

Conclusion

Transcriptionally active papillomavirus did not associate with glottic laryngeal cancer.

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