

# HISTOLOGY AND HISTOPATHOLOGY

ISSN: 0213-3911  
e-ISSN: 1699-5848

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DOI: 10.14670/HH-11-929

Article type: ORIGINAL ARTICLES

Accepted: 2017-09-06

Epub ahead of print: 2017-09-06

**Title: PREVALENCE OF HIGH RISK HPV DNA IN ESOPHAGUS IS HIGH IN BRAZIL BUT NOT RELATED TO ESOPHAGEAL SQUAMOUS CELL CARCINOMA**

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**Running title:** HPV IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA

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**Keywords:** Human Papillomavirus; Esophageal Neoplasms; Risk Factors, Cancer Epidemiology, Esophageal cancer.

**ABSTRACT**

**Background:** The first publication that associated Human Papillomavirus (HPV) infection and esophageal cancer was published in 1982. However, data are still contradictory and require further investigation. The aim of this study was to identify high risk HPV DNA in esophageal tissue of patients with and without esophageal squamous cell carcinoma (ESCC) and correlate HPV presence with classical risk factors. **Methods:** Invited patients signed the informed consent form, and interviews were conducted in order to obtain information about sociodemographic and lifestyle behavior. During endoscopy, esophageal biopsies were collected from case and controls. Multiplex polymerase chain reaction genotyping was conducted on endoscopic biopsies to identify HPV types and HPV-16 was further evaluated by specific PCR real time. **Results:** Among 87 cases, 12 (13.8%) had tumors harboring high risk HPV DNA and among 87 controls, 12 (13.8%) had high risk HPV DNA (OR:1.025 [CI:0.405:2.592]). Variables regarding consumption of alcohol and use of tobacco continued to characterize risk factors even after adjustments by presence or absence of high risk HPV. **Conclusion:** HPV was demonstrated to be frequently and similarly associated to normal and malignant esophageal tissues, but not as an independent risk factor to esophageal cancer. **Impact:** To contribute to the Brazilian population data on this subject, which is still contradictory.

## INTRODUCTION

Currently, esophageal cancer (EC) is an important public health problem worldwide. According to estimates by GLOBOCAN 2012, EC is the eighth most common cancer worldwide and the sixth most common cause of cancer death (Ferlay et al., 2013).

Risk factors related to, and those suspected of being involved with EC, have been widely studied; however, little has been achieved since the classic studies of Wynder *et al.* (Wynder and Bross, 1961) and Silber (Silber, 1985), suggesting an association of some chemicals (nitrosamines, mycotoxins, cigarette smoke, excessive intake of alcohol and opium abuse), physical (solid and hot food) and nutritional deficiencies (particularly vitamins A, B, C and certain trace elements such as molybdenum and zinc) with the development of this malignant neoplasm (Wynder and Bross, 1961; Silber, 1985).

Recently, evidence suggests that human papillomavirus (HPV) may play an etiological role in esophageal carcinogenesis, in at least one subtype of esophageal carcinoma, the squamous cell carcinoma; the number of studies in this area have increased steadily, as evidenced in several reviews (Syrjanen, 1987; Syrjänen et al., 1996; Syrjanen, 2000a,b; Syrjanen 2002; Syrjänen, 2003). The first descriptions of oral lesions associated with HPV were preceded by reports that suggested the involvement of viruses in the development of benign (Syrjanen et al., 1982) and malignant (Syrjanen, 1982) lesions of the squamous epithelium of the esophagus. These initial observations were based on the discovery of morphological similarities between HPV lesions in the genital tract (warts) and esophageal lesions (papillomas) (Syrjanen et al., 1982; Syrjanen, 1982).

Although the first report on the presence of HPV in esophageal squamous cell carcinoma (ESCC) occurred more than 30 years ago (Syrjanen et al., 1982), its real prevalence is still poorly known and its role in carcinogenesis is questionable. Although the number of studies and interest in the subject have increased in recent years, the literature is scarce and controversial

(Kamangar et al., 2009). Data accumulated reflects a trend linking HPV and EC in high risk areas, whereas in low risk areas such association was not evident (Antunes et al., 2013).

Knowing that esophageal carcinogenesis is complex, dependent on different risk factors and considering the controversial results described in reports that correlate high risk HPV infection with the development of ESCC, we aimed, in this study, to characterize individuals without cancer and patients with ESCC in relation to the presence of high risk HPV DNA and well known risk factors for both EC as well as for HPV infection.

## **MATERIALS AND METHODS**

This is an observational case-control study. Cases were defined as patients of both genders, aged above 18 years, who were admitted to the Barretos Cancer Hospital (BCH), with histopathological confirmation of ESCC, clinical indication for endoscopy and no previous treatment for cancer. Controls were defined as individuals without cancer of the esophagus, having had clinical indication for endoscopy for benign disease of the digestive system, examined in the Endoscopy Department from Medical Specialties Ambulatory (AME, acronym in Portuguese) in Barretos, São Paulo, Brazil. The sample size of that prospective and controlled study was based on earlier published work (Guo et al., 2012). Patients with limited understanding of research objectives during the consent, submitted to adjuvant and/or neoadjuvant therapy previous to sample collection, mental disability, unfavorable clinical conditions to undergo medical procedures, or insufficient amount of sample and/or inadequate quality were excluded.

- **Endoscopic examination and sample collection**

The procedure for conducting the Digestive Endoscopy followed the routine of the Department of Endoscopy at BCH and Ambulatory Medical Specialties in Barretos, using sedation and flexible video endoscopes (Olympus 180, Japan; Fuginon 4400, Japan). The

collection of biological samples was prospectively performed from esophageal biopsies. Samples were fixed in formalin and embedded in paraffin. The biological material was processed by the Department of Pathology, at BCH for histopathological diagnosis.

- **HPV detection and characterization**

Specimens were digested with proteinase K-SDS 1% and HPV DNA was obtained by organic extraction (Green and Sambrook, 2012). HPV DNA was measured in all samples using type-specific PCR bead-based multiplex genotyping (TS-MPG) assays that combine multiplex polymerase chain reaction (PCR) and bead based Luminex technology (Luminex Corp., Austin, TX, USA), as described elsewhere (Nunes et al., 2016). This methodology is able to identify 21 HPV types including 13 of high-risk and 3 of low risk oncogenic, accordingly (Munoz et al., 2006; Gheit et al., 2007).

As a positive control for the quality of the template DNA, primers targeting the  $\beta$ -globin gene were included. The assays were performed with 10 $\mu$ L DNA template in a 96-well format in 25- $\mu$ L/well reaction volume. HPV multiplex PCR was performed with QIAGEN Multiplex PCR Kit (Qiagen, Dusseldorf, Germany), according to manufacturer's instructions. Each reaction consisted of 45 cycles: 94°C for 30 seconds, 63°C for 3 minutes, and 72°C for 90 seconds. The first cycle was preceded by incubation at 95°C for 15 minutes and the last one was extended for 10 minutes at 72°C. Negative controls consisted of reaction mixture without DNA.

The hybridization assay was performed according to Schmitt et al. (2006) (Schmitt et al., 2006). For each HPV type-specific probe, mean fluorescence intensity (MFI) values obtained when no PCR product was added to the mixture of hybridization, was considered as background. The cutoff was calculated by adding 5 MFI for 1.1 X the value of median found. We considered positive values higher than 20 MFI.

- **Statistical analyses**

Covariates associated with HPV detection and ESCC were analyzed and the results were expressed as odds ratio (OR) by simple logistic regression. Confirmatory model were analyzed in multiple logistic regression between alcohol and tobacco interaction, where time and consumption were considered, using the HPV detection as variable of interest. The respective intervals with 95% confidence (95% CI) were also presented. The significance level was 0.05 (5%). For tabulation and statistical analysis we used IBM® SPSS® Statistics 20.0.1 software for Windows (IBM Corporation, Route 100, Somers NY 10589).

## **RESULTS**

During the period between February 2013 and August 2014, 174 individuals were enrolled, of which 87 (50.0%) were patients with ESCC (cases) and 87 (50.0 %) was individuals without cancer (controls). Cases and controls were matched (1 case : 1 control) by gender and age ( $\pm$  3 years). The population of the study had the following characteristics: male (78.2%; n = 136), self-reported white skin (67.9%; n = 110); marital status (69.5%; n = 121); low level of schooling (16.7% n = 29) were illiterate and 71.3% (n = 124) attended up to middle school. Age ranged from 41-83 years (mean = 60.6 years, SD = 10.1 years; median = 60.0 years).

Table 1 shows the results of the comparison between cases and controls by high risk HPV DNA, correlated with social status, consumption of alcohol, tobacco and sexual habits variables.

Regarding the combined effect of tobacco use and alcohol consumption over the course of time, individuals who consumed alcohol for less than 15 years and tobacco for more than 15 years had 9.6 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco for less than 15 years; individuals who consumed alcohol more than 15 years and tobacco for less than 15 years had a 5.5 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco less than 15 years; individuals who consumed alcohol and

tobacco for more than 15 years had a 10.4 fold increased risk to develop ESCC relative to those who consumed alcohol and tobacco for less than 15 years (Table 1).

There was no statistically significant difference between the variables age at first intercourse and number of partners (Table 1). Active oral sexual intercourse showed statistically significant differences ( $p = 0.017$ ). Individuals who reported engaging in oral sex once in their lives had a lower risk ( $OR = 0.4$ ) of having ESCC when compared to individuals who reported never having performed oral sex (Table 1).

In the multivariate analysis, using a confirmatory model including the Interaction of tobacco and alcohol, considering time and consumption, and included the HPV detection with interest variable, the HPV didn't show an increased risk to ESCC. The results of the multivariable regression analyses are shown in Table 2.

## **DISCUSSION**

The results herein reported revealed a high prevalence of high risk HPV DNA in esophageal mucosa; however, these occurrences did not seem to have the same causality observed in cervix carcinoma. This data is very interesting and intriguing because they stimulate provocative queries about the behavior of high risk HPV DNA in the esophageal tissue. Different risk factors were evaluated, especially those widely known to facilitate HPV infection. History of oral sexual intercourse, e.g., was not a discriminatory variable, indicating that for cases and controls, the presence of high risk HPV DNA can be a constant independent of the meaning of any injury. On the contrary, it was observed that alcohol and tobacco consumption still prevail as the classic risk factors for developing ESCC.

This was an observational case-control study; such a design is generally used for slow progressive disease and allows the evaluation of various risk factors for a particular outcome (Cousens and Balthazar 1995). However, these studies are subject to criticism because they

may contain limitations as related to the control group selection and also the interpretation of the results may be hampered by confounding factors (Pereira, 1995).

Selection of participants for the “case” group was performed at the Endoscopy Department of the BCH, an exclusively specialized oncology center. We included individuals for the “control” group in the Endoscopy Department of the AME, which is also located in the municipality of Barretos, São Paulo, Brazil. The differences between the general characteristics of the two groups generate results that cannot be sufficiently explained as what occurred with oral sex intercourse, evaluated as a protector factor. In order to balance the divergence between institutions and to assemble homogeneous groups, patients were matched by gender and age ( $\pm 3$  years).

Many studies about EC achieved data from retrospective cases and scarcely reported information about the population characteristics enrolled. The differential of the current work was the prospective collection of samples and detailed questionnaire about individual characteristics, which allows for the rigorous selection of the biopsy area and the range of risk factors evaluated. Consequently, several reports worldwide have described heterogeneous data regarding HPV prevalence in the esophageal tissue that varied from the absence of virus (Ashworth et al., 1993; Rugge et al., 1997; Gao et al., 2006; Koh et al., 2008; Antunes et al., 2013), to prevalences up to 100% (Li et al., 2001) in ESCC. In control population, some studies also report the absence of the virus (Lenhart et al., 1991; Ashworth et al., 1993; Cooper et al., 1995; Fidalgo et al., 1995; Lambot et al., 2000; Souto Damin et al., 2006; Koh et al., 2008; Antonsson et al., 2010) and others reported prevalences as high as 59% (Li et al., 2001). It should be considered that this variation in prevalence can be explained by multiple factors such as the methodological differences used to detect HPV, the number of cases analyzed, as well as environmental, geographic and demographic differences among the populations evaluated (Takahashi et al., 1998; Kawaguchi et al. 2000; Matsha et al., 2002; Syrjanen, 2002; Far et al., 2007; Malik et al., 2011; Zhang et al., 2011; Syrjanen, 2013).

In the Brazilian territory, the prevalence of HPV in ESCC was analyzed in four studies, ranging from 0% to 16% (Weston and Prolla 2003; Souto Damin et al., 2006; Herbster et al., 2012; Antunes et al., 2013). Several studies, including those performed in Brazil, have in common the fact that these were conducted retrospectively, performed with different HPV detection techniques and quite conflicting results. All the data supported that HPV, somehow, is not involved with the esophagus carcinogenesis, which is corroborated, in part, by our findings. Furthermore, our study showed the same positivity indexes for high risk HPV DNA, in controls and cases, also observed in other studies. The high sensitivity of the methodologies herein used, and the strict control of prospectively collected samples, are determinant variables to explain these differences among the results observed in Brazilian regions. It is important to emphasize, however, that in Brazil, HPV-related to esophageal cancer, does not have the same meaning as reported in other countries, such as China, for example (Guo et al., 2012).

HPV prevalence observed among cases is high when compared to global data accumulated so far, but reflect and corroborate the results described in many studies found in the literature. Studies conducted in South Africa, India, Greece and Japan found similar prevalences as we obtained, ranging from 15% to 25% (Williamson et al., 1991; Agarwal et al., 1998; Khurshid et al., 1998; Lyronis et al., 2005). In individuals without cancer, however, there is a higher prevalence rate when compared only to data published in Brazilian literature to date (Weston and Prolla, 2003; Souto Damin et al., 2006; Antunes et al., 2013).

It is well-known that three forms of HPV infection may occur: clinical, subclinical and latent. The latent virus can be diagnosed only by molecular techniques targeting HPV-DNA (Richart and Wright, 1991). Therefore, we hypothesize that this may be the form found in controls in this study. The mechanisms by which the HPV remains in this state of latency remain largely unknown, but it is believed that immunological factors are decisive for this condition. Often, HPV infection may regress spontaneously, but we have no further knowledge

about how long the virus could remain in this state, nor about the rate of progression of this form of latency (Burd, 2003).

Alcohol consumption was considered a major risk factor for ESCC. The International Agency for Research on Cancer (IARC) classifies alcohol as a cause of esophageal cancer (IARC 2008) and ecological and case-control classical studies established that alcohol has a strong causal role in the development of ESCC in most regions of the world (Tuyns, 1970; Audigier et al., 1975; Tuyns et al., 1979; Tuyns, 1983). Tobacco use was also considered a risk factor for ESCC in this study, mainly when smoking hand-rolled cigarettes was considered separately. We can also observe the so called “dose response effect”, in that the risk increased as the amount consumed also increased.

The difference between study groups engaged in active oral sex may be related to selection bias of the control population. As much as has been paired by sex and age to the homogenization of the groups, it is observed that the control group is markedly more urbanized, which, despite potential controversies, may directly reflect the data on the sexual habits obtained in this study.

HPV was demonstrated to be frequently associated to normal and malignant esophageal tissues despite the fact that esophageal HPV presence does not appear to be a risk factor for ESCC development, esophagus seems to be an important HPV-reservoir, in health or disease.

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**List of abbreviations**

AME: Medical Specialties Ambulatory  
BCH: Barretos Cancer Hospital  
CI: Confidence Interval  
EC: Esophageal cancer  
ESCC: Esophageal squamous cell carcinoma  
HPV: Human papillomavirus  
OR: Odds Ratio  
PCR: Polymerase chain reaction

**Ethical Aspects and Consent to Participate**

This study was approved by the Ethics Committee at BCH with register number 134471. Patients were referred to a private room and informed about the purpose of the study, procedures for biological sampling and the necessary information requested on the data collection instrument. Patients who agreed to participate signed the Informed Consent form. Participants, then, answered a questionnaire containing sociodemographic information and explaining the known risk factors for the HPV infection and EC. All interviews were conducted by previously trained members of the HPV Research Group at BCH - Pius XII Foundation. Interviews provided information to characterize the duration and type of exposure.

**Acknowledgements**

The authors would like to thank the BCH, Medical Specialties Ambulatory (AME) Clinic of Barretos, HPV Teams at BCH and Molecular Biology Laboratory, Center for Translational Research in Oncology, São Paulo Cancer Institute (ICESP), São Paulo, Brazil.

**Funding statement**

CNPq Universal for providing supplies to the largest study, of which this study is a part of, entitled "The role of human papillomavirus (HPV) as the etiologic agent of esophageal cancer. A cross-sectional study, case-control and longitudinal at Barretos Cancer Hospital"; (Grant number 482666/2012-9 to ALF); INCT HPV [Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) [Grant number 08/57889-1 to LLV]; Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Grant number 573799/2008-3 to LLV)].

**Conflict of interest:**

Nothing to declare.

## Tables

Table 1 – Odds ratio associate with patients characteristics (n= 174)

Variable Category	Cases		Controls		OR [95% CI] Univariate
	n	%	n	%	
<b>High Risk HPV</b>					
Negative	75	86.2	75	86.2	Reference
Positive	12	13.8	12	13.8	1.00 [0.42 : 2.36]
<b>Sociodemographic variables</b>					
<b>Sex</b>					
Female	19	21.8	19	21.8	Reference
Male	68	78.2	68	78.2	1.00 [0.48 : 2.05]
<b>Age</b>					
≤ 60 years	46	52.9	42	48.3	Reference
> 60 years	41	47.1	45	51.7	0.82 [0.45 : 1.51]
<b>Race (self-reported) *</b>					
White	59	71.1	51	64.6	Reference
Non-white	24	28.9	28	35.4	0.74 [0.37 : 1.44]
<b>Marital status</b>					
Not married	33	37.9	20	23.0	Reference
Married	54	62.1	67	77.0	<b>0.48 [0.25 : 0.94]</b>
<b>Education</b>					
Illiterate	15	17.2	14	16.1	Reference
Up to middle school	63	72.4	61	70.1	0.96 [0.42 : 2.16]
High school / Graduate	9	10.3	12	13.8	0.70 [0.22 : 2.16]
<b>Life Habits</b>					
<b>Interaction time consumption of tobacco and alcohol</b>					
Alcohol <15 years and Tobacco <15 years	3	3.4	21	24.1	Reference
Alcohol <15 years and Tobacco ≥15 years	11	12.6	8	9.2	<b>9.6 [2.11 : 43.75]</b>
Alcohol ≥15 years and Tobacco <15 years	15	17.2	19	21.8	<b>5.52 [1.38 : 22.10]</b>
Alcohol ≥15 years and Tobacco ≥15 years	58	66.7	39	44.8	<b>10.40 [2.9 : 37.29]</b>
<b>Interaction consumption of tobacco and alcohol *</b>					
Alcohol <30 g alcohol/day and Tobacco <40 packs/year	57	66.3	67	77.9	Reference
Alcohol <30 g alcohol/day and Tobacco ≥40 packs/year	12	14.0	6	7.0	2.35 [0.83 : 6.66]
Alcohol ≥30 g alcohol/day and Tobacco <40 packs/year	11	12.8	10	11.6	1.29 [0.51 : 3.26]
Alcohol ≥30 g alcohol/day and Tobacco ≥40 packs/year	6	7.0	3	3.5	2.35 [0.56 : 9.82]
<b>Sexual habits</b>					
<b>Age at first intercourse *</b>					
≤15 years old	29	34.9	25	29.1	Reference
>15 - ≤17 years old	21	25.3	22	25.6	0.79 [0.35 : 1.79]
>17 years old	33	39.8	39	45.3	0.71 [0.35 : 1.46]
<b>Partner number *</b>					
≤ 5	34	41.0	44	50.6	Reference
> 5	49	59.0	43	49.4	1.47 [0.80 : 2.70]
<b>Total</b>	<b>87</b>	<b>100</b>	<b>87</b>	<b>100</b>	

\* There are missing values.

**Table 2 – Multivariable analyses**

<b>Variable Category</b>	<b>p</b>	<b>OR [95% CI] Univariate</b>
<b>High Risk HPV</b>		
Negative	0.959	Reference
Positive		1.025 [0.405 : 2.592]
<b>Interaction time consumption of tobacco and alcohol</b>		
Alcohol <15 years and Tobacco <15 years	<b>0.007</b>	Reference
Alcohol <15 years and Tobacco ≥15 years	<b>0.005</b>	<b>9.423 [1.974 : 44.981]</b>
Alcohol ≥15 years and Tobacco <15 years	<b>0.016</b>	<b>5.572 [1.372 : 22.627]</b>
Alcohol ≥15 years and Tobacco ≥15 years	<b>0.001</b>	<b>9.581 [2.592 : 35.421]</b>
<b>Interaction consumption of tobacco and alcohol *</b>		
Alcohol <30 g Alcohol/day and Tobacco <40 packs/year	0.866	Reference
Alcohol <30 g Alcohol/day and Tobacco ≥40 packs/year	0.489	1.468 [0.495 : 4.355]
Alcohol ≥ g Alcohol/day and Tobacco <40 packs/year	0.890	0.935 [0.358 : 2.441]
Alcohol ≥ g Alcohol/day and Tobacco ≥40 packs/year	0.614	1.460 [0.335 : 6.359]

**Abstract word count:** 207 words

**Manuscript word count (excluding references):** 2,513 words

**Tables count:** 2 tables

HISTOLOGY AND HISTOPATHOLOGY  
(non-edited manuscript)