No evidence of HPV DNA in esophageal squamous cell carcinoma in a population of Southern Brazil

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Received: July 18, 2013 Revised: August 3, 2013
Accepted: August 17, 2013
Published online: October 21, 2013

Abstract

AIM: To investigate the association between human papillomavirus (HPV) and esophageal squamous cell carcinoma (ESCC) in southern Brazil.

METHODS: We studied 189 esophageal samples from 125 patients from three different groups: (1) 102 biopsies from 51 patients with ESCC, with one sample from the tumor and another from normal esophageal mucosa distant from the tumor; (2) 50 esophageal biopsies from 37 patients with a previous diagnosis of head and neck squamous cell carcinoma (HNSCC); and (3) 37 biopsies from esophageal mucosa with normal appearance from 37 dyspeptic patients, not exposed to smoking or alcohol consumption. Nested-polymerase chain reaction (PCR) with the MY09/11 and GP5/6 L1 primers was used to detect HPV L1 in samples fixed in formalin and stored in paraffin blocks. All PCR reactions were performed with a positive control (cervicovaginal samples), with a negative control (Human Genomic DNA) and with a blank reaction containing all reagents except DNA. We took extreme care to prevent DNA contamination in sample collection, processing, and testing.

RESULTS: The histological biopsies confirmed the diagnosis of ESCC in 52 samples (51 from ESCC group and 1 from the HNSCC group) and classified as well differentiated (12/52, 23.1%), moderately differentiated (27/52, 51.9%) or poorly differentiated (7/52, 13.5%). One hundred twenty-eight esophageal biopsies were considered normal (51 from the ESCC group, 42 from the HNSCC group and 35 from dyspeptic patients). Nine had esophagitis (7 from the HNSCC and 2 from dyspeptic patients). Of a total of 189 samples, only 6 samples had insufficient material for PCR analysis: 1 from mucosa distant from the tumor in a patient with ESCC, 3 from patients with HNSCC and 2 from patients without cancer. In 183 samples (96.8%) GAPDH, G3PDH and/or β-globin were amplified, thus indicating the adequacy of the DNA in those samples. HPV DNA was negative in all the 183 samples tested: 52 with ESCC, 9 with esophagitis and 122 with normal esophageal mucosa.

CONCLUSION: There was no evidence of HPV infection in different ESCC from southern Brazil.

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Key words: Esophageal cancer; Esophageal squamous cell carcinoma; Human papillomavirus; Head and neck cancer; Polymerase chain reactions; Nested-polymerase
INTRODUCTION

Worldwide, esophageal cancer is the eighth most common cancer and the sixth most common cause of death from cancer, with an estimated incidence of 482,000 new cases and 407,000 deaths in 2008[1]. Esophageal squamous cell carcinoma (ESCC) is still the most common type worldwide and its known risk factors are smoking and excessive alcohol consumption, poor nutritional and socio-economic status, exposure to polycyclic aromatic hydrocarbons (PAH), low consumption of fruits and vegetables, ingestion of hot beverages, genetic factors, history of caustic injury in the esophagus, and history of head and neck squamous cell carcinoma (HNSCC)[2-4].

In Brazil, the highest rates of esophageal cancer occur in the country’s southernmost state, Rio Grande do Sul, where the rate of incidence is considered moderate with 18.01 cases per 100,000 men and 6.60 cases per 100,000 women[5]. In the south of Brazil the most important risk factors are smoking and excessive alcohol consumption[6], however, the consumption of a hot beverage made with the infusion of Ilex paraguayensis (also known as yerba mate) is also a risk factor[7]. This beverage is often consumed at high temperatures and contains high levels of PAH[8].

The role of human papillomavirus (HPV) in the development of ESCC remains controversial[9-12]. Two studies conducted in southern Brazil, each using a different technique, showed different results regarding the association between ESCC and HPV[13,14]. Therefore, to clarify the association between HPV infection and ESCC in southern Brazil, we looked for the presence of HPV DNA in esophageal biopsies from: (1) primary tumor and mucosa without neoplasia from patients with ESCC; (2) Lugol stained and unstained areas in patients with HNSCC; and (3) normal appearing mucosa from patients not exposed to tobacco or alcohol.

MATERIALS AND METHODS

Patients and tissues

We evaluated 189 consecutive esophageal samples, collected in Santa Maria, a city in the central region of Rio Grande do Sul, the most southern state in Brazil, from 2008 to 2011. We included 51 samples from esophageal tumors and 51 samples from non-tumoral areas of the esophagus of patients with ESCC. We analyzed 50 esophageal biopsies (32 from Lugol stained areas, 17 from unstained areas and 1 from tumor) from patients diagnosed with HNSCC. Neither the patients with ESCC nor those with HNSCC had received chemotherapy or radiotherapy before the sample collection. We also collected biopsies from the normal middle esophagus of thirty-seven non-smoking and non-alcohol drinking dyspeptic patients who underwent upper GI endoscopy.

We collected demographic data (sex, age, place of birth, occupation) and information regarding smoking habits, alcohol consumption and previous history of cancer. The samples were fixed in neutral buffered formalin, embedded in paraffin, cut and stained with Hematoxilin-Eosin. For DNA extraction, one slice at least 5 μm thick, as recommended for polymerase chain reaction (PCR) amplification, was cut from the paraffin-embedded tissues[15]. To minimize the risk of contamination, the materials used to process the sample stored in the paraffin block were completely disposable and used once per sample. In addition, the microtome sample holder was washed with absolute alcohol and the blade was replaced before each block was cut. Different rooms were used for DNA extraction, preparing the DNA solution, adding the DNA samples to the PCR solution and electrophoresis analysis. The rooms could only be accessed through antechambers with a single flow of material. All PCR reactions were performed with a positive control (cervicovaginal samples), with a negative control (Human Genomic DNA, Cat. No. G304A, Promega, Madison, WI) and with a blank reaction containing all reagents except DNA. The same method was also employed to test for the presence of HPV DNA in thirty-five samples of primary tumor from patients with HNSCC.

DNA Extraction and PCR

Once the samples were de-waxed, the DNA was extracted using the Qiagen QIAamp DNA Mini Kit (Valencia, CA) according to manufacturer’s instructions. DNA quantification and purity were determined by optical density in a spectrophotometer (Thermo Scientific NanoDrop 2000).

The DNA (25-80 ng) from each sample was amplified by PCR. The integrity of the DNA samples was observed by amplifying the human conserved genes GAP-
The sequences of the HPV L1 gene were amplified by nested-PCR using two general primer sets: MY09/MY11 (MY09: 5’-GTCCMARRG-GAWACTGATC-3’, MY11: 5’-GCMCAGGGWCATA-AYAATGG-3’) in the first amplification step to produce a 450 bp fragment; and GP5/GP6 (GP5: 5’-TTTGT-TACTGTGGTAGATAC-3’, GP6: 5’-ACTAAATGT-CAAATAAAAAG-3’) in the second step to produce 150 bp fragments of the PCR product. In the first step, PCR was performed with a reaction mixture containing 50 μL, including 5.0 μL of the genome from the extracted DNA sample, 5.0 μL 10× PCR buffer, 5.0 μL of dNTP (2.5 mmol/L), 1.2 μL of MgCl2 (50 mmol/L), 0.5 μL of Taq DNA polymerase and 0.2 μL (500 pmol/μL) of the MY09 and MY11 primers. In the second step, which also had a final volume of 50 μL, 1.0 μL (500 pmol/μL) of the GP5 and GP6 primers was used. The PCR mixture was subjected to 40 amplification cycles, each consisting of an initial denaturation step at 94 ℃ for 30 s, annealment at 56 ℃ for 1 min and extension at 72 ℃ for 1 min. The PCR products were separated by electrophoresis on 2% agarose gel and visualized by staining with ethidium bromide by electrophoresis (Figure 1).

Ethics
The study was approved by the Research Ethics Committee of the Federal University of Santa Maria and of the Federal University of Rio Grande do Sul, Brazil. Informed consent was obtained from each participant before they underwent upper GI endoscopy.

Statistical analysis
The variables were expressed as mean and SD or numbers and percents. Associations would have been considered statistically significant when a two-sided P value was ≤ 0.05. All statistical analyses were performed with the aid of SPSS 11 (Statistical Package for Social Sciences).

RESULTS

Patient characteristics
We included 125 individuals divided in three groups (Table 1): (1) 51 patients with ESCC, 43 male (84.3%) with a mean age of 60.1 ± 10.3 years; (2) 37 patients with previous diagnosis of HNSCC, 34 males (91.9%) with a mean age of 57.8 ± 8.4 years; and (3) 37 dyspeptic patients, 12 males (32.4%) with a mean age of 56.8 ± 17.7 years.

Histology
We studied 189 esophageal biopsy samples. All fifty-two biopsies from tumoral areas (51 from the ESCC group and 1 from the HNSCC group) were diagnosed as squamous cell carcinoma and classified as well differentiated (12/52, 23.1%), moderately differentiated (27/52, 51.9%) or poorly differentiated (7/52, 13.5%). None of the 51 samples of esophageal mucosa distant from the tumor in the ESCC group showed malignancy. In the patients with HNSCC, we performed 55 esophageal biopsies. Their histological analysis showed the following findings: 42 were normal (76.4%), 7 had esophagitis (12.7%), 1 contained ESCC (1.8%) and five had insufficient tissue for histological analysis (9.1%). In the biopsies of normal esophagus in the dyspeptic patients, 35 were considered normal and 2 had mild esophagitis.

PCR analysis
The average DNA concentration in the samples was 214.68 ng/μL (range: 8-1313), with a mean ratio between the absorbance readings at 260 nm and 280 nm wavelengths of 2.12 (range 1.15-5.95). GAPDH, G3PDH and/or β-globin were amplified in 183 (96.8%) esophageal biopsies, showing that the DNA was adequate for analysis. These conserved human genes were not amplified in only six samples, all with normal esophageal mucosa (1 from mucosa distant from the tumor in a patient.
with ESCC, 3 from patients with HNSCC and 2 from patients without cancer). The PCR results were negative for HPV DNA in all the esophageal biopsies (52 with ESCC, 9 with esophagitis and 122 with normal esophageal mucosa), as well as in all biopsies from the primary tumor of head and neck cancer.

**DISCUSSION**

The current study used a nested primer-based PCR test to identify HPV DNA in esophageal samples from individuals from a moderate risk area for ESCC in southern Brazil. Our results showed no evidence of HPV DNA in any of the samples of the ESCC and from non-tumoral areas of the esophagus, in the esophageal mucosa of patients with HNSCC, or in the esophageal mucosa from patients without risk factors for ESCC.

Some studies using a bovine model have found that the bovine papillomavirus is essential in the early stages of carcinogenesis of the upper digestive tract, but is not required for progression to the status of malignancy. Consequently, we believe that our technique was sufficiently sensitive to accurately detect the presence of HPV DNA in tissue samples from the aero-digestive tract.

In southern Brazil, Weston and Prolla analyzed 40 ESCC samples and 10 benign esophageal biopsies from patients without cancer. Using the Hybrid Capture II test, they detected HPV DNA in only one case of ESCC and in one benign specimen. Subsequently, Souto Damin et al. reported detecting HPV in 15.75% of ESCC patients (26/165) and in none of the specimens of benign esophagus (0/26) using auto-nested PCR with the GP5+/GP6+ consensus primer pair. Some features of our study that differed from these two previous HPV studies conducted in southern Brazil are: (1) the extreme care we took to prevent DNA contamination throughout the specimen processing and testing; (2) the use of a more highly sensitive HPV detection method; and (3) the assessment of possible precursor lesions in the non-tumoral mucosa of esophagi with cancer and in the esophageal mucosa of patients with head and neck cancer.

A tissue study similar to ours was recently reported from a high-risk area of China. The authors analyzed tumor samples from 272 patients with ESCC who underwent esophagectomy at the Yaocun Commune Hospital in Linxian, in north-central China. The patients came from various regions of China, with different incidence rates of ESCC and different mortality rates for cervical cancer. HPV DNA was tested on fresh frozen tumor tissue and tumor samples fixed with formalin and stored in paraffin using PCR with the PGMY L1 and SPF10 L1 consensus primers, respectively. Adopting careful measures to avoid contamination of the samples, similar to those used in the present study, these authors also found no cases with convincing evidence of carcinogenic HPV activity.

There are some limitations to the present study, such as the lack of information regarding sexual habits, socio-economical background, and dietary habits of the patients. Furthermore, the study was performed on a relatively small number of patients, and the material was not uniformly available from all sites of the esophagus.

| Table 1 Clinical and demographic characteristics and risk factors of the patients n (%) |
|---------------------------------------------------------------|
| **ESCC** (n = 51) | **HNSCC** (n = 37) | **Not exposed** (n = 37) |
| Age (yr) | 42.79 ± 8.4 | 57.8 ± 8.4 | 56.8 ± 17.7 |
| Sex | 43 (84.3) | 34 (91.9) | 12 (32.4) |
| Smoking | 3 (6.8) | 25 (67.6) |  |
| Alcohol use | 42 (82.4) | 35 (94.6) |  |
| Never smoked | 5 (9.8) | 2 (5.4) | 10 (27.0) |
| Active smokers and alcohol users (> 10 yr) | 16 (31.4) | 4 (10.8) | 35 (94.6) |
| Ex-alcohol users (> 10 yr) | 18 (35.3) | 18 (48.6) |  |
| Never smoked or drunk alcohol | 3 (5.9) | 0 (0) | 26 (70.3) |

ESCC: Esophageal squamous cell carcinoma; HNSCC: Head and neck squamous cell carcinoma.
economic characteristics and the consumption of the beverage *yerba mate*, but there is no reason to think that having such information would have changed the PCR results of this study.

Our results confirm previous observations in other regions that report the absence of an association between esophageal mucosa infection by HPV and ESCC, and suggest that, in southern Brazil, this virus is not an important risk factor for squamous cell carcinoma of the esophagus.

ACKNOWLEDGMENTS

The authors thank the following individuals for their assistance: Patricia Chaves Brites for useful suggestions in the human papillomavirus analysis, and Drs Leandro Bizarro Muller, Eduardoz Buzatti Souto, Daniela Costa, Stela Maria Motta, Alexandre Rampazzo and João Carlos Cantarelli Jr for their collaboration in the upper endoscopy. Special acknowledgment is given to Dr Sanford M. Dawsey for his helpful comments on the manuscript and for his help in the English revision.

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