HPV-16/18 detection does not affect the prognosis of head and neck squamous cell carcinoma in younger and older patients

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Abstract. Recently, high-risk human papillomavirus (HPV) has emerged as a possible agent associated with head and neck squamous cell carcinoma (HNSCC) in younger patients. Therefore, the purpose of the present study was to assess the effect of age on the distribution of HPV-16/18 in HNSCC, together with the impact of the virus on patient prognosis. A longitudinal prospective study was used adjusted for age, gender, TNM staging, smoking status and alcohol consumption. HPV was detected by PCR with consensus primers. Results showed there was no difference in the frequency of HPV-16/18 positivity when younger patients were compared to the older patients. No association was found among high-risk HPV positivity, gender, smoking habit and anatomical site. High-risk HPV was associated with advanced TNM in bivariate analyses; however, it did not impact on survival. Only TNM staging was associated with risk of mortality. Our study supports the theory that age does not affect the presence of HPV-16/18 in HNSCC and has no impact on patient prognosis. The incidence of HNSCC among patients under the age of 45 years is reportedly on the increase worldwide. The factors associated with HNSCC in younger adults are not well established. Findings of this study indicate that HPV-16/18 may not play a role in HNSCC patients under the age of 45 years.

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer and causes 350,000 cancer mortalities worldwide each year (1,2). HNSCC comprises malignant epithelial neoplasms that arise in the paranasal sinuses, nasal cavity, oral cavity, pharynx and larynx, and generally affects males between the sixth and ninth decades of life following long-term exposure to smoking and alcohol consumption (3). However, an increase in the incidence of head and neck cancer among patients under the age of 45 years has been reported worldwide (4-10). Some studies suggest that in younger patients, HNSCC arises regardless of the classical risk factors (4-8,10). HPV infection has been suggested as a possible etiologic factor for such cases (11-16). HPV was detected in a variety of HNSCCs (11-15,17) and it was suggested to be associated with lesions of potential malignisation in the head and neck (18,19); however, divergent results are found in the literature regarding the role of HPV presence in HNSCC (20,21).

Taking these facts into consideration, we investigated whether there is any change in the distribution of HPV-16/18 in younger patients compared to older ones. In addition, we assessed the effect of the virus on patient prognosis. To test these hypotheses we performed a longitudinal prospective study.

Patients and methods

Patients. In total, 75 patients diagnosed with HNSCC recruited from a database of head and neck surgeries that occurred between 1996 and 2007 in Montes Claros, Brazil, were included in the current study (4). Patients younger than 45 years old were selected (n=25). The older patients (n=50) were randomly selected in a proportion of 2:1 adjusted for gender, TNM staging, anatomical site, smoking and alcohol intake. The patients were from the same geographical area and evaluated/treated by the same practitioner.

Clinical data. The mean age was 42.1 years (SD 3.17; range, 33-45) for younger HNSCC patients and 62.2 years (SD 8.0; range, 49-82) for older HNSCC patients. Skin colour was not used as a physical descriptor since it is a poor predictor of genomic ancestry in Brazil (22,23). The current investigation was approved by the local Ethics Committee. Information on age, gender, tobacco smoking history, alcohol consumption history, medical history, tumour site, TNM clinical staging and survival was obtained from medical files.

All patients were staged according to the UICC TNM classification of malignant tumours (1997) (24). HNSCC lesions were classified according to the primary site as described in the international classification of diseases (ICD-10) for oncology. The anatomical sites reviewed in this study included: i) 28 (37.3%) mouth and perioral region sites (C00, C01, C02, C04, C05, C06.0 and C06.2); ii) 22 (29.3%) oropharynx (C09-C10) sites; and iii) 25 (23.4%) hypopharynx-larynx sites (C12, C13 and C32). The sites were classified according to anatomical site (anterior: Oral mucosa, tongue, retromolar trigon, mouth floor, jugal mucosa and gengival; border/posterior: Base tongue, oropharynx and hypopharynx-larynx). The patients had histologically confirmed HNSCC based on the World Health Organisation criteria (WHO, 1997) (25,26). Patients with a diagnosis of carcinoma *in situ* or multiple head and neck carcinomas were excluded. Ethics approval for this study was obtained from the local ethics committee (468/06).

HPV identification. HPV-DNA sequences were first polymerase chain reaction (PCR)-amplified by L1 (F: 5'-GCM CAGGGWCATAAYAATGG-3' and R: 5'-CGTCCMAAR GGAWACTGATC-3', where, M=A or C, R=A or G, W=A or T, Y=C or T) and then by HPV-16 (F: 5'-AAGGCCAACTA AATGTCA-C-3' and R: 5'-CTGCTT TTATACTAACCGG-3') and HPV-18 (F: 5'-ACCTTAATGAAAAACCACGA-3' and R: 5'-CGTCGTTTAGAGTCGTTC-3'). β-globin gene primers were used as an internal control. The primer sequences were described by Katiyar *et al* (27). PCR was performed in a total volume of 25 μl containing approximately 100 ng genomic DNA as a template, 0.5 μl of each primer (20 pmol/μl), 2.5 μl dNTP-mix (25 mM of each, Amresco, Ohio, CA, USA), 2.5 μl 10X PCR buffer, 1.25 μl magnesium chloride (50 mM) and 2.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA) (Fig. 1). Reactions were performed using positive (cultured virus) and negative (PCR without DNA) controls.

Electrophoresis. The PCR products were verified on a 6.5% polyacrylamide gel that was electrophoresed at a constant voltage of 120 V for 1.5 h and stained with silver nitrate. Electrophoresis results were estimated against a 100-bp ladder.

Statistical analysis. The statistical significance of differences between case and control group distributions for HPV positivity was evaluated using Fisher's test or the Chi-squared test. Survival time was calculated from the date of diagnosis to the time of the last follow-up visit or to the time of mortality. Using these criteria, the records of each patient were reviewed from 0 to 2500 days. Mortalities were the result of locoregional and/or metastatic disease. Mortalities that occurred without evidence of recurrence were excluded from analysis. Survival time was presented by the means of the Kaplan-Meier method for the variables. Variables were included in the Cox proportional hazards multivariate model. In accordance with the literature, categorical variables considered as references were those associated with a reduced risk of mortality. Analyses were assessed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA), and statistical significance was set at $p<0.05$.

Table I. The distribution of clinical parameters.

	Frequencies	
Clinical parameters	n	%
Age		
Older	50	66.7
Younger	25	33.3
Gender		
Male	64	85.3
Female	11	14.7
Tobacco habit		
Yes	72	96.0
No	3	4.0
Alcohol habit		
Yes	70	93.3
No	5	6.7
TNM staging		
I/II	9	12.0
III/IV	66	88.0
Lesion site		
Anterior	28	37.3
Posterior	47	62.7
Anatomical site		
Hypopharynx	8	10.7
Larynx	17	22.7
Oropharynx	22	29.3
Oral cavity	28	37.3

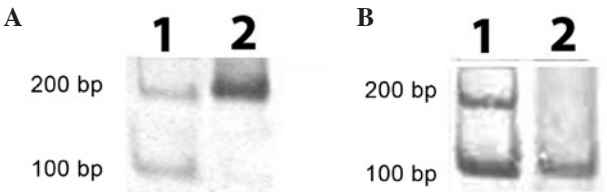

Figure 1. (A) PCR for HPV-16. Lane 1, 100-bp molecular marker. Lane 2, HPV-16-positive (217 bp). (B) PCR for HPV-18. Lane 1, 100-bp molecular marker. Lane 2, HPV-18-positive (100 bp). PCR, polymerase chain reaction; HPV, human papillomavirus.

Results

The descriptive data of the population used in the current study are shown in Table I. The majority of patients (96%) in the

Table II. The distribution of clinical and molecular parameters according to a positive HPV status.

	HPV16$^-$ (%)	HPV16$^+$ (%)	P-value	HPV-18$^-$ (%)	HPV-18$^+$ (%)	P-value	HPV-16/18$^-$ (%)	HPV-16/18$^+$ (%)	P-value
Age									
Older	38 (69.1)	12 (60.0)		26 (63.4)	24 (70.6)		19 (63.3)	31 (68.9)	
Younger	17 (30.9)	8 (40.0)	0.318	15 (36.6)	10 (29.4)	0.342	11 (36.7)	14 (31.1)	0.399
Gender									
Male	58 (86.6)	6 (75)		34 (82.9)	30 (88.2)		26 (86.7)	38 (84.4)	
Female	9 (13.4)	2 (25)	0.333	7 (17.1)	4 (11.8)	0.378	4 (13.3)	7 (15.6)	0.533
Smoking habit									
Yes	54 (98.2)	18 (90.0)		39 (95.1)	33 (97.1)		29 (96.7)	43 (95.6)	
No	1 (1.8)	2 (10.0)	0.172	2 (4.9)	1 (2.9)	0.464	1 (3.3)	2 (4.4)	0.650
TNM staging									
I/II	7 (12.7)	2 (10.0)		7 (17.1)	2 (5.9)		7 (23.3)	2 (4.4)	
III/IV	48 (87.3)	18 (90.0)	0.552	34 (82.9)	32 (94.1)	0.129	23 (76.7)	43 (95.6)	**0.018**
Lesion site									
Anterior	20 (36.4)	6 (30.0)		15 (36.6)	11 (32.4)		10 (33.3)	16 (35.6)	
Posterior	35 (63.6)	14 (70.0)	0.411	26 (63.4)	23 (67.6)	0.445	20 (66.7)	29 (64.4)	0.522
Anatomical site									
Hypopharynx	5 (9.1)	3 (15)		2 (4.8)	6 (18.2)		2 (6.6)	6 (13.3)	
Larynx	10 (18.2)	7 (35)		13 (31.0)	4 (12.1)		8 (26.7)	9 (20.0)	
Oropharynx	18 (32.7)	4 (20)		10 (23.8)	12 (36.4)		8 (26.7)	14 (31.1)	
Oral cavity	22 (40.0)	6 (30)	0.319	17 (40.4)	11 (33.3)	0.105	12 (40.0)	16 (35.6)	0.725

HPV$^+$, positivity for human papillomavirus; HPV$^-$, absence of human papillomavirus. Bold indicates a statistically significant result.

study population were smokers and consumed alcohol (93.3%). Moreover, 88% presented with advanced TNM staging.

Table II shows the distribution of clinical and molecular parameters according to HPV status. There is no change in the frequency of HPV-16/18 related to patient age. No association was found among high-risk HPV positivity, gender, smoking habit and anatomical site. In combination, high-risk HPV was associated with advanced TNM in bivariate analyses (Table II); however, it did not impact on the survival (Table III). Anatomical site and age did not interfere with the survival. Only TNM staging was associated with risk of mortality (Table III).

Discussion

Currently, there are no biological mechanisms that could justify the high-risk HPV predilection for a specific anatomical site in the head and neck. The role of high-risk HPV in HNSCC has recently been highlighted in the oropharynx (13), oral cavity (14), larynx (15), hypopharynx (11,12) and in HNSCC metastasis (17). The relevance of the high-risk HPV-E6/E7 protein for the carcinogenesis of multiple head and neck sites was demonstrated in transgenic mice (28-30). In addition, there is no consensus in the literature to detect HPV infection (20). In agreement with these facts, we did not observe high-risk HPV predilection for anatomical sites. The absence of consensus is justified by the differences in the study sample. It

Table III. Cox regression analyses in HNSCC patients with a follow-up of 2,500 days.

			95% CI	
	P-value	OR	Lower	Upper
TNM				
I/II	Reference			
III/IV	**0.015**	6.052	1.423	25.742
Age				
Younger	Reference			
Older	0.394	1.316	0.700	2.474
Site				
Anterior	Reference			
Posterior	0.317	0.741	0.412	1.333
HPV-16/18				
HPV$^-$	Reference			
HPV$^+$	0.886	0.957	0.527	1.739

OR, odds ratio; HPV, human papillomavirus; HNSCC, head and neck squamous cell carcinoma; CI, confidence interval. The model was adjusted to the best-fit model. Bold indicates a statistically significant result.

is well known that there are limitations for paraffin-embedded tissues, particularly as the fixation makes it difficult to purify RNA from paraffin-embedded tissues (17,31,32). Although non-quantitative PCR-based methods do not allow for the determination of infectious activity, these methods are beneficial as they provide greater sensitivity and specificity than *in situ* hybridisation techniques (20). Evidence suggests that the p16 immunohistochemistry could be used as a survival biomarker in head and neck cancer (33) and aid in high-risk HPV detection (18).

A possible association between HPV infection and the development of HNSCC in younger patients has been proposed (16). In addition, it has been postulated that HPV-positive tumours are a subgroup of HNSCC that is distinct from tobacco- and alcohol-induced carcinomas (19). An absence of $p16_{CDKN2A}$ mutations as well as the expression of p16 is the characteristic feature of high-risk HPV-positive tumours, in contrast to the inactivation of the $p16_{CDKN2A}$ gene in HPV-negative tumours (34). p16 protein is important in the regulation of the G1/S phase cell-cycle checkpoint (33,35,36) and it has been reported that p16-positive tumours have a better prognosis than p16-negative tumours (33). Therefore, p16 expression may explain the reason for HPV-positive tumours showing a favourable prognosis as compared to HPV-negative tumours. In the current study, HPV-16/18 positivity was not associated with age or survival. The explanation for these findings may be the rigorous selection criteria (age, gender, TNM staging and anatomical site) or that the simultaneous presence of some of these independent variables alters the evolution of cancer in a different way. Moreover, the majority of our patients were smokers, thus our data are in agreement with previous studies (21). Genetic and epigenetic factors are associated with a susceptibility to cancer development at a young age (4,37). Alternatively, genetic and epigenetics factors may play a stronger role than HPV-16/18 infection in the development of HNSSC at an early age (38-42). In the current study, we did not observe differences in survival associated with age, in agreement with previous studies (4,37).

The mechanisms underlying the association between high-risk HPV and HNSCC have been unclear. Studies using SCC samples shed light on the role of high-risk HPV infection in epigenetic regulation (43). For example, it was suggested that HPV-16 induces the methylation of $p16_{CDKN2A}$ (44); however, as oncogenic HPV neutralises phosphorylated Rb (PRb)-mediated control of the cell cycle, there would be no advantage for the HPV-infected host cell to block the same signalling pathway at another checkpoint by downregulating $p16_{CDKN2A}$ (45-47).

In conclusion, our study supports the theory that there is no change in the frequency of HPV-16/18 positivity in younger patients when compared to the older ones. In addition, HPV-16/18 presence did not change the HNSCC prognosis.

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