Expression of cell cycle proteins according to HPV status in oral squamous cell carcinoma affecting young patients: a pilot study

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Objective. Tobacco and alcohol consumption are considered the main risk factors for oral squamous cell carcinoma (OSCC); however, the role of these factors in patients younger than 40 years is controversial, so it has been suggested that genomic instability and high-risk human papillomavirus (HR-HPV) infection may be contributing factors to oral carcinogenesis at a young age. Therefore, the aim of this study was to evaluate the immunoexpression of cell cycle proteins according HPV status in OSCC affecting young patients.

Methods. A tissue microarray construction based on 34 OSCC samples from young patients (<40 years old) was subjected to immunohistochemical reactions for Ki67, cyclin D1, C-ErbB2, p21, Myc, epidermal growth factor receptor, p53, and p16 antibodies.

Results. The clinicopathologic features and the immunoexpression of all tested proteins were similar in both groups. Patients with HPV-related OSSC tended to have better cancer-specific survival (CSS; 39% vs 60% 5-y CSS), and overall survival (OS; 29.2% vs 60% 5-year OS). However, this difference was not statistically significant.

Conclusion. No significant difference exists in the expression of cell cycle proteins studied between HR-HPV DNA–positive and HR-HPV DNA–negative OSCC affecting young patients. (Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:317–325)

Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral malignancies,[1] and the mortality and morbidity rates are high because of particularly late diagnoses, aggressive local invasion, and a high risk of regional lymph node metastases.[2] OSCC typically affects men in the fifth and sixth decades of life[1]; however, a higher prevalence among patients younger than 40 years has been reported in recent years worldwide.[3-6]

OSCC is a multifactorial disease for which tobacco and alcohol consumption are considered the main risk factors[1]; however, the role of these factors in young patients is controversial because of the short time of exposure in this population or no exposure at all.[7] High-risk human papillomavirus (HR-HPV) plays an important role in the development of cervical,[8] anal,[9] and oropharyngeal[10] cancer, but its function in OSCC is still considered uncertain.[11] Nevertheless, a previous study from our group reported a higher frequency of HR-HPV DNA in OSCC of young patients, suggesting that this oncogenic virus may be a contributing factor to carcinogenesis in this age group.[12]

On the other hand, increased genomic instability was also pointed out by an international collaborative study on young patients with OSCC.[13] In this context, genetic alterations involved in neoplastic transformation include activation of proto-oncogenes and inactivation of tumor suppressor genes. Proto-oncogenes are genes encoding proteins that regulate the proliferation and differentiation of cells and several mechanisms that can turn them into oncogenes. The protein products of oncogenes can become inducers of tumorigenesis.[14] On the contrary, tumor suppressor genes negatively modulate neoplastic transformation, encoding proteins that inhibit progression at different checkpoints of the cell cycle.[14] Several tumor suppressor genes may suffer loss of function and therefore favor the development of tumors. Abnormalities in the cell cycle proteins Ki67, cyclin D1, C-ErbB2, p21, Myc, epidermal growth factor receptor (EGFR), p53, and p16 have been commonly identified in OSCC.[15]

Statement of Clinical Relevance

The clinicopathologic features, survival rates, and immunoexpression of cell cycle proteins in human papillomavirus (HPV)-positive and HPV-negative oral squamous cell carcinoma (OSCC) affecting young patients are equal. Moreover, p16 immunoexpression is independent from the high-risk DNA HPV presence in OSCC.

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Therefore, the aim of this study was to evaluate the clinicopathologic features and immunoexpression of selected cell cycle proteins in HPV-related OSCC affecting young patients compared with those in HPV-negative OSCC.

PATIENTS AND METHODS

Tissue samples

This retrospective series analyzed selected cases of OSCC affecting patients younger than 40 years old treated at A.C. Camargo Cancer Center (São Paulo, Brazil), in the period from 1968 to 2011. Only tumors of the oral cavity were included: lateral border of the tongue, floor of the mouth, and retromolar trigone and buccal mucosa.

Clinical data concerning demographic features (age, sex, and affected site), tumor stage, risk habits (tobacco and alcohol consumption), therapeutic modality (surgery, radiotherapy, and/or chemotherapy), and follow-up status were retrieved from patients' medical charts. Clinical stage was grouped as early (CS I and II) or advanced (CS III and IV),[16] whereas histologic differentiation was classified as well differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) tumors.[1] Overall survival (OS) time was obtained by the difference in time between treatment and a patient's death as a result of any cause or the patient's last follow-up, whereas cancer-specific survival (CSS) represented the time difference between treatment and a patient's death as a result of the tumor or the patient's last follow-up.

HPV detection

HPV status of all tumor samples was conducted and published by Kaminagakura et al.[12] DNA was extracted according to standard protocols for extraction from archival formalin-fixed, paraffin-embedded tissue sections based on the antigen retrieval principle.[16] Briefly, 5-µm sections of each block were placed in 1.5-mL sterilized tubes for later deparaffinization with xylene and ethanol and digestion with proteinase K. Subsequently the samples were subjected to organic extraction with phenol/chloroform/isopropanol alcohol. The aqueous phase was precipitated in 100 µL of ammonium acetate, 7.5 M and 800 µL of ethanol 100% for 2 hours in a freezer –70°C. The precipitate was centrifuged for 15 minutes at 12,000 rpm and then washed with 1 mL of 70% ice-cold ethanol and again centrifuged at 12,000 rpm for 15 minutes. This step was repeated 1 time. The precipitate was dried and resuspended in 50 µL of Tris-EDTA buffer (TE) pH 7.5.

DNA quality was established by amplification of a fragment of the human β-globin gene using PCO3 þ/PCO4 þ primers. To HPV DNA detection of 17 types (HPV6, HPV11, HPV16, HPV18, HPV31, HPV33, HPV35, HPV39, HPV42, HPV45, HPV51, HPV52, HPV53, HPV54, HPV55, HPV56 and HPV58), generic primers GP5 þ/GP6 þ specific for the L1 gene were used,[17] followed by electrophoresis on 7% polyacrylamide gel of polymerase chain reaction (PCR) products and silver staining.[18] Specimens with a band around 150 base pairs with positive signals on genotyping by dot blot hybridization using radioactive probes[19] were considered positive for HPV DNA.

Tissue microarray construction

Tissue microarrays were constructed using the Tissue Microarrayer, model MTA-I (Beecher Instruments, Silver Springs, MD, USA). Cylindrical cores of 1.0 mm diameter were taken from the tumor invasion front of the original block and then transferred into the paraffin receptor block in duplicate.

Immunohistochemistry

Three-micrometer-thick sections were dewaxed with xylene and hydrated in a series of ethanol solutions. Antigen retrieval and incubation with 3% hydrogen peroxide to quench the endogenous peroxidase was further performed. Then, incubations overnight with primary antibodies were performed at 4°C. Antibody clones, dilutions, antigen retrieval, sources, and positive controls are shown in Table I. The slides were exposed to either Post Primary Block (NovoLink Max Polymer Leica Biosystems, Milton Keynes, UK) or avidin-biotin complex and horseradish peroxidase reagents (LSAB Kit,

Table I. Antibodies used for the immunohistochemical analysis

Antibody	Clone	Dilution	Antigen Retrieval	Source	Positive Control
Ki67	MIB-1	1:300	Citric acid	Dako	Tonsil
Cyclin D1	RBT14	Ready	EDTA/Tris	Biosb	Tonsil
C-ErbB2		1:1500	Citric acid	Dako	Breast carcinoma
p21	SX118	1:50	EDTA/Tris	Dako	Breast carcinoma
Myc	9 E.10.3	1:50	EDTA/Tris	Thermo	Burkitt lymphoma
EGFR	EGFR.25	1:50	Citric acid	Leica	Placenta
p53	DO-7	1:300	Citric acid	Dako	Carcinoma
p16	E6 H4	Ready	CC1	Ventana*	Cervical carcinoma

EGFR, epidermal growth factor receptor.
*Automated immunohistochemistry.

DakoCytomation, Carpinteria, CA, USA). DAB chromogen (Diaminobenzidine Tetrahydrochloride, Sigma, St Louis, MO, USA) was used to visualize the reactions, followed by counterstaining with Carazzi hematoxylin. Negative controls were obtained by omitting the specific primary antibody.

Immunohistochemical analysis

The immunohistochemical stained slides were scanned into high-resolution images using the Aperio Scanscope CS Slide Scanner (Aperio Technologies Inc., Vista, CA, USA). All digital images obtained in .svs format were visualized with ImageScope software (Aperio Technologies). The Nuclear Staining V9 Algorithm was used to analyze the nuclear markers (Ki67, cyclin D1, p21, Myc, and p53), and positive nuclear staining was expressed as a percentage. The expression of nuclear markers was defined following the literature as positive expression when more than 10% of cells displayed nuclear staining and negative expression when there was no stained nuclei or immunoexpression was present in fewer than 10% of tumor cells.[20]

Membrane markers (C-ErbB2 and EGFR) and p16 (nucler and cytoplasmic marker) were analyzed using the PixelCount V9 Algorithm, and staining was automatically quantified according to previously established input parameters.[21] Reactivity was classified as weak (scored as 1), moderate (scored as 2), or strong staining (scored as 3). The final score of each case was calculated as the sum of the percentage of each category multiplied by their intensity scores using the formula ([%weak × 1] + [%moderate × 2] + [%strong × 3]), obtaining a score from 100 to 300. For negative cases, a score of 100 was established as a reference. For statistical purposes, the median value of the final immunostaining results was used to split cases into 2 groups, more than and less than the median, representing low and high expression levels, respectively, of each membrane and cytoplasmic marker analyzed.[22]

Statistical analysis

Associations between clinicopathologic features and protein expression were determined by χ^2 and Fisher tests. Comparisons between quantitative variables were performed with the nonparametric Mann-Whitney U test. Survival curves were generated by the Kaplan-Meier method. The significance level adopted was P value < .05, and the SPSS Statistics Software Version 23.0 (IBM Corp., Armonk, NY, USA) was employed to perform all statistical analyses.

Ethical approval

Ethical guidelines were followed in accordance with the ethical standards of the Research Ethics Committees of AC Camargo Cancer Center (Brazil). Samples and clinicopathologic data were handled in a coded fashion (2199/16).

RESULTS

Thirty-four samples from patients younger than 40 years had tissue available for HPV detection studies, of which 24 (70.6%) were HR-HPV DNA negative, and 10 (29.4%) were HR-HPV DNA positive. The sociodemographic and clinicopathologic features are shown in Table II.

Table II. Sociodemographic and clinicopathologic features

	HR-HPV Negative	HR-HPV Positive	
Feature	n (%)		P
Age			
Mean	34	35.1	.467
Median	34.5	37.5	
Range	20-40	20-40	
Sex			
Male	18 (75)	6 (60)	.431
Female	6 (25)	4 (40)	
Tobacco consumption			
Yes	20 (90.9)	6 (66.7)	.131
No	2 (9.1)	3 (33.3)	
Alcohol consumption			
Yes	18 (81.8)	4 (44.4)	.077
No	4 (18.2)	5 (55.6)	
Anatomic site			
Oral tongue	8 (33.3)	5 (50)	.468
Floor of the mouth	10 (41.7)	2 (20)	
Other	6 (25)	3 (30)	
T classification			
T1/T2	5 (20.8)	3 (30)	.666
T3/T4	19 (79.2)	7 (70)	
N classification			
N0	0 (0)	0 (0)	ND
N1-N3	24 (100)	10 (100)	
Clinical stage			
I/II	4 (16.7)	1 (10)	1
III/IV	20 (83.3)	9 (90)	
Histologic differentiation			
I	17 (70.8)	7 (70)	.235
II	3 (12.5)	3 (30)	
III	4 (16.7)	0 (0)	
Surgical margins			
Negative	16 (72.7)	6 (88.9)	.639
Positive	6 (27.3)	1 (11.1)	
Treatment			
Surgery	7 (29.2)	4 (40)	.778
Radiotherapy	1 (4.2)	0 (0)	
Surgery + radiotherapy	15 (62.5)	6 (60)	
Surgery + radiotherapy + chemotherapy	1 (4.2)	0 (0)	
Recurrence			
Yes	15 (62.5)	6 (60)	.891
No	9 (37.5)	4 (40)	

HR-HPV, high-risk human papillomavirus; *NA,* not available; *ND,* not determined.

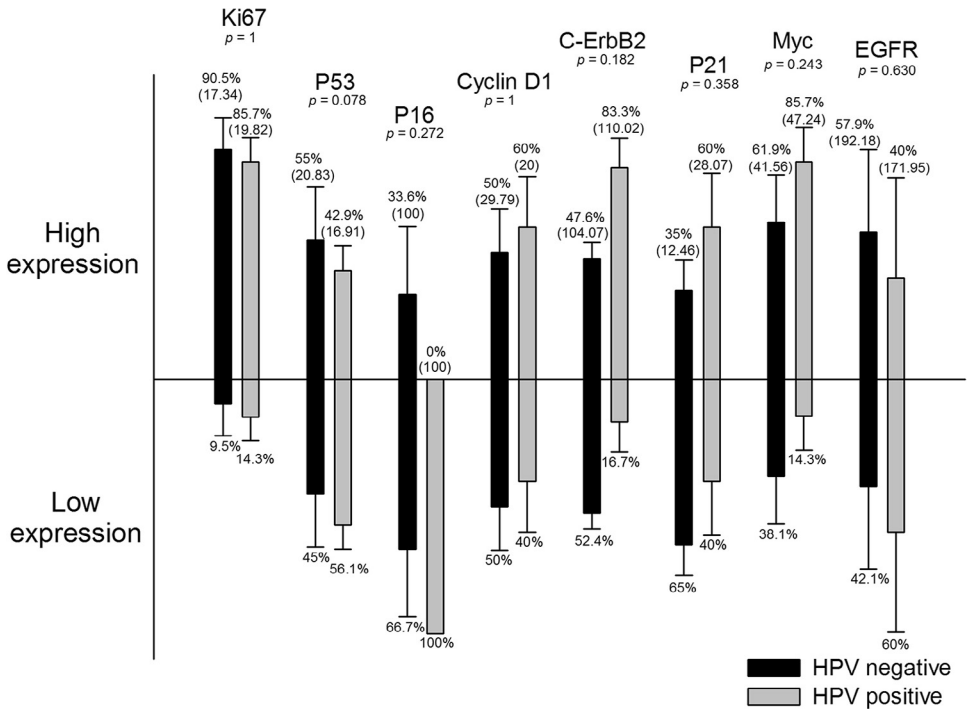

Fig. 1. Comparison of immunohistochemical expression of cell cycle proteins between patients with high-risk human papillomavirus (HR-HPV) DNA–negative and HR-HPV DNA–positive tumors. *EGFR,* epidermal growth factor receptor.

Ki67 immunoreactivity revealed a median proliferative rate of 17.34% (±12.57) in HR-HPV DNA–negative tumors and 19.83% (±9.47) in HR-HPV DNA–positive tumors. The first group had a higher proliferative index (90.5%) than the last one (85.7%), but this difference was not statistically significant ($P = .672$). Similar results were found with EGFR antibody; this protein was expressed in the membrane and cytoplasm of neoplastic cells of HR-HPV DNA–negative tumors indicating a higher expression than HR-HPV DNA–positive tumors (57.9% vs 40%, respectively) ($P = .630$) (Figure 1).

Higher expression for cyclin D1, C-ErbB2, p21, and Myc were found in HR-HPV DNA–positive tumors compared with HR-HPV DNA–negative ones: 60%, 83.3%, 60%, and 85.7%, respectively, in the positive group and 50%, 47.6%, 35%, and 61.9% in the negative group; nevertheless, the differences were not statistically significant ($P = 1$, $P = .182$, $P = .358$, and $P = .243$, respectively) (Figure 1).

Tumor suppressor proteins p53 and p16 had a higher expression in HR-HPV DNA–negative tumors than in HR-HPV DNA–positive tumors. Tumor suppressor protein p53 was highly expressed in 55% vs 42.9% of cases ($P = .678$) and p16 in 33.6 vs 0% of tumors ($P = .272$) (Figure 1). Immunohistochemical staining patterns of the all markers evaluated are shown in Figure 2.

The 5-year survival was higher in HR-HPV DNA–positive than in HR-HPV DNA–negative patients; however, the difference was not statistically significant. Recurrence rates were similar in both groups (62.5% vs 60%, $P = .891$). The CSS rate was 60% in patients with HR-HPV DNA–positive tumors and 39% in those with HR-HPV DNA–negative tumors ($P = .254$). The OS rate was 60% in patients with positive tumors and 29.2% in those with negative ones ($P = .175$) (Figure 3).

Sixty-three percent of cases were HR-HPV DNA positive/EGFR positive or HR-HPV DNA negative/EGFR negative; 29.4% HR-HPV DNA negative/EGFR positive; and 7.8% HR-HPV DNA positive/EGFR negative. The last group had a higher OS than the others, and patients with HR-HPV DNA–negative/EGFR-positive tumors had the worst survival (50% vs 38.8% at 5 years). However, the difference was not statistically significant ($P = .833$) (Figure 4).

DISCUSSION

HPV mainly infects skin and mucosas, and so far, approximately 100 subtypes have been identified, which are classified as low-risk (HPV-6 and HPV-11, associated with benign lesions) and high-risk (HPV-16 and HPV-18, known to be carcinogenic)[23] genotypes. Its viral genome contains 3 regions: (1) region E (early), constituted by 8 genes, E1-E8; (2) region L (late), composed of L1 and L2; and (3) the regulatory region. The oncoprotein E7 has the ability to link and form a high-affinity complex with several proteins, including the retinoblastoma protein, which activates E2 F transcription factors important in controlling the transition from the G1 to S phase of the

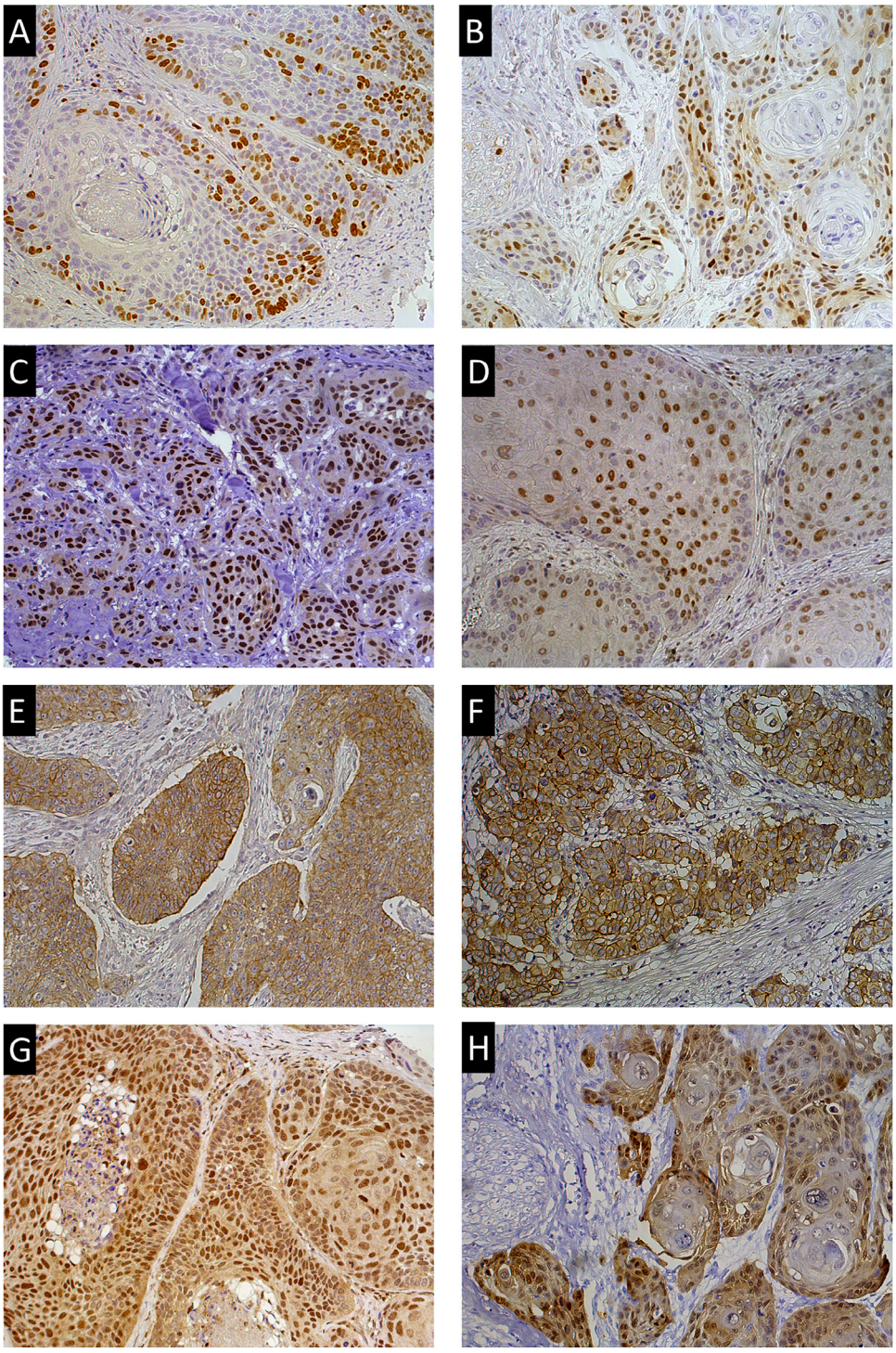

Fig. 2. Immunohistochemical staining patterns of the studied markers. Immunopositivity was detected in nuclei of tumour cells for (**A**) Ki 67, (**B**) Cyclin D1, (**C**) p53 and (**D**) p21, in the membrane and cytoplasm for (**E**) EGFR and (**F**) C-ErbB2 and in the nuclei and cytoplasm for (**G**) Myc and (**H**) p16.

cell cycle. Moreover, E6 can form a complex with or degrade the p53 tumor suppressor gene, responsible for cellular genomic integrity protection.[24] The final result of these interactions is a potential for suppression of cell cycle checkpoints, which favors DNA damage, cell immortalization, transformation of cell lines, inhibition of apoptosis, and ultimately carcinogenesis.[25]

HR-HPV is strongly associated with cervical,[8] anal,[9] and oropharyngeal[10] cancers; however, its role in oral cancer is still controversial,[11] probably because most of

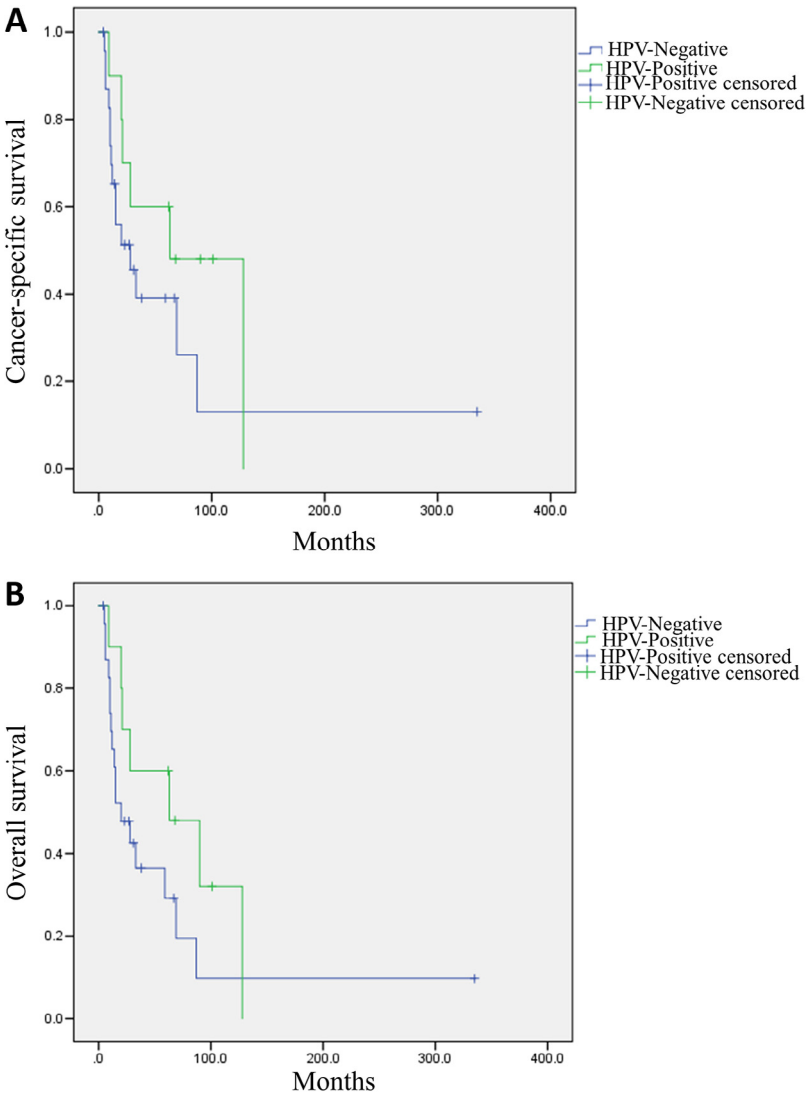

Fig. 3. Five-year-survival in HR-HPV DNA-positive and HR-HPV DNA-negative patients. (**A**) Cancer-specific survival and (**B**) overall survival.

the investigations have been using different methodologies to detect HPV in tumor samples from different anatomic sites of the head and neck region.[26] In 2003, The International Agency for Research on Cancer published a multicenter study that investigated the association of HPV in 1415 oral cancers. The PCR testing was performed on fresh biopsy specimens and antibodies against HPV16 L1, E6, and E7 proteins in blood, and the results indicated a small group of oral cancers that appeared to be related to HPV.[27] Lingen et al.[28] identified the etiologic fraction for HR-HPV (E6/7) in 5.9% of OSCC samples from 4 North American reference hospitals. Moreover, HR-HPV–positive tumors were significantly associated with male patients, early tumor stage, poor histopathologic tumor differentiation, and basaloid-like morphologic phenotype. Similar, Kaminagakura et al.[12] detected a low prevalence of HR-HPV DNA in OSCC (19.2%); nevertheless, when the analysis was carried out by age group, 68.2% of patients were younger than 40 years, suggesting the possibility of this virus being a contributing factor to carcinogenesis in younger patients.

Salazar et al.[29] reported an important difference in the presence of traditional risk factors between HPV-negative and positive OSCC, where the former had a higher incidence of tobacco consumption than the latter. In the present study, similar results were noted, where tobacco and alcohol consumption were associated with 90.9% and 81.8% of HR-HPV DNA–negative samples, respectively, whereas these habits were present in 66.7% and 33.3% of HR-HPV DNA-positive tumors. These findings suggest that HPV may be a tumor driver in OSCC of young patients without the traditional risk factors.

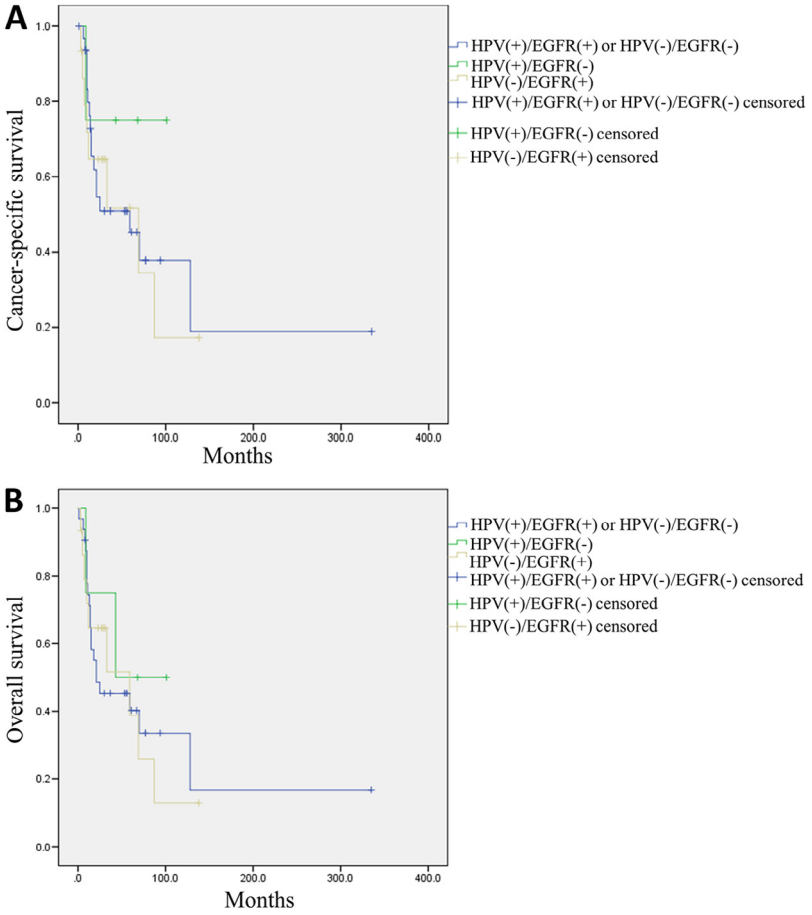

Fig. 4. Comparison of 5-year-survival according EGFR and HR-HPV DNA status. (**A**) Cancer-specific survival and (**B**) Overall survival.

A different anatomic site distribution was also identified between both HPV status groups; whereas the floor of the mouth was the most compromised localization in patients with HR-HPV DNA–negative tumors, the HR-HPV DNA–positive tumors affected the tongue more commonly. This variance can be explained by the anatomic proximity between the posterior two-thirds of the tongue and the oropharynx, where HPV has more affinity because of the juxtaposition between the epithelium and lymphoid tissue in the tonsils and the tonsillar crypt epithelium, which provides an exposed layer of basal epithelial cells, similar to the female genital tract.[30]

OSCC is a complex disease that develops in the combination of individual genetic predisposition, exposure to environmental carcinogens, and a reduction in function of the intrinsic factors for DNA protection, which in combination promote the malignant behavior of epithelial cells.[31] The genetic alterations involve dysregulation of the cell cycle, this being a fundamental hallmark of cancer progression.[32] Many molecules are involved in this process, beginning with membrane receptors. Members of the ErbB family are receptors for growth factors composed of 19 subclasses, including c-erbB-1 (EGFR) and c-erbB-2, which bind the epidermal growth factor (EGF) and other similar molecules to mediate cell growth, differentiation, and survival.[33]

Signals from the cell surface are trasmitted to intracellular targets by intracellular signal transducers such as the protein product of the oncogene H-ras: P21 WAF1/Cip1. p21 is regulated by p53 and perform their biological functions by binding to and inhibiting the activity of cyclin-dependent kinases (CDKs), in particular, functions of checkpoints in the cell cycle phases, being a regulator.[34]

Beyond the intracellular signal transducers, the transcription factor Myc has the ability to bind to promoter regions of DNA and initiate various functions that alter cell proliferation.[35] Once the cell cycle is activated, several proteins are involved in its regulation, and Ki67 is an important nuclear protein with functions in the organization of nucleolar chromatin and maintenance of the mitotic spindle.[36] Moreover, cyclin D1, the product of CCND1, regulates the cell cycle in response to extracellular mitogens. It is synthesized and accumulated in the nucleus

in the G1 phase of the cell cycle and dimerizes with CDK4 and CDK6, enabling progression to the S phase of the cell cycle.[37] These proto-oncogenes can mutate; increase gene expression, chromosomal translocations, or retroviral insertion; and turn into oncogenes.

Tumor suppressor proteins negatively modulate neoplastic transformation, inhibiting progression through cell cycle checkpoints, where they exhibit changes in DNA and others promote apoptosis.[32] The tumor suppressor protein p53 has the regulatory function of avoiding duplication of damage to DNA that regulates the cell cycle, DNA repair, and apoptosis, as well as inhibiting tumor formation.[38] Protein p16 is encoded by the gene CDKN2 A CDK, which acts by binding to CDK4/CDK6, preventing their interaction with cyclin D. The interaction CDK4/cyclin D6 forms a complex that phosphorylates the retinoblastoma protein, resulting in the release of E2 F and new transcription regulatory genes of the cell cycle. Protein p16 has an important role in cell cycle regulation, especially in preventing the progression from G1 to S phase.[39] Tumor suppressor proteins may suffer loss of function and become inducers of tumor agents that favor the development of tumors by blocking their inhibitory function,[40] as may occur in HPV infection. The block of retinoblastoma protein by the viral oncoprotein E7 leads to an increase of p16; hence, p16 immunoexpression has been correlated with HPV infection in tonsil carcinoma.[41]

The relation between p16 immunoexpression and HPV infection in OSCC is still controversial.[12,42] In the present investigation, all HPV-DNA–positive tumors were negative for the p16 antibody, sugessting that p16 immunoexpression is not indicative of HPV-DNA presence in OSCC. These findings are in contrast with results published by our group on the same sample,[12] where the association between HPV and p16 was verified, probably because of differences in the technique and methodology used to analyze the immunohistochemical reaction of p16 (different clone). Nevertheless, less than 50% of the HPV-positive OSCC samples were p16 positive.[12,42]

The study of these proteins failed to identify any difference in the biological capabilities acquired during the complex development of carcinogenesis in OSCC between HR-HPV DNA–positive and HR-HPV DNA–negative tumors. Once we know that these processes are similar, the next query is about the clinical behavior in HR-HPV DNA–positive tumors; several studies have reported better survival rates and responses to radiotherapy/chemotherapy in patients with head and neck cancers who are HR-HPV DNA positive compared with those who are HR-HPV DNA negative.[43] Our results indicated a trend toward the patients with HR-HPV DNA–positive tumors having better 5-year CSS and OS. An interesting finding was the analysis of survival by HPV status combined with EGFR expression, because patients who were HR-HPV DNA positive/EGFR negative had higher survival rates than those who were HR-HPV DNA negative/EGFR positive. This is in agreement with Kumar et al.,[44] who also reported HPV and EGFR to be good markers in response to organ-sparing therapy in advanced oropharyngeal cancer. However, in the present investigation, the differences were not statistically significant, probably because of the small number of analyzed cases.

The small number of samples analyzed was one of the limitations of this study, wherein larger sample sizes would probably lead to more robust results. However, this was a consequence of the rareness of the studied sample. In spite of this, our data are original in characterizing the impact of HPV tumor status on molecular pathways of oral carcinogenesis in young patients.

HR-HPV E6/7 mRNA expression was not investigated in our study because of tumor tissue unavailability, which limited the confirmation of our results by the detection of HPV oncogene transcription.

In summary, our results suggest that in OSCC no significant difference exists in the expression of cell cycle proteins between HR-HPV DNA-positive and negative tumors. Further studies are required to understand the prognostic value of HPV and EGFR and its effect as a therapy target.

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