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Human papillomavirus in carcinomas of the tongue: clinical and prognostic implications

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Abstract. It is not clear whether the presence of human papillomavirus (HPV) in squamous cell carcinomas of the tongue (SCCT) is of etiopathogenic and clinical significance. This study was designed to establish the incidence of HPV in SCCT and to determine the influence of HPV detection on clinical parameters and the prognosis. Clinical and histopathological data of 64 patients with SCCT were collected. Thirty benign lesions of the tongue were analyzed in parallel, in order to compare the HPV incidence and genotypes in these lesions with those of SCCT. Paraffin blocks of all cases were collected and PCR was carried out using SPF10 primers and the INNO-LiPA genotyping methodology. HPV was detected in 26.2% of the patients. Hybridization results showed that all patients except one had high-risk (HR)-HPV. HPV56 was the most common (42.1%), followed by HPV18 (26.3%), HPV16 (10.5%), HPV66 (10.5%), HPV39 (5.3%), and HPV51 (5.3%). The odds ratio of HR-HPV infection in cases vs. controls was statistically significant (9.45, 95% confidence interval 1.18–75.46). Among the results of the univariate analysis correlating the presence of HR-HPV with different clinical parameters, only mortality showed a statistically significant correlation, being higher in HR-HPV patients (odds ratio 3.97, 95% confidence interval 1.07–14.7).

Key words: oral cancer; tongue cancer; squamous cell carcinomas of the tongue; HPV; human papillomavirus; PCR; genotype; survival.

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Squamous cell carcinoma of the tongue (SCCT) is the most frequent oral cancer.[1,2] In approximately two-thirds of cases, the lesion is located in the oral tongue, ahead of the circumvallate papillae,[3] and in the other third the lesion is located at the base of the tongue (pharyngeal tongue).[4] Most cases of SCCT, around 55–70% of cases, occur at the lateral border of the tongue.[4] In addition, SCCT have the highest incidence of metastatic dissemination at the time of diagnosis amongst all oral squamous cell carcinomas (OSCC); approximately half of the cases have lymph node metastasis at the time of diagnosis.[4,5] Surgical excision of the tumour with wide margins, often combined with ipsilateral or bilateral regional lymphadenectomy, is the elective therapy for SCCT. This therapy is sometimes combined with adjuvant radiotherapy or adjuvant chemotherapy, or a combination of both. The 5-year survival of patients with SCCT varies between 15% and 70%, depending mainly on the

size of the tumour and on the presence of metastasis.[6]

Smoking and alcohol intake are known risk factors that influence the development of oral cancer.[6,7] During the last few years, human papillomavirus (HPV) has been detected in a variable proportion of OSCC, and HPV has been postulated to have an important etiopathogenic influence on carcinogenesis in some head and neck squamous cell carcinomas, especially in oropharyngeal carcinomas.[7–9]

HPVs are viruses belonging to the *Papillomaviridae* family, and have the capacity to infect basal cells of excoriated epidermal tissues. Once these cells have phagocytosed the viruses, they are transported to the nucleus,[10] where they are multiplied by episomal replication. In some cases the viral genome is integrated into the host genome, producing a cell-cycle deregulation that has carcinogenic potential.[11]

The HPV oncogenic potential has been demonstrated in anogenital carcinomas, especially in carcinomas of the uterine cervix, where the presence of HPV is considered to be necessary for malignant transformation.[8] The detection of HPV in OSCC observed in different meta-analyses varies between 23.5% and 46.5%.[12–14] The implication of HPV in the development of oropharyngeal carcinomas is compelling, particularly in carcinomas of the tonsil. However, evidence of the influence of HPV in the development of tongue carcinomas is far less conclusive, and it is not clear whether the presence of HPV in these tumours is of etiopathogenic and prognostic significance.[7,15–18]

The aggressive therapy that is required for the treatment of carcinomas of the tongue, and the poor prognosis for patients in whom these types of tumour present, have prompted the search for possible etiologic factors that will assist in the development of effective prevention strategies, or in the use of less aggressive and more effective therapies. The present study was designed to establish the incidence of HPV in SCCT using a sensitive method, and to determine the clinical influence that this may represent.

Materials and methods

Patients, tissue samples, and diagnosis

A retrospective search of all cases of SCCT diagnosed or treated in the oral and maxillofacial surgery department of the hospital from 2002 to 2010, identified 64 cases. Clinical data were obtained from the clinical histories of the patients held in the city tumour registry and, when necessary, by means of telephone or personal interview with the patient. Clinical data obtained were: age, gender, size and location of the lesions, alcohol intake and tobacco smoking habits, regional lymph node involvement, treatment procedures, recurrence and second primary tumour appearance, and mortality. Cancer staging was performed in accordance with the 2002 American Joint Committee on Cancer sixth edition staging criteria.[19]

To compare the incidence of HPV in carcinomas versus benign lesions of the tongue, samples of 30 benign lesions of the tongue diagnosed from 2002 to 2010 were selected. Glass slides, paraffin blocks, and histopathological reports were obtained for all the cases from the files of the hospital pathology department. In addition, 30 biopsy samples of the benign tongue lesions were selected. This retrospective study was approved by the hospital ethics committee.

Tissue preparation and nucleic acid isolation

Three 5-μm-thick sections from each paraffin block were placed in sterile Eppendorf tubes. To prevent possible cross-contamination between samples during the polymerase chain reaction (PCR) procedure, each microtome was cleaned with 70% ethanol before cutting the blocks, and each block was cut using a new disposable microtome blade. Tissue sections were deparaffinized using xylene, and washed with ethanol. The tissue sections were digested overnight with proteinase K in a volume of 250 μl at 56 °C. Proteinase K was heat-inactivated at 95 °C for 10 min. A 1/10 dilution of the sample was used for the PCR (10 μl).

HPV DNA PCR detection and genotyping

Broad-spectrum HPV DNA amplification was performed using the short PCR fragment (SPF10) primer set with the INNO-LiPA HPV genotyping technology of Innogenetics NV (Gent, Belgium). The SPF10 primers amplify a 65-bp fragment of the L1 region of the HPV genome. For HPV amplification, a 9-min denaturation step at 94 °C was followed by 40 cycles of amplification using 1.5 IU DNA polymerase (AmpliTaq Gold DNA Polymerase; Applied Biosystems, Foster City, CA, USA) in a thermocycler (GeneAmp 9700; Applied Biosystems). These cycles included denaturation at 94 °C for 30 s, primer annealing at 52 °C for 45 s, and chain elongation at 72 °C for 45 s. The final elongation step was prolonged to 7 min. To confirm DNA amplification, a second PCR using primers for the human β-globin gene was conducted (primers Hg063/064).[20] Amplification products were first tested for the presence of HPV DNA by DNA enzyme-immunoassay (DEIA), which consists of hybridization with conserved probes in a microtiter-plate assay format (Universal DNA ELISA Kit; Labo Bio-medical Products BV, Rijswijk, Netherlands). SPF10 amplimers from DEIA HPV-positive samples were subsequently analyzed by reverse hybridization in an HPV line-probe assay, LiPA25 system version 1 (Labo Bio-medical Products), at high stringency, generating a type-specific hybridization pattern. In this assay, 10 μl of denatured HPV PCR product was hybridized to the genotype-specific probes immobilized as parallel lines on a nitrocellulose strip. After the washing step, the products of hybridization were detected by a colour reaction with alkaline phosphatase–streptavidin conjugate and substrate (5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium), which results in a purple precipitate. The results of the hybridization were assessed visually by comparison to the standard grid. The HPV LiPA25 version 1 permits specific detection of 25 HPV types: HPV 6, 11, 16, 18, 31, 33, 34, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 66, 68/73, 70, and 74. These assays were performed automatically using the Auto-LiPA system (Innogenetics Diagnostica Iberia, Barcelona, Spain) for 48 strips.

Statistical analysis

Basic descriptive statistics methods were used to characterize the study patients, including the mean and standard deviation (SD) for continuous variables and the absolute frequency and percentage for discrete variables. On the basis of HPV detection, patients were divided into two groups: high-risk (HR)-HPV-positive and HR-HPV-negative; the variables observed in each group were compared. Before parametric tests were performed, the normal distribution of the continuous variables was ascertained by means of the Kolmogorov–Smirnov test. For the univariate analysis, the Mann–Whitney *U*-test was applied to compare continuous variables and Fisher's exact test was used for contrasting categorical variables. A *P*-value of less than 0.05 was considered to indicate a statistically significant difference. Finally, a multivariate, non-conditional logistic