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Prevalence of human papillomavirus in laryngeal and hypopharyngeal squamous cell carcinomas in northern Spain

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ABSTRACT

Background: Recent studies support a role for human papillomavirus (HPV) in oropharyngeal squamous cell carcinomas (SCCs); however, the significance of HPV in non-oropharyngeal head and neck cancers is uncertain. The aim of this study was to determine the prevalence of HPV in a large cohort of laryngeal and hypopharyngeal SCCs in northern Spain.

Materials and methods: Clinical records and paraffin-embedded tumor specimens of 124 consecutive patients surgically treated for laryngeal (62 cases) and hypopharyngeal (62 cases) SCCs between 2002 and 2007 were retrieved. All cases were histologically evaluated, and presence of HPV was assessed by p16-immunohistochemistry followed by GP5+/6+-PCR-based DNA detection. Samples positive in both assays were subjected to HPV genotyping and HPV E6 transcript analysis.

Results: Seventeen cases (14%) were positive for p16 immunostaining, of which 2 (1 larynx, 1 hypopharynx, 1.6% of total series) were found positive for HPV DNA by subsequent GP5+6+-PCR. Both SCCs contained HPV type 16 and showed HPV16 E6 mRNA expression.

Conclusions: HPV is only occasionally involved in laryngeal and hypopharyngeal SCC patients in northern Spain.

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1. Introduction

Most head and neck cancers are squamous cell carcinomas (SCCs), which are related with tobacco smoking and excessive alcohol consumption. Human papillomavirus (HPV) infection, particularly HPV type 16 (HPV16), has been acknowledged as an etiological factor in a subset of head and neck SCCs [1]. Head and neck SCCs caused by HPV mainly comprise oropharyngeal SCCs, such as tonsillar cancer, with unique clinical and pathological features and prognostic significance [2,3]. In tumors of other anatomical sites of the upper aerodigestive tract (oral cavity, larynx, hypopharynx), HPV presence has been reported in several studies, but controversy exists regarding its role in carcinogenesis. Thus, the clinical significance of HPV infection in non-oropharyngeal head and neck SCCs remains uncertain.

Although recently overshadowed by its involvement in oropharyngeal carcinogenesis, the classical location of HPV infection in the upper aerodigestive tract has been the larynx, with laryngeal papillomatosis caused by low-risk HPV types 6 and 11. The reported prevalence of HPV DNA in laryngeal carcinomas ranges widely (from 0% to 58%) being approximately 25% in meta-analysis [3–6]. Similarly, few studies have addressed the relationship of HPV with hypopharyngeal carcinomas, and also a wide variation (from 3% to 74%) in HPV prevalence has been reported in these cancers [3–5,7]. There are various possible reasons to explain this disparity, such as geographical differences, prevalence of smoking and drinking in the studied populations, anatomical subsite of the tumor, sample selection, time period of the study and/or distinct methods employed for HPV detection. The presence of HPV DNA in a tumor does not necessarily indicate that the virus is biologically active to drive and contribute to tumor development or progression. Viral DNA may well be a 'bystander' since HPV DNA

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can be detected in non-tumor and even normal tissue from the upper aerodigestive tract [5,8]. The most informative test for a biologically relevant association between HPV infection and cancer is the analysis of viral E6/E7 oncogene transcripts in tumor specimen, but this is technically challenging when using low-quality mRNA from archival formalin-fixed paraffin-embedded (FFPE) samples. Accordingly, Smeets et al. [9] proposed a test algorithm to assess HPV involvement using FFPE materials, comprising of p16 (INK4A, CDKN2A) immunohistochemistry (IHC) followed by HPV-DNA GP5+/6+ PCR on the p16-positive cases. This approach has recently been validated: comparison of mRNA E6/E7 RT-PCR and p16 IHC/HPV-DNA PCR on both frozen and FFPE tissue samples of 82 patients showed a concordance of 98% [12]. This test algorithm is based in the fact that all HPV-positive cases show overexpression of p16 protein. As the HPV E7 oncogene product inhibits the activity of retinoblastoma protein (pRb), p16 is upregulated *via* the loss of the negative feedback control of pRb expression. Thus p16 expression may serve as a surrogate marker of HPV related cellular transformation [1,9]. In contrast, p16 expression is usually absent in tobacco-related HNSCCs due to inactivation of the *p16*^{INK4A} gene, although some cases do not harbor this inactivation and could show p16 overexpression. In addition, p53 IHC was also included in this study since most HPV-related cases have an intact *TP53* gene and normally show negative p53 protein expression due to its rapid proteosome degradation, whereas *TP53* gene mutations (very common in tobacco-related HNSCCs) enable protein stabilization, accumulation, and p53 protein detection by IHC [1].

Here, we analyzed the possible association of HPV infection with laryngeal and hypopharyngeal SCC using FFPE specimen collected in a Spanish study population ($n = 124$) and the above mentioned test algorithm [9]. The cases that were positive in both assays were further subjected to genotyping of GP5+/6+ PCR products and HPV E6 transcript analysis.

2. Materials and methods

2.1. Patients and tissue specimens

Surgical tissue specimens from 124 consecutive patients with laryngeal (62 cases) or hypopharyngeal (62 cases) SCC who underwent surgical treatment at the Hospital Universitario Central de Asturias between 2002 and 2007 were retrospectively collected, following institutional review board guidelines. Informed consent was obtained from all patients. Representative tissue sections were obtained from archival, formalin-fixed and paraffin-embedded (FFPE) blocks and the histological diagnosis was confirmed by an experienced pathologist (MFF).

2.2. Tissue microarray (TMA) construction and DNA extraction

Five morphologically representative areas were selected from each individual tumor paraffin block: two for DNA isolation and three for the construction of a TMA. To avoid cross-contamination, two punches of 2 mm diameter were taken first, using a new, sterile punch (Kai Europe GmbH, Solingen, Germany) for every tissue block, and stored in Eppendorf tubes at room temperature prior to DNA extraction. Subsequently, three 1 mm cylinders were taken to construct TMA blocks, as described previously [10]. A total of 5 TMAs were created, containing three tissue cores of each of the 124 laryngeal and hypopharyngeal carcinomas. In addition, each TMA included three cores of normal epithelium (pharynx) as an internal negative control and three cores of a HPV-positive cervix carcinoma as a positive control (Fig. 1A).

Special care was taken to obtain high-quality DNA from the FFPE tissues by applying an elaborate extraction protocol [11], which includes thorough deparaffinization with xylene, methanol washings to remove all traces of the xylene, and a 24-h incubation in 1 mol/L sodium thiocyanate to reduce cross-links. Subsequently, the tissue pellet was digested for 3 days in lysis buffer with high doses of proteinase K (final concentration, 2 $\mu g/\mu L$, freshly added twice daily). Finally, DNA extraction was done by using the QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

2.3. Immunohistochemistry

The TMAs were cut into 3-μm sections and dried on Flex IHC microscope slides (DakoCytomation, Glostrup, Denmark). Immunohistochemistry was performed in all 124 samples using an automatic staining workstation (Dako Autostainer, Dako Cytomation, Glostrup, Denmark) with the Envision system and diaminobenzidine chromogen as substrate. The following primary antibodies were used: anti-p53 clone DO-7 (DAKO, Glostrup, Denmark), anti-p16 clone E6H4 (Roche mtm laboratories AG, Heidelberg, Germany).

P16 and p53 immunostainings were evaluated by two independent observers (MFF and JPR). P16 immunostaining was scored as negative (0), weak to moderate staining (1+: 10–75% of diffuse nuclear and cytoplasmic stained cells) or strong staining (2+: more than 75% diffuse nuclear and cytoplasmic stained cells). Scores ≥ 1 were considered as p16-positive expression (Fig. 1B). P53 immunostaining was evaluated as positive when $>10\%$ of the malignant cells showed nuclear staining (Fig. 1C).

2.4. HPV DNA detection and genotyping by GP5+/6+-PCR-EIA and luminex assay

Following the algorithm of Smeets et al. [9], only those cases showing positive p16-immunostaining were subjected to high-risk HPV DNA detection and genotyping as described previously [9,12]. In short, isolated DNA was subjected to GP5+/6+-PCR with an enzyme-immuno-assay (EIA) read-out for detection of 14 high-risk HPV types (*i.e.* HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68). Subsequent genotyping of EIA-positive cases was performed by bead-based array on the Luminex platform. Sample quality after DNA extraction was controlled by β-globin PCR [12].

2.5. Detection of HPV16 E6 mRNA

EIA-positive cases were subjected to detection of HPV16 E6 mRNA as previously described [9]. In short, RT-PCR with EIA read-out was performed to detect the most abundant splice variant within the HPV16 E6 open reading frame, namely E6*I. For each clinical sample, RT-PCR without reverse transcriptase and β-glucuronidase RT-PCR were performed as controls.

2.6. HPV DNA detection by *in situ* hibridization (ISH)

In an attempt to further confirm the results and avoid false-negative cases, ISH with biotinylated HPV DNA probes considered to react with HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68 (Y1443, DakoCytomation, Glostrup, Denmark) was performed on all 124 laryngeal and hypopharyngeal carcinomas using 3 μm formalin-fixed, paraffin-embedded tissue sections of the TMAs, according to the manufacturer's instructions. The results were evaluated by two independent observers (MFF and JPR). Focal DAB staining in the tumor nuclei indicated the presence of HPV.

3. Results

3.1. Patient characteristics

All patients had a single primary tumor and received no treatment prior to surgery. No patient had distant metastases at

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