Detection and Quantification of Human Papillomavirus in Benign and Malignant Parotid Lesions

GÉRALDINE DESCAMPS1*, ANAËLLE DURAY1*, ALEXANDRA RODRIGUEZ4, GILBERT CHANTRAIN4, CHRISTOPHE E. DEPUYDT2, PHILIPPE DELVENNE3 and SVEN SAUSSEZ1,4

1Laboratory of Anatomy, Faculty of Medicine and Pharmacy, University of Mons, Mons, Belgium;
2Laboratory for Clinical Pathology and Molecular Biology (LaboLokeren, Campus Riatol), Antwerp, Belgium;
3Department of Pathology, CHU Sart-Tilman, University of Liège, Liège, Belgium;
4Department of Oto-Rhino-Laryngology, CHU Saint-Pierre, Free University of Brussels, Brussels, Belgium

Abstract. Background/Aim: Human papillomavirus (HPV) is implicated in head and neck squamous cell carcinomas. However, the causal role of HPV in carcinoma of the parotid gland remains uncertain and less documented. This study aimed to determine the potential implication of HPV in the development of benign and malignant lesions of the parotid gland. Materials and Methods: Paraffin-embedded biopsies were obtained from 40 patients with benign parotid gland tumors and 39 patients with parotid gland carcinomas. The 79 samples were evaluated for the presence of HPV DNA using both GP5+/GP6+ consensus Polymerase Chain Reaction (PCR) and type-specific E6/E7 PCR to detect 18 HPV types. Results: Our results showed a low prevalence of HPV, with only three HPV-positive cases among the 40 benign tumors and one infected carcinoma in the malignant population. Conclusion: No association between the presence of HPV DNA and the development of parotid gland tumors was found in our study.

Since Syrjänen et al. first suggested that human papillomavirus (HPV) might be involved in oral carcinogenesis in 1983, studies have been dedicated to examining the presence of HPV infection in the upper aerodigestive tract (1). The presence of HPV is increasingly accepted as being an independent risk factor in the development of head and neck cancer. Indeed, over the last 10 years, it has been proven that a subset of oropharyngeal squamous cell carcinomas (OSCCs), including tonsil carcinomas, is associated with high-risk HPV infection. A case control study comparing 100 patients with OSCC and 200 controls demonstrated that oral HPV infection was strongly associated with oropharyngeal carcinoma among patients who did not have the classical risk factors of tobacco and alcohol use (2).

Although the implication of HPV in oral carcinogenesis is well documented, scant information is available regarding its relation to salivary gland tumors (SGTs). To our knowledge, only one study has analyzed the presence of HPV in SGTs (3). Nine parotid specimens, comprising seven tumors, one lymphoepithelial cyst and one lipoma, were analyzed using Polymerase Chain Reaction, quantitative PCR (qPCR) and in situ PCR. Their results showed that seven out of the nine lesions were infected by HPV16 and/or HPV18 oncogenic types, suggesting a possible involvement of the virus in the parotid lesions (3).

Disruptions in the pathway of retinoblastoma proteins (pRb) are frequently observed during tumorigenesis; however, studies dedicated to the expression of p16 have had conflicting results (4-6). The overexpression of p16 is widely reported as a consequence of HPV infection, suggesting that this protein is a powerful surrogate marker of HPV infection (7). On the other hand, many reports have shown that the loss of p16 is an early event in the development of human tumors, including head and neck squamous cell carcinomas (8-10).

Based on these results, we investigated the prevalence of HPV DNA in a series of 79 parotid tumors using General Primers (GP)5+/GP6+ consensus PCR and subsequent genotyping using E6/E7 type-specific PCR for 18 HPV types. In addition, we analyzed the immunohistochemical expression of p16 in the 39 malignant parotid tumors.

Materials and Methods

Histopathological and clinical data. A total of 79 tumor cases, comprising 40 patients with benign lesions (SGTs) and 39 patients...
with malignant lesions who underwent surgery aimed at curative tumor resection, were studied (see Table I for clinical data). Tumor specimens were obtained by a retrospective compilation from the records of the pathology departments of the Hôpital Erasme (M.R., Brussels, Belgium), the CHU Saint-Pierre-Institut Bordet (N.S., Brussels, Belgium) and the CHU Sart-Tilman (L.D., Liège, Belgium). The Institutional Review Boards of these hospitals approved the study. Hematoxylin- and eosin-stained sections of the 79 tumors were examined by two pathologists to confirm the diagnosis. Paraffin blocks that presented the highest proportion of tumor, avoiding the necrotic area frequently observed in the central part of the tumor, were selected for use in the studies.

**DNA extraction.** The formalin-fixed, paraffin-embedded tissue samples were sectioned (10×5 μm), deparaffinized, and digested with proteinase K overnight at 56°C. DNA was purified using the QIAamp DNA Mini Kit (Qiagen, Benelux, Belgium) according to the protocol recommended by the manufacturer.

**Detection of HPV using PCR amplification.** HPV was detected using PCR with GP5+/GP6+ primers (synthesized by Eurogentec, Liege, Belgium). The GP5+/GP6+ primers amplify a consensus region located within the L1 region of the HPV genome. PCR for HPV-L1 DNA amplification was performed in a 25-μl reaction mixture containing 2 μl of extracted DNA, 2.5 μl of 1×PCR buffer, 0.025 U of Taq DNA polymerase (Roche, Mannheim, Germany), 200 μM dNTPs and 0.5 pmol of each primer. The cycling conditions for the PCR were as follows: denaturation was performed at 94°C for 1 minute, annealing was performed at 55°C for 1 minute and 30 seconds, and extension was performed at 72°C for 2 minutes for a total of 45 amplification cycles. The first cycle was preceded by a 7-minute denaturation step at 94°C. The last cycle was followed by an additional 10-minute extension step at 72°C. Aliquots (10 μl) of each PCR product were separated via electrophoresis on a 1.8% agarose gel and stained with ethidium bromide to visualize the amplified HPV-L1 DNA fragments.

**Real-time quantitative PCR amplification of type-specific HPV DNA.** All DNA extracts were tested for the presence of 18 different HPV genotypes using TaqMan-based real-time quantitative PCR that targeted type-specific sequences of the following viral genes: 6 E6, 11 E6, 16 E7, 18 E7, 31 E6, 33 E6, 35 E6, 39 E7, 45 E7, 51 E6, 52 E7, 53 E6, 56 E7, 58 E6, 59 E7, 66 E6, 67 L1, and 68 E7. For the real-time quantitative PCR assays, the analytical sensitivity ranged from 1 to 100 copies and was calculated using standard curves for the 18 type-specific PCRs constructed with plasmids containing the entire genome of the various HPV types. Real-time quantitative PCR for the detection of β-globin was performed in each PCR assay to verify the quality of DNA in the samples and to measure the amount of DNA analysed (11). The following HPV types tested were considered high risk: 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, and 66.

**Immunohistochemistry.** All samples were fixed in 4% buffered formaldehyde for 24 h, dehydrated and embedded in paraffin. Immunohistochemistry was performed on 5-μm-thick sections mounted on silane-coated glass slides. Before starting the immunohistochemistry protocol, deparaffinized tissue sections were placed in a 0.01 M citrate buffer (pH 6.0) and briefly pre-treated in a microwave for 2×5 minutes at 900 W. The sections were then incubated with a solution of 0.06% hydrogen peroxide for 5 minutes to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS; 0.04 M Na₂HPO₄, 0.01 M KH₂PO₄ and 0.12 M NaCl, pH 7.4) and successively exposed to solutions containing avidin (0.1 mg/ml in PBS) and biotin (0.1 mg/ml in PBS) for 5 minutes periods to avoid false-positive staining reactions resulting from the presence of endogenous biotin. After a thorough washing with PBS, the sections were incubated for 15 minutes with a solution of 0.5% casein in PBS and sequentially exposed to solutions of the specific primary antibody (anti-p16; Abcam, Cambridge, UK) for 1 hour, the corresponding biotinylated secondary antibody (polyclonal goat anti-mouse IgG) for 30 minutes and the avidin-biotin-peroxidase complex (ABC kit; DakoCytomation, Glostrup, Denmark) for 45 minutes, all at room temperature. The samples were subjected to thorough washing steps to remove unbound proteins in between incubation steps. The antigen-dependent presence of the peroxidase complex in the sections was visualized following incubation with the chromogenic substrates containing diaminobenzidine and H₂O₂. After rinsing, the sections were counterstained with luxol fast blue and mounted in a synthetic medium. To exclude antigen-independent

Table I. Clinical data.

<table>
<thead>
<tr>
<th></th>
<th>Benign salivary gland tumors</th>
<th>Malignant salivary gland tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (F/M)</td>
<td>21/19</td>
<td>22/17</td>
</tr>
<tr>
<td>Age, years (range)</td>
<td>43.8 (15-76)</td>
<td>52.4 (19-88)</td>
</tr>
<tr>
<td>Localization</td>
<td>40 Parotid tumors</td>
<td>3 Submandibular specimens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 Oral cavity specimens</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29 Parotid specimens</td>
</tr>
<tr>
<td>Treatment</td>
<td>40 Superficial parotidectomies</td>
<td>23 Superficial parotidectomies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 Total parotidectomies</td>
</tr>
<tr>
<td></td>
<td></td>
<td>7 Neck dissections</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 Local (oral cavity or submandibular) resections</td>
</tr>
<tr>
<td>Histology</td>
<td>40 Pleomorphic adenomas</td>
<td>15 Adenoid cystic carcinomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 Mucoepidermoid carcinomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9 Carcinoma ex-pleomorphic adenomas</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6 Acinic cell carcinomas</td>
</tr>
</tbody>
</table>

**ANTICANCER RESEARCH J2: xxx-xxx (2012)**
staining, the incubation step with primary/secondary antibodies was omitted from the protocol in control samples. In all instances, these controls were negative. Assessment of p16 immunoreactivity was performed by two investigators who were blinded to the clinical details of the patients.

**Results**

**HPV status in benign parotid lesions.** From the 40 benign tumors analyzed, HPV was detected via PCR analysis with GP5+/GP6+ primers in three pleomorphic adenomas. Among these three cases, one was infected by the type 16 oncogenic HPV (viral load: <4 copies per cell) and two by a low-risk HPV. Two tumors were positive for the consensus PCR and negative for the qPCR and were thus considered to be infected with low-risk HPV types.

**HPV status in malignant parotid lesions.** Among the 39 malignant tumors analyzed, only one acinic cell carcinoma was positive via the qPCR. The HPV was also the high-risk HPV16 genotype. The remaining parotid tumors were negative for HPV infection.

**p16 expression as determined via immunohistochemistry.** Among the 39 cases with malignancy, a weak p16 signal was detected in six tumors (four adenoid cystic carcinomas and two carcinomas ex-pleomorphic adenoma) but none of the cases were HPV positive. In addition, the percentage of p16 positive epithelial cells was less than 10%. The only HPV positive case was also p16 negative.

**Discussion**

The potential association of HPV with head and neck cancer is now well documented. However, only one study has examined the role of HPV in parotid tumors; therefore, conclusions were difficult to draw. Nevertheless, questions remain regarding the transmission route of HPV to the parotid gland. Although sexual transmission is the main cause of HPV infection in head and neck cancer, viremia could be proposed as the best hypothesis to explain the entry of HPV into the parotid. Indeed, many studies have reported HPV in these organs except the heart and the kidney. Indeed, all p16-positive parotid tumors were negative for HPV; these results could illustrate the typical tobacco-related loss of p16. p16 inactivation has been demonstrated as an early and frequent event in oral carcinogenesis.

In conclusion, our data do not support the existence of a prominent role for HPV infection in parotid carcinogenesis.

**Acknowledgments**

GD and AD are Ph.D. students supported by a grant from the FNRS (Bourse Télévie).

**References**


