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

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Abstract. *Background/Aim: Human papillomavirus (HPV) is implicated in head and neck squamous cell carcinomas. However, the causal role of HPV in carcinomas 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 from 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 of 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 shown 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+/(GP)6+ 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 of 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).

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Table I. *Clinical data*.

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

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-Tilmant (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 tumors, avoiding the necrotic area frequently observed in the central part of the tumor, were selected for analysis in the studies.

DNA extraction. The formalin-fixed, paraffin-embedded tissue samples were sectioned (10×5 μm), de-paraffinized, 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 min, annealing was performed at 55˚C for 1 min and 30 s, and extension was performed at 72˚C for 2 min 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-min 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 analyzed DNA (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, de-paraffinized tissue sections were placed in a 0.01-M citrate buffer (pH 6.0) and briefly pre-treated in a microwave for 2×5 min at 900 W. The sections were then incubated with a solution of 0.06% hydrogen peroxide for 5 min to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (PBS; 0.04 M Na_2HPO_4, 0.01 M KH_2PO_4 and 0.12 M NaCl, pH 7.4) and were successively exposed to solutions containing avidin (0.1 mg/ml in PBS) and biotin (0.1 mg/ml in PBS) for 5-min 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 min 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 h, the corresponding biotinylated secondary antibody (polyclonal goat anti-mouse IgG) for 30 min and the avidin-biotin-peroxidase complex (ABC kit; DakoCytomation, Glostrup, Denmark) for 45 min, 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 H_2O_2. After rinsing, the sections were counterstained with luxol fast blue and mounted in a synthetic medium. To exclude antigen-

independent staining, the incubation step with primary/secondary antibodies was omitted from the protocol in the 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* qPCR. The HPV was also of 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 are 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 plasma viremia in women with HPV16 or 18 in their cervical tissue (12). The detection rates of HPV viremia vary from one study to another according to the differences in study populations, sample types and primers and probes used. HPV was also detected in a variety of other tumor types, with percentages up to 97% for colon cancer, 80% for lung cancer and 74% for breast cancer (13). In fact, the virus has been found in all organs except the heart and the kidney. In most cases, viremia was the argument of choice to explain the presence of HPV in these organs. Hennig *et al*. and Tseng *et al*. proposed that HPV can be transported through the bloodstream to the breast and the lung (14-15). As suggested by Gnanamony *et al*., the presence of HPV in the bloodstream could be interpreted as the presence of disseminated tumor cells in the circulation (12).

The *p16* tumor suppressor gene is often up-regulated in HPV-induced cancer because it is negatively-regulated by pRb. The overexpression of p16 is frequently reported, qualifying this protein as a representative marker of HPV infection (4). While many studies found a correlation between p16 overexpression and high-risk HPV infection in head and neck cancer, this association was not confirmed in our study. 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 (8). In 2010, p16 overexpression was suggested as an effective surrogate marker of HPV infection, oropharyngeal origin and better prognosis (4-7). These contradictory results reflect the fact that more studies are needed before considering p16 as a reliable marker.

In the current study, we investigated the presence of HPV genotypes in 79 parotid lesions with a type-specific PCR and a subsequent qPCR. We found that only four tumors were infected by two high-risk HPV types and two low-risk HPV types. In conclusion, our data do not support the existence of a prominent role for HPV infection in parotid carcinogenesis.

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References

1 Syrjänen K, Syrjänen S, Lamberg M, Pyrhonen S and Nuutinen J: Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. Int J Oral Maxillofac Surg *12*: 418-424, 1983.
2 D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH and Gillison ML: Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med *356*: 1944-1956, 2007.
3 Vageli D, Sourvinos G, Ioannou M, Koukoulis GK and Spandidos DA: High-risk human papillomavirus (HPV) in parotid lesions. Int J Biol Markers *22*: 239-244, 2007.
4 Weinberger PM, Yu Z, Haffty BG, Kowalski D, Harigopal M, Brandsma J, Sasaki C, Joe J, Camp RL, Rimm DL and Psyrri A: Molecular classification identifies a subset of human papillomavirus associated oropharyngeal cancers with favorable prognosis. J Clin Oncol *24*: 736-747, 2006.
5 Duray A, Descamps G, Arafa M, Decaestecker C, Remmelink M, Sirtaine N, Ernoux-Neufcoeur P, Mutijima E, Somja J, Depuydt CE, Delvenne P and Saussez S: High incidence of high-risk HPV in benign and malignant lesions of the larynx. Int J Oncol *39*: 51-59, 2011.

6 Duray A, Descamps G, Decaestecker C, Remmelink M, Sirtaine N, Lechien J, Ernoux-Neufcoeur P, Bletard N, Somja J, Depuydt CE, Delvenne P and Saussez S: Human papillomavirus DNA strongly correlates with a poorer prognosis in oral cavity carcinoma. Laryngoscope *122*: 1558-1565, 2012.
7 Smith EM, Rubenstein LM, Hoffman H, Haugen TH and Turek LP. Human papillomavirus, p16 and p53 expression associated with survival of head and neck cancer. Infect Agent Cancer *5*: 4, 2010.
8 Reed AL, Califano J, Cairns P, Westra WH, Jones RM, Koch W, Ahrendt S, Eby Y, Sewell D, Nawroz H, Bartek J and Sidransky D: High frequency of p16 (CDKN2/MTS-1/INK4A) inactivation in head and neck squamous cell carcinoma. Cancer Res *56*: 3630-3633, 1996.
9 Weber A, Langhanki L, Schütz A, Wittekind C, Bootz F and Tannapfel A. Alterations of the INK4A-ARF gene locus in pleomorphic adenoma of the parotid gland. J Pathol *198*: 326-334, 2002.
10 Ernoux-Neufcoeur P, Arafa M, Decaestecker C, Duray A, Remmelink M, Leroy X, Herfs M, Somja J, Depuydt CE, Delvenne P and Saussez S: Combined analysis of HPV DNA, p16, p21 and p53 to predict prognosis in patients with stage IV hypopharyngeal carcinoma. J Cancer Res Clin Oncol *137*: 173-181, 2011.
11 Depuydt CE, Boulet GA, Horvath CA, Benoy IH, Vereecken AJ and Bogers JJ: Comparison of MY09/11 consensus PCR and type-specific PCRs in the detection of oncogenic HPV types. J Cell Mol Med *11*: 881-91, 2007.
12 Gnanamony M, Peedicayil A, Subhashini J, Ram TS, Rajasekar A, Gravitt P and Abraham P: Detection and quantitation of HPV 16 and 18 in plasma of Indian women with cervical cancer. Gynecol Oncol *116*: 447-51, 2010.
13 Petersen I and Klein F: HPV in non-gynecological tumors. Pathologe *29*: 118-122, 2008.
14 Hennig EM, Suo Z, Thoresen S, Holm R, Kvinnsland S and Nesland JM: Human papillomavirus 16 in breast cancer of women treated for high-grade cervical intraepithelial neoplasia (CIN III). Breast Cancer Res Treat *53*: 121-135, 1999.
15 Tseng CJ, Pao CC, Lin JD, Soong YK, Hong JH and Hsueh S: Detection of human papillomavirus types 16 and 18 mRNA in peripheral blood of advanced cervical cancer patients and its association with prognosis. J Clin Oncol *17*: 1391-1396, 1999.

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