

Human papillomaviruses are not involved in the etiopathogenesis of salivary gland tumors

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SUMMARY

Background. Tumor-related high risk human papillomaviruses (HPV) 16 and 18 have been repeatedly detected in head and neck cancers, particularly, they are well known risk factors in squamous cell carcinoma of the oropharyngeal and tonsillar region. Little is known, however, about the possible role of HPV in salivary gland tumors.

Methods. Fifty-five cases of benign and malignant salivary gland tumors were tested using p16 immunohistochemistry followed by HPV DNA polymerase chain reaction (PCR) using SPF, CPSGB, GP5+/GP6+ primers, and type specific primers for HPV 16, 18, 31, 33, 35, 45 in the cases with strong immunohistochemical expression for p16 protein (score 3+).

Results. Only 5 tumors of 55 (9 %) were completely devoid of any p16 staining, and in 10 cases (18 %), less than 25 % of tumor cells stained (score 1+). In the majority of cases (35 of 55; 64 %) there was a patchy nuclear and cytoplasmic strong staining in 26 to 50 % of tumor cells (score 2+). In five cases (9 %), strong nuclear and cytoplasmic staining in more than 51% of tumor cells was detected (score 3+). However, none of the p16-positive cases showed any evidence of high-risk HPV by PCR.

Conclusions. The results of the study indicate that HPV, in particular oncogenic types 16 and 18, are not involved in the etiology of benign and malignant epithelial tumors of salivary glands. Therefore, it is likely that salivary gland tumors belong to the category of tissues in which the p16 positive immunohistochemistry is not biologically relevant to the oncogenic role of HPV infection.

Keywords: salivary gland tumor – HPV – human papilloma virus – p16 immunohistochemistry – PCR

Lidské papilomaviry se neúčastní v etiopatogeneze nádorů slinných žláz

SOUHRN

Onkogenní typy 16 a 18 lidských papilomavirů (HPV z angl. Human Papillomavirus) byly opakovaně prokázány v části dlaždicobuněčných karcinomů v oblasti hlavy a krku. HPV tak představuje prokázaný rizikový faktor v etiopatogenezi částí dlaždicových karcinomů orofaryngeální a tonsilární oblasti. Velmi málo je naopak známo o možné roli HPV v nádorech slinných žláz.

Metody. Na vlastním souboru 55 pacientů s benigními a maligními nádory slinných žláz prezentujeme pokus o detekci biologicky relevantní HPV infekce při využití algoritmu kombinujícího imunohistochemický průkaz proteinu p16 proteinu a molekulárně genetický průkaz HPV DNA pomocí polymerázových řetězových reakcí (PCR) za použití SPF, CPSGB, GP5+/GP6+ primerů, a typově specifických primerů pro detekci HPV 16, 18, 31, 33, 35 a 45.

Výsledky. Ve skupině 55 tumorů slinných žláz, pouze 5 případů (9 %) bylo zcela negativních na průkaz p16 proteinu a v 10 nádorech se barvilo méně než 25 % nádorových buněk (skóre 1+). Ve většině případů (35/55; 64 %) jsme prokázali silnou jaderně-cytoplasmatickou pozitivitu p16 proteinu v 26 – 50 % nádorových buněk (skóre 2+). V 5 případech (9 %), více než 51 % nádorových buněk expri-movalo p16 protein silně (skóre 3+). Žádný z těchto p16-protein silně pozitivních případů však nevykazoval přítomnost vysoce rizikových onkogenních HPV pomocí PCR.

Závěry. Výsledky naší studie ukazují, že HPV 16 a 18 nejsou etiologicky asociovány s nádory slinných žláz. Zdá se proto, že exprese p16 proteinu v salivárních nádorech není diagnosticky užitečným zástupným markerem biologicky relevantní transformující infekce HPV.

Klíčová slova: nádor slinných žláz – HPV – lidský papilomavirus – p16 protein – PCR

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Oncogenic human papillomavirus (HPV), a well-known causative agent in cervical cancer, has been repeatedly detected also in head and neck cancers, especially in squamous cell carcinoma (SCC) of

the tonsils (1). The association of high risk HPV 16 and 18 with oropharyngeal cancer is well documented and the HPV-related oropharyngeal and oral SCC is increasingly recognized as a distinct entity among head and neck cancers for its favorable clinical outcome (2). In addition, the occurrence of HPV can also cause a number of benign lesions in the oral cavity, pharynx and larynx. However, little is known about the possible link of HPV with salivary gland tumors. The involvement of HPV 16 and 18 in salivary gland tumors was described in only two papers and one abstract so far, with controversial results (3-5). While Atula et al. (3,4) did not detect the presence of HPV in any of 38 benign and malignant salivary gland tumors, Vageli et al. (5) have reported the presence of HPV infection in 7 of 9 cases of parotid gland tumors using PCR

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with the GP5+/GP6+ primers and confirmed the results by PCR with specific primers for each type of HPV. In particular, high viral loads of high risk HPV 16 and 18 were found in the oncocytoma, pleomorphic adenoma, and Warthin tumor according to these authors (5). Therefore, they have concluded that high-risk HPV may be involved in salivary gland tumor oncogenesis (5).

In the present study, we investigated the possible involvement of tumor-related HPV by means of p16 immunohistochemistry on 55 samples of benign and malignant epithelial salivary gland tumors of different types, followed by polymerase chain reaction (PCR) for HPV DNA in p16 positive cases.

MATERIALS AND METHODS

Histochemical and immunohistochemical study

For histological and immunohistochemical studies, paraffin blocks and re-cuts were available in 55 cases of epithelial benign and malignant tumors of minor and major salivary glands. The tumors were categorized according to the 2005 WHO classification (6). All non-epithelial tumors were excluded. Benign tumors comprised of pleomorphic adenoma (14x), Warthin tumor (9x), myoepithelioma (4x), and oncocytoma, cystadenoma and basal cell adenoma in one each case. Malignant salivary gland tumors included polymorphous low grade adenocarcinoma PLGA (4x), cribriform adenocarcinoma of tongue (CAT) (7x), adenoid cystic carcinoma of minor glands (6x), salivary duct carcinoma (2x), mucoepidermoid carcinoma (2x), acinic cell carcinoma (2x), and hyalinizing clear cell carcinoma and carcinosarcoma ex pleomorphic adenoma in one each case.

For conventional microscopy, the excised tissues were fixed in formalin, routinely processed, embedded in paraffin, cut, and stained with hematoxylin-eosin.

Immunohistochemistry was carried out for p16 protein on 4- μ m-thick sections cut from formalin-fixed, paraffin embedded tissue blocks using a monoclonal antibody to p16 CINtec Histology V-kit on a Ventana BenchMark ULTRA automated immunostainer (Ventana, mtm Laboratories AG, Germany) according to standard protocol. Detection involved Ventana's ultraView Universal DAB Detection Kit that uses a cocktail of enzyme-labeled secondary antibodies that locate bound primary antibodies. The complex was then visualized with hydrogen peroxide substrate and 3-3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen. No biotin was involved. Antigen retrieval, standard on the machine, used the Ventana CC1, EDTA-Tris, pH 8.0 solution. The slides were counterstained with Mayer's hematoxylin. A known p16 expressing head and neck squamous cell carcinoma case was used as a positive control, and sections of normal tonsil were used for negative controls with each run. Staining was nuclear and cytoplasmic, and was graded for its extent as follows: 0 = negative; 1+ = 1 to 25 % of cells positive; 2+ = 26 to 50 % cells positive; 3+ = more than 51 % cells positive.

Molecular genetic study

DNA for molecular genetic investigation was extracted from formalin-fixed, paraffin-embedded tissues by the NucleoSpin[®] Tissue Kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. Moreover, special precautions were taken to prevent HPV DNA microcontamination.

Detection of HPV

The HPV DNA detection was performed using a set of several PCRs with different primers to cover a wide detection range of predominantly HR/LR HPV types. The following primer systems were used: SPF, CPSGB (7), GP5+/GP6+ (8,9), and type specific primers for HPV 16, 18, 31, 33, 35, 45 (10,11).

An assay with SPF primers was run according to the manufacturer's protocol of a commercial product INNO-LiPA HPV Genotyping kit Extra (Innogenetic NV, Belgium).

PCR using CPSGB primers was performed according to the original article with some modification, concretely, the reaction mix for PCR reaction in a total volume of 25 μ l consisted of 12.5 μ l of HotStar Taq MM (Qiagen, Hilden, Germany), 0.5 μ l of each primer (25 μ M), 100-200 ng of DNA template, and PCR water. The amplification program consisted of 15 minutes of denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 1,5 minute and extension at 72°C for 2 minutes. The last cycle was extended by a 7-minute elongation at 72°C.

PCR using GP5+/GP6+ was run in modification according to van den Brule et al. (9) with modified ramping times between the temperatures used for denaturation, annealing, and elongation. Briefly, reagents in a total volume of 25 μ l were mixed as follows: 12.5 Fast Start Taq MM (Roche Diagnostics, Mannheim, Germany), 1 μ l of each primer (25 μ M), 1.5 μ l of MgCl₂ (25mM), 100-200 ng of DNA template, and PCR water. The amplification program consisted of denaturation at 94°C for 10 minutes, followed by 45 cycles of denaturation at 94°C for 20 seconds (\rightarrow 33 % of standard cool-down temperature), annealing at 38°C for 30 seconds (\rightarrow 44 % of standard warm-up temperature) and extension at 71°C for 80 seconds (\rightarrow 62 % of standard warm-up temperature). The last cycle was extended by a 7-minute elongation at 72°C.

To avoid false negative findings (because of loss of L1 or E1 region due to the process of HPV integration into the host genome) PCR in multiplex format targeting HPV oncogenes E6, and E7 of 6 most prevalent HR-HPV types, namely type 16, 18, 31, 33, 35, 45 was performed. PCR conditions were as follows: reaction mix in a total volume of 25 μ l consists of 12.5 Fast Start Taq MM (Roche Diagnostics, Mannheim, Germany), 1 μ l of each primer (10 μ M), 100-200 ng of DNA template, and PCR water. The amplification program consisted of 10 minutes of denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds and extension at 72°C for 1 minute. The last cycle was extended by a 7-minute elongation at 72°C.

All PCR were run on the cyclor GeneAmp PCR System 9700 (PE Applied Biosystem, Forster City, CA). Amplicons were analyzed in 2% agarose gel with ethidiumbromide. Positive and negative controls were included in every single run.

RESULTS

Only 5 tumors of 55 (9 %) were completely devoid of any p16 staining, and in 10 cases (18 %) less than 25 % of tumor cells were stained (score 1+). In the majority of cases (35 of 55; 64%) there was a patchy nuclear and cytoplasmic strong staining in 26 to 50 % of tumor cells (score 2+) (Fig. 1A, 1B). In five cases (9 %), strong nuclear and cytoplasmic staining in more than 51% of tumor cells was detected (score 3+) (Fig. 2A– 2D). However, none of the p16-positive salivary gland tumor cases showed any evidence of high-risk HPV by PCR (Fig. 3).

DISCUSSION

At present, the causal role of HPV in cervical cancer has been well established. High-risk HPV (mostly 16 and 18) are identified almost in all squamous cell carcinomas (SCC) of the uterine cervix (11). Moreover, recently several papers showed that HPV is also involved in the pathogenesis of a subset of squamous cell carcinomas (SCC) of the head and neck (1,2,12) leading to dis-

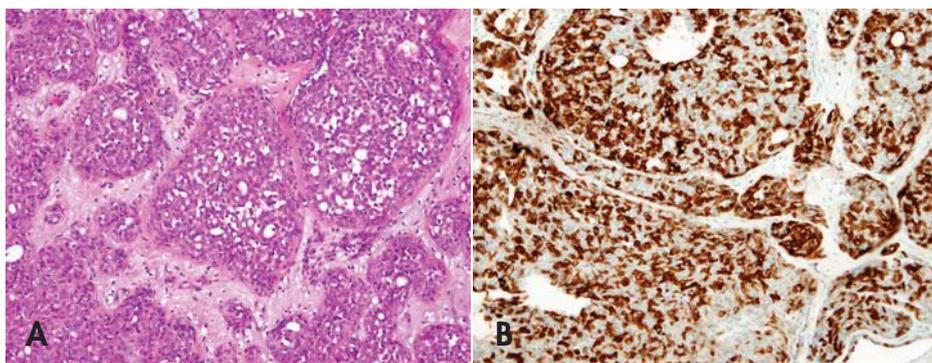


Fig. 1. Cribriform adenocarcinoma of tongue (CAT). A: Hematoxylin and eosin (HE), 200x. B: Positive p16 immunohistochemical staining with patchy pattern (score 2+), 200x.

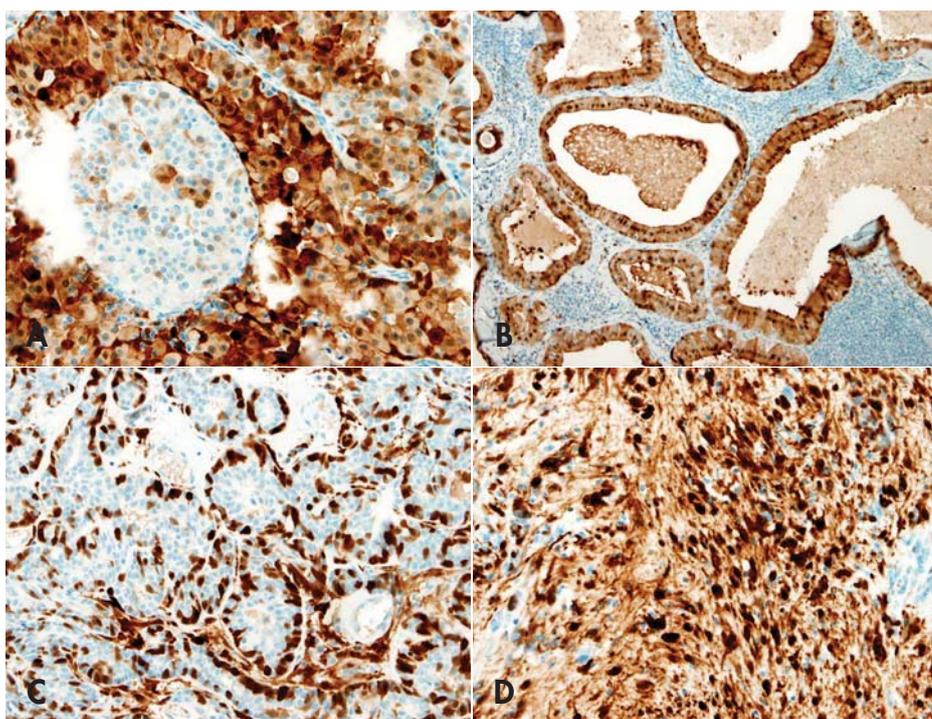


Fig. 2. p16 immunostaining in a variety of salivary gland tumors. A: Diffuse strong staining in oncocytoma of parotid gland (score 2+). Immunohistochemistry, 400x. B: Diffuse p16 staining in epithelial cell of benign Warthin tumor (score 3+). Immunohistochemistry, 200x. C: Strong patchy nuclear and cytoplasmic staining limited to abluminal cells of basal cell adenoma (score 2+). Immunohistochemistry, 200x. D: Diffuse staining in cellular pleomorphic adenoma of tongue (score 3+). Immunohistochemistry, 200x.

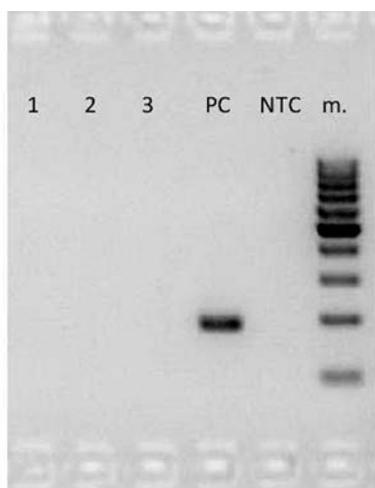


Fig. 3. Polymerase chain reaction (PCR). No positivity on agarose gel in any of three salivary gland tumor samples (Lanes 1 to 3). PC means positive control (HPV 18) and NTC is non template control.

tinct molecular, morphological and clinical features different if compared with HPV-negative SCCs. HPV-associated SCCs of the head and neck tend to show a better prognosis, different response to therapy, and are more likely to affect younger patients. Therefore, the correct assessment of HPV status of head and neck cancers is clinically relevant. Despite the importance of precise detection of the HPV status of head and neck cancers, no consensus has been reached so far on the optimal way to identify HPV-associated tumors (biologically and clinically relevant oncogenic HPV infection). The methods include immunohistochemistry with p16 antibody, HPV polymerase chain reaction (PCR) testing, and HPV in situ hybridization (ISH) (13).

Very little is known about the role of HPV in salivary gland tumors. The involvement of HPV 16 and 18 in salivary gland tumors was described in only few papers so far, with controversial results (3–5). In particular, Vageli et al. (5) have reported the presence of HPV infection in 7 of 9 cases of parotid gland tumors using PCR, and concluded that high-risk HPV may be involved in salivary gland tumor oncogenesis (5). More recently, we have studied a series of 33 lymphadenomas of salivary glands and none of the 11 cases analyzed for high risk HPV using in situ hybridization was positive (14). In our recent study of 55 cases of benign and malignant tumors of both major and minor salivary glands, p16 immunostaining was carried out in order to examine a possible role of high-risk HPV 16 and 18 in etiology of salivary gland tumors. In con-

trast to Vageli et al. (5), we failed to demonstrate biologically relevant HPV infection in salivary gland tumors using PCR testing despite p16 overexpression in most cases.

Several authors emphasized earlier, that a hallmark of the presence of HPV in cancer could be found in nuclear and cytoplasmic strong overexpression of the p16 protein, so that p16 could be considered a useful surrogate marker for HPV infection (2,13). However, p16 is overexpressed in a subset of tumors apparently lacking evidence of the presence of HPV DNA (2). The mechanism of p16 protein overexpression in salivary gland tumors has not been clearly defined. There are only few studies that have looked at this and they produced conflicting results (15,16). Protein p16 is a tumor suppressor protein that inhibits cyclin-dependent kinase 4A. In tumors with biologically active HPV, overexpression of p16 arises as a consequence of pRb inactivation by the HPV E7 oncoprotein. A strong and diffuse immunostaining pattern for p16 is therefore considered as a highly sensitive surrogate marker of HPV-associated tumors. It is, however, not always so, as other pathways may also lead to p16 overexpression. In our

series, we demonstrated overexpression of p16 protein immunohistochemically in the majority of the studied tumors (range 15 - 90 % of tumor cells positive). However, if detection of DNA of a wide spectrum of high risk/low risk HPV types was performed, all the cases were negative by PCR.

The significance of detection of HPV in some tumors seems to be controversial, as a p16 and HPV positivity has been reported in different lesions but the role of HPV in their etiopathogenesis remains elusive. According to recent studies, the most appropriate way to detect a biologically relevant association between HPV infection and the development of head and neck tumors is the measurement of E6/E7 HPV expression (17).

In conclusion, the results of the present study indicate that HPV, in particular oncogenic type 16 and 18, are not involved in the etiology of benign and malignant epithelial tumors of salivary glands. Moreover, it seems likely that p16 positive immunohistochemistry in salivary gland tumors does not represent a marker of an oncogenic transforming role of HPV infection.

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