ORIGINAL ARTICLE

Expression of cyclin-dependent kinase inhibitor 2A 16, tumour protein 53 and epidermal growth factor receptor in salivary gland carcinomas is not associated with oncogenic virus infection

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It is known that human papillomavirus (HPV) infection can cause squamous cell neoplasms at several sites, such as cervix uteri carcinoma and oral squamous carcinoma. There is little information on the expression of HPV and its predictive markers in tumours of the major and minor salivary glands of the head and neck. We therefore assessed oral salivary gland neoplasms to identify associations between HPV and infection-related epidermal growth factor receptor (EGFR), cyclin-dependent kinase inhibitor 2A (CDKN2A/p16) and tumour protein p53 (TP53). Formalin-fixed, paraffin-embedded tissue samples from oral salivary gland carcinomas ($n=51$) and benign tumours ($n=26$) were analysed by polymerase chain reaction (PCR) analysis for several HPV species, including high-risk types 16 and 18. Evaluation of EGFR, CDKN2A, TP53 and cytomegalovirus (CMV) was performed by immunohistochemistry. Epstein–Barr virus (EBV) was evaluated by EBV-encoded RNA *in situ* hybridisation. We demonstrated that salivary gland tumours are not associated with HPV infection. The expression of EGFR, CDKN2A and TP53 may be associated with tumour pathology but is not induced by HPV. CMV and EBV were not detectable. In contrast to oral squamous cell carcinomas, HPV, CMV and EBV infections are not associated with malignant or benign neoplastic lesions of the salivary glands.

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INTRODUCTION

Several viruses are associated with neoplastic diseases of the head and neck, particularly human papillomavirus (HPV), human herpesvirus-4/Epstein–Barr virus (EBV) and human herpesvirus-5/cytomegalovirus (CMV). HPV is frequently found in squamous cell carcinomas,^{1–3} while EBV is detectable in nasopharyngeal carcinomas, lymphomas and post-transplant lymphoproliferative disease.^{4–5} CMV, which is associated with sialadenitis, has recently been shown to be detectable in mucoepidermoid carcinomas.^{6–7}

HPV consists of several subtypes, which are classified according to their carcinogenic potential, including low-risk HPV (e.g., types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72) and high-risk HPV (types 16, 18, 31, 26, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82).^{1–2} After cellular infection, HPV manipulates the intracellular signalling associated with cell cycle regulation and differentiation via several viral proteins (e.g., E5, E6 and E7). These proteins interact with host cell factors such as cyclin-dependent kinase inhibitor 2A (CDKN2A/p16), tumour protein p53 (TP53/p53) and epidermal growth factor receptor (EGFR). Stimulation of EGFR by extracellular cytokines or by intracellular HPV-derived protein E5 induces activation of intracellular signalling cascades that lead to proliferation. Cyclin-dependent

kinase 4 (CDK4) is a component of the protein kinase complex that regulates the G₁–S cell cycle phase. CDK4 inactivates retinoblastoma 1 (RB1) by phosphorylation, preventing the active (hypophosphorylated) RB1 protein from binding to E2F transcription factor 1 (E2F1). CDKN2A inhibits CDK4 and thereby regulates RB1–E2F1 binding. HPV protein E7 interferes with this regulatory cascade by binding to RB1 (thus, E2F1 can no longer bind to RB1). In addition, E7 binds to the E2F1–DNA complex and induces RB1-independent gene transcription. The binding of E7 to RB1 results in a compensatory increase in CDKN2A expression, but this accumulation of CDK4-inhibiting protein is ineffective due to the presence of E7. CDKN2A also protects transcription factor TP53 from degradation, but HPV protein E6 counteracts this function by binding to TP53 and inducing increased degradation of the protein. Through these mechanisms, viral proteins mediate uncontrolled activation of cell division and inhibition of apoptosis, which can ultimately result in neoplastic transformation of the infected cell population.^{1–2}

It has been reported that HPV can be detected in benign salivary gland tumours and adenocarcinomas.^{8–12} CDKN2A expression has been observed in salivary gland tumours without detection of HPV.^{13–14} We hypothesised that screening a large number of HPV

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types by a sensitive and specific polymerase chain reaction (PCR) assay may reveal an association between viral infection and CDKN2A, TP53 and EGFR expression in salivary gland tumours.

MATERIALS AND METHODS

Patient cohort and tumour samples

We selected tissue samples from salivary gland neoplasms ($n=77$) and non-neoplastic salivary gland tissues ($n=65$). Non-neoplastic controls consisted of 58 normal glands in the tumour margins and 7 unrelated cases without adenocarcinomas. Tumour types were as follows: adenoid cystic carcinomas ($n=20$), adenocarcinomas not otherwise specified (NOS) ($n=17$), mucoepidermoid carcinomas ($n=11$), carcinomas ex pleomorphic adenomas ($n=3$), pleomorphic adenomas ($n=4$) and Warthin's tumours ($n=22$). Surgical resection and subsequent routine pathological examination were performed between 2000 and 2006.

The retrospective analysis of archived tissue was approved by our local ethics committee.

Analysis of HPV infection in salivary gland tumours

HPV was analysed in all tumours by PCR analysis (Chipron, Berlin, Germany) as previously described.^{15–16} Briefly, DNA was extracted from tumour blocks (>80% tumour cells) in formalin fixed-paraffin embedded (FFPE) tissue sections with the DNEasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany). A total of 50 ng of DNA (determined with a Micro-Volume UV-Vis Nanodrop 2000 spectrophotometer; Thermo Scientific, Wilmington, DE, USA) was used for multiplex PCR for 32 high- and low-risk HPV samples (types 6, 11, 16, 18, 31, 33, 35, 39, 42, 44, 45, 51–54, 56, 58, 59, 61, 62, 66–68, 70, 72, 73, 81–84, 90, 91). The sensitivity of the PCR for each of the above-mentioned HPV types is reported by the manufacturer to be 20 virus copies per μg of extracted DNA (Chipron, Berlin, Germany). The controls were FFPE tissue-derived DNA from HPV-positive cervix samples and DNA-free water.

For the evaluation of EBV and CMV, two tissue microarrays (each with 60 samples) were used, which contained all the carcinoma, pleomorphic adenoma and control samples. Deparaffinised and rehydrated FFPE tissue sections (approximately 2–3 μm) were processed in an automated staining system (Benchmark ULTRA; Ventana Medical Systems, Tucson, AZ, USA). Infection of tumour cells by EBV and/or CMV was tested by *in situ* hybridisation (EBV-encoded RNA; Ventana Medical Systems, Tucson, AZ, USA) and immunohistochemistry (CMV; Thermo Fisher Scientific, Schwerte, Germany), respectively. Controls were FFPE samples from an EBV+lymphoma patient and a CMV+colitis patient.

Protein expression analysis of HPV-associated human genes

Carcinoma and pleomorphic adenoma samples underwent cell cycle and receptor protein expression analysis using a tissue microarray. Sections (approximately 2–3 μm) were used for automated immunohistochemistry (Benchmark ULTRA, Ventana Medical Systems, Tucson, AZ, USA) of CDKN2A (Santa Cruz Biotechnology, Santa Cruz, CA, USA), TP53 (Dako, Glostrup, Denmark) and EGFR (Zytomed, Berlin, Germany).

Immunohistochemistry was scored semi-quantitatively as described previously¹⁷: 0 (no apparent reaction), 1+ (positivity in <30% of tumour cells), 2+ (positivity in $\geq 30\%$ but <60%) and 3+ (positivity in $\geq 60\%$). Immunostaining intensity was scored as 0 (absent), 1+ (weak), 2+ (intermediate) or 3+ (strong).

Statistical analysis

Overall survival was obtained from the medical records. Survival analysis was performed with Prism 5.0 (GraphPad Software, San Diego, CA, USA) by applying the log-rank (Mantel–Cox) test. P values <0.05 were considered statistically significant.

RESULTS

Clinical characteristics of patients with salivary gland tumours

The median patient age was 63 years (24 females/53 males). All patients were of European descent. Tumours were located in the major salivary glands of the head and neck (parotid $n=45$, submandibular $n=3$ and sublingual gland $n=1$) or in minor salivary glands of the oral cavity ($n=28$). Carcinoma grades were G1 in 7/51 (14%), G2 in 30/51 (59%) and G3 in 14/51 (27%) of the cases. The median follow-up period in carcinoma cases was 111 months, and the tumour-free survival rate was 71%. In this small patient cohort, patients with mucoepidermoid carcinoma had a significantly better survival rate than patients with other types of salivary gland tumours ($P<0.05$). The characteristics of the study group are summarised in Table 1.

Salivary gland tumours are not associated with HPV infection

HPV was tested by PCR in carcinomas and benign salivary gland tumours but could not be detected in any of these samples. None of the tumour samples under investigation showed infection with EBV (no EBV-encoded RNA hybridisation signals) or CMV (no immunostaining).

A subfraction of tumour samples exhibited expression of cell cycle and receptor proteins (Figure 1). The most frequent tumour types with positive expression were adenoid cystic carcinomas for CDKN2A/p16

Figure 1 Expression of cell cycle factors and EGFR in salivary gland carcinomas. (a) Representative histomorphology of an HPV-negative adenoid cystic carcinoma expressing CDKN2A/p16 in a subfraction of tumour cells. (b) Adenoid cystic carcinoma with TP53/p53 expression. (c) Adenoid cystic carcinoma showing EGFR expression. (d) Mucoepidermoid carcinoma with partial EGFR positivity. Original magnification of $\times 200$ in **a**, **c** and **d** and $\times 400$ in **b**, as well as in inserts in **a**, **c** and **d**. Microscopic images were produced with a BZ-9000 slide scanner (Keyence, Neu-Isenburg, Germany). CDKN2A, cyclin-dependent kinase inhibitor 2A; EGFR, epidermal growth factor receptor; HPV, human papillomavirus; TP53, tumour protein p53.

Table 1 Characteristics of patient cohort

Characteristics	Diagnosis						
	Adenoid cystic carcinoma	Adenocarcinoma, NOS	Mucoepidermoid carcinoma	Adenocarcinoma ex pleomorphic adenoma	Pleomorphic adenoma	Warthin's tumour	Controls without adenocarcinomas
Gender-females	15/20 (75%)	9/17 (53%)	6/11 (55%)	1/3 (33%)	2/4 (50%)	4/22 (18%)	5/7 (71%)
Age/years	63 (21-78)	73 (30-87)	59 (26-83)	67.5 (54-81)	66.5 (43-63)	61 (27-81)	56 (7-76)
pT (n)	T1 (7), T2 (4), T3 (3), T4 (6)	Tx (1*), T1 (5), T2 (5), T3 (3), T4 (3)	Tx (1*), T1 (7), T2 (1), T3 (1), T4 (1)	T2 (1), T3 (1), T4 (1)			
pN (n)	Nx (11), N0 (7), N1 (1), N2b (1)	Nx (8), N0 (3), N1 (3), N2b (2), N2c (1)	Nx (7), N0 (4)	Nx (1), N0 (1), N1 (1)			
M (n)	Mx (19), M1 (1)	Mx (15), M0 (2)	Mx (8), M0 (2), M1 (1)	Mx (3)			
G (n)	G1 (0), G2 (17), G3 (3)	G1 (1), G2 (8), G3 (8)	G1 (5), G2 (3), G3 (3)	G1 (1), G2 (2)			
Therapy**							
S	13	7	9	2	4	22	7
S+R	6	6	1	1			
S+R+C	1	1	1				
Not known		3					
Follow-up/years	2.95 (0.1-7.3)	0.95 (0-5.7)	0.75 (0-10)	3.75 (1.3-6.2)			
Alive without disease	14/20 (70%)	10/17 (59%)	8/11 (73%)	1/3 33%			

NOS, not otherwise specified.

* Due to biopsy sampling, in two cases pT stage could not be determined.

** S, surgery; R, radiotherapy; C, chemotherapy.

Table 2 Expression of cell signalling factors are not related to HPV infection in salivary gland carcinomas

Diagnosis	CDKN2A/p16 (positive tumours)	TP53 (positive tumours)	EGFR (positive tumours)
Adenoid cystic carcinoma	72% (n=13/18, 2 not evaluable)	61% (n=11/18, 2 not evaluable)	56% (n=10/18, 2 not evaluable)
Adenocarcinoma, NOS	53% (n=9/17)	69% (n=11/16, 1 not evaluable)	38% (n=6/16, 1 not evaluable)
Mucoepidermoid carcinoma	64% (n=7/11)	46% (n=5/11)	91% (n=10/11)
Adenocarcinoma ex pleomorphic adenoma	67% (n=2/3)	0% (n=0/2, 1 not evaluable)	50% (n=1/2, 1 not evaluable)

CDKN2A, cyclin-dependent kinase inhibitor 2A; HPV, human papillomavirus; NOS, not otherwise specified; PCR, polymerase chain reaction; TP53, tumour protein p53. In none of the tumours HPV could be detected by PCR analysis. Further details on the percentage of positive tumour cells and staining intensity are summarized in Supplementary Table 1.

and adenocarcinomas, NOS for TP53/p53 (Table 2). EGFR was mainly expressed in mucoepidermoid carcinomas (Table 2). A detailed description of the percentage of positive tumour cells and the intensity of immunostaining is provided in Supplementary Table 1.

DISCUSSION

Viruses have been demonstrated to be the mediators of neoplastic proliferation in squamous tumour types of the head and neck,¹⁻² while adenocarcinomas are rarely associated with viruses.¹⁰⁻¹² Recently, CMV was analysed by immunohistochemistry in mucoepidermoid carcinomas; remarkably, it was detected in these carcinoma cells.⁷ If CMV has a major causal role in neoplastic proliferation, it would be

expected that the majority of carcinoma cells would be positive. In contrast, it has been shown that the luminal carcinoma cells were particularly positive for immunostaining,⁷ and in our cohort, none of the carcinomas, including the mucoepidermoid carcinomas, showed positivity for CMV. In another study, PCR analysis did not reveal CMV in salivary tumours.¹⁸ EGFR and other cell cycle factors were expressed in these tumour samples,⁷ but it is not clear whether these factors are directly related to CMV detection or are a result of aberrantly increased proliferation. Because CMV results have not yet been confirmed by *in situ* hybridisation or PCR, CMV infection cannot be regarded as a major pathogenic driver in adenocarcinomas of the head and neck.

EBV can be found in nasopharyngeal carcinomas and lymphoepithelial carcinomas.¹⁹ Indolent EBV infection of B cells is very common among adults, and the oro-nasal cavity is the main anatomical site of virus entry. EBV persists in ductal epithelial cells of salivary glands and can be involved in aberrant proliferation, such as in sporadic B-cell lymphoma or Hodgkin lymphoma and post-transplant lymphoproliferative disease.^{4–5} Although EBV is localised within ductal epithelial cells and can mediate neoplastic proliferation, we and others have shown that salivary gland tumours in the Caucasian population are negative for this virus.²⁰ Regarding the role of ethnic background, the Inuit are known to have a higher risk for developing EBV-positive high-grade salivary gland carcinomas and have higher frequencies of EBV-positive nasopharyngeal carcinomas, similar to Asians and North Africans.²¹ The pathobiology of this ethnic predisposition is currently unknown, and it is also not known why nasopharyngeal carcinomas are usually associated with EBV, while oropharyngeal carcinomas are associated with HPV, despite their close anatomical relationship. HPV has been analysed by *in situ* hybridisation and PCR in oral, nasal and parotid gland tumours.^{8,10–12} In one study, 7% of cases ($n=2/27$) were found to be positive for HPV subtype 16 and revealed a concomitant strong and diffuse expression of CDKN2A.¹⁰ These two tumours were high-grade adenoid cystic carcinomas of the nasal cavity, and the ethnic background of these two patients was not specified.¹⁰ The correlation of HPV and CDKN2A presence in oropharyngeal carcinoma and cervical carcinoma has shown that strong and diffuse CDKN2A positivity is an indicator of underlying HPV infection.^{22–23} However, CDKN2A was also strongly and diffusely detectable in HPV-negative, high-grade adenoid cystic carcinomas and other salivary gland tumours, indicating that this kinase inhibitor can be increased independent of HPV status.^{10,13} Corresponding to the lack of HPV infection in our cohort, tumour expression of CDKN2A, TP53 and EGFR was mainly weak to moderate and often patchy. Previous studies have demonstrated similar expression of these three factors in salivary gland carcinomas and the frequency of positive tumour types in our analysis is similar to findings in other cohorts.^{24–26} For example, the frequency of EGFR-positive mucoepidermoid carcinomas was 91% in our cohort and 82% in the analysis of Shang *et al.*²⁵ Taken together, these findings show an aberrant expression of cell cycle factors and EGFR in a subfraction of tumour cells refers to the deregulated cell homeostasis and not to virus infection.

In another recently published study, PCR analyses of mucoepidermoid carcinomas from American patients revealed that 36% ($n=35/98$) of samples were HPV-positive, mainly HPV subtype 16 and, less frequently, HPV 18 or HPV 16+18.¹¹ In our cohort of salivary gland carcinomas (European patients), we used a sensitive and specific PCR method¹⁵ but did not identify any HPV-positive cases among a variety of different tumours types, including 20 adenoid cystic carcinomas and eleven mucoepidermoid carcinomas. Another study demonstrated only occasional HPV-positive parotid gland carcinomas ($n=1/39$, 3%).¹² Similar to our results, in three other studies which included more than 100 salivary gland carcinoma cases, no HPV-positive case could be identified.^{27–29}

While salivary gland carcinomas show no general association with HPV infection, some reports have indicated that benign salivary gland neoplasms are frequently positive. In particular, greater than 50% of HPV-positive Warthin's tumours were identified in small cohorts.^{8–9} In contrast, another study that included a larger number of benign parotid tumours reported the identification of less than 10% of HPV-positive cases ($n=3/49$, 7.5%).¹² We analysed a set of Warthin's tumours and pleomorphic adenomas and expected that a subfraction would

be HPV-positive according to previous results,^{8–9} but no association was found. Therefore, similar to carcinomas, HPV is not a general mediator of aberrant proliferation in benign salivary gland neoplasms.

In the minority of salivary gland tumours that are HPV-positive, the transmission route of HPV infection is not fully understood. Extrapolating from studies on squamous tumours,^{1–2} sexual transmission could be one risk factor. Viraemia after HPV entry in an anatomical site other than the salivary glands is an alternative hypothesis. Immunosuppression, particularly by co-infection with human immunodeficiency virus, could be another factor that increases the potential of HPV to infect non-squamous cells.³⁰ In contrast to salivary gland neoplasms, cervical cancer data show that squamous carcinomas are often infected by HPV, as are a subfraction of adenocarcinomas.³¹ Similar to adenocarcinomas of the cervix, HPV can also be detected in adenocarcinomas of the lung, but the frequency of HPV-associated lung carcinoma is lower and appears to be related to ethnicity (higher in Asia, lower in parts of Europe and America).^{32–33} The exact entry mechanism that allows HPV to enter glandular but not squamous cells is still uncertain. Recent experiments in squamous cells suggest that HPV binds nonspecifically to glycoproteins (particularly heparan-sulfonated proteoglycans) and that this complex activates EGFR.^{34–35} The EGFR-activated mTOR/PI3K signalling cascade leads to translocation of intracellular annexin A2 to the extracellular leaflet of the plasma membrane. At the site of HPV/EGFR/annexin A2 interaction, endosome formation is induced and ultimately leads to internalisation of the virus.³⁴ Chronic stress with (transient) squamous metaplasia of salivary duct cells could increase the likelihood of HPV entry. *In situ* precursor lesions of HPV-positive cervical adenocarcinomas are often located adjacent to cervical squamous intraepithelial dysplasia/neoplasms.³⁴ Therefore, infection of a common adenosquamous precursor cell at the site of transition between squamous and glandular epithelial cells is possible. Furthermore, a HPV-positive adenosquamous carcinoma of the tongue has been described.³⁶

In summary, HPV can be found in a few nasal-type high-grade adenocarcinomas,¹⁰ in a few benign and malignant parotid gland tumours¹² and in some mucoepidermoid carcinomas.¹¹ In contrast to oropharyngeal carcinomas, HPV is not generally associated with salivary gland tumours. Similar to HPV findings, infection with either EBV or CMV is not a major driver of aberrant cell proliferation in salivary gland carcinomas.

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