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ORIGINAL INVESTIGATION

Human polyomavirus and human papillomavirus prevalence and viral load in non-malignant tonsillar tissue and tonsillar carcinoma

Stephan Herberhold[1,2] · Martin Hellmich[3] · Marcus Panning[4] · Eva Bartok[5] · Steffi Silling[6] · Baki Akgül[6] · Ulrike Wieland[6]

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Abstract Human papillomaviruses (HPVs) are an acknowledged cause of a subset of oropharyngeal cancers, especially of tonsillar cancer. Similar to HPV, some human polyomaviruses (HPyVs), such as Merkel cell polyomavirus (MCPyV), have an oncogenic potential. Recently, several novel HPyVs have been discovered. The aim of our study was to determine viral DNA prevalence and viral DNA load of 13 different HPyVs in benign and malignant tonsillar tissue and to compare the data with those found for HPV. A total of 78 biopsies of palatine tonsils with a histologic diagnosis of non-malignant disease (chronic tonsillitis, tonsillar hyperplasia, $n = 40$) or tonsillar squamous cell carcinoma ($n = 38$) were included in the study. HPyV DNA prevalence and viral load were determined by virus-specific quantitative real-time PCRs. JCPyV (1/40, 2.5%) and WUPyV (3/40, 7.5%) were only found in non-malignant tonsillar tissue. HPyV7 and HPyV10 were only detected in one (2.6%) and seven (18.4%) of the 38 cancer biopsies, respectively. Both MCPyV (8/38, 21.1 vs. 4/40, 10.0%) and HPyV6 (2/38, 5.3 vs. 1/40, 2.5%) were found more frequently in cancer samples than in non-malignant tissue, but the differences were not significant. BKPyV, KIPyV, TSPyV, HPyV9, STLPyV, HPyV12 and NJPyV were not discovered in any of the samples. HPyV loads found in HPyV DNA-positive biopsies were very low with no difference between non-malignant and malignant samples (median load <0.0001 HPyV DNA copies per beta-globin gene copy, respectively). In contrast to HPyV, high-risk HPV types (HPV16/HPV18) were found significantly more frequently in tonsillar cancers than in non-malignant tonsillar tissue (17/38, 44.7 vs. 2/40, 5.0%, $p < 0.001$). Furthermore, high-risk HPV DNA loads were significantly higher in the cancer compared to the non-malignant samples (median load 11.861 vs. 7×10^{-6} HPV DNA copies per beta-globin gene copy, $p = 0.012$). While both HPV and HPyV may persist in tonsillar tissue, our data on HPyV DNA prevalence and load do not support a role of HPyV in tonsillar carcinogenesis, neither alone nor as co-infecting agents of HPV.

Keywords Human polyomavirus · Human papillomavirus · Tonsillitis · Tonsillar hyperplasia · Tonsillar carcinoma

✉ Baki Akgül
baki.akguel@uk-koeln.de

1 ENT Department, Head and Neck Surgery, University of Bonn, Bonn, Germany

2 Present Address: ENT Department, Johanniter-Kliniken Bonn, Bonn, Germany

3 Institute of Medical Statistics, Informatics and Epidemiology, University of Cologne, Cologne, Germany

4 Institute for Virology, Medical Center – University of Freiburg, Freiburg, Germany

5 Institute of Clinical Chemistry and Clinical Pharmacology, University of Bonn, Bonn, Germany

6 Institute of Virology, National Reference Center for Papilloma- and Polyomaviruses, University of Cologne, Fürst-Pückler-Str. 56, 50935 Cologne, Germany

Introduction

Along with noxious agents such as alcohol or tobacco, high-risk (HR) human papillomavirus (HPV) types of the genus alpha such as HPV16 or HPV18 play an independent and important role in the etiology of squamous cell carcinoma (SCC) of the oropharynx [1–3]. HPVs are small, non-enveloped, oncogenic DNA viruses that infect basal

Table 1 Patients' characteristics

Characteristic	All biopsies $n = 78$	Histological diagnosis		p value
		Non-malignant tonsillar tissue[a] $n = 40$	Tonsillar cancer $n = 38$	
Sex				
Male	47 (60.3%)	16 (40.0%)	31 (81.6%)	<0.001
Female	31 (39.7%)	24 (60.0%)	7 (18.4%)	
Age				
Median age (IQR)	47.5 (20.0–62.0)	20.0 (10.0–27.5)	62.0 (57.0–70.0)	<0.001
Mean age (SD, range)	41.4 (24.0, 2.0–81.0)	21.0 (14.1, 2.0–65.0)	62.9 (8.2, 47.0–81.0)	
Smoking				
Yes	37 (47.4%)	9 (22.5%)	28 (73.7%)	<0.001
No	37 (47.4%)	31 (77.5%)	6 (15.8%)	
No data available	4 (5.1%)	0	4 (10.5%)	

IQR interquartile range, *SD* standard deviation

[a] Chronic tonsillitis or tonsillar hyperplasia

and stem cells of cutaneous and mucosal squamous epithelium. 40–70% of oropharyngeal cancers, mainly tonsillar and base of the tongue carcinomas, are attributed to HR-HPV infection [4, 5]. Compared to patients with HPV-negative oropharyngeal cancer, patients with HPV-associated oropharyngeal cancer are more frequently non-smokers, non-drinkers, male and of younger age [1, 4, 6]. Worldwide, approximately 400,000 new cases of oropharyngeal cancer and cancer of the oral cavity are diagnosed per year [7]. In Germany alone, roughly 5300 patients will die of oropharyngeal cancer and cancer of the oral cavity each year [8]. Depending on the stage of the tumor, treatment in Western Europe generally involves surgery and chemoradiation treatment. Both treatment response and survival rates are more favorable for HPV-positive compared to HPV-negative oropharyngeal cancer [9–12]. In recent years, the development of vaccination programs and immunotherapies has opened up new perspectives in the prevention and treatment of oropharyngeal cancer [1, 13, 14].

Similar to HPV, polyomaviruses are small, non-enveloped DNA viruses with a possible oncogenic potential. To date, 13 human polyomaviruses (HPyVs) have been discovered [15–18]. Serological studies suggest that HPyVs are widespread and that infections occur early in life [16, 19–22]. Some HPyVs such as BKPyV and JCPyV can cause severe disease in immunosuppressed patients as nephropathy (BKPyV), hemorrhagic cystitis (BKPyV) and progressive multifocal leukoencephalopathy (JCPyV) [23–26]. Merkel cell polyomavirus (MCPyV) is the only HPyV that can cause disease in non-immunosuppressed patients and is the principal etiology of Merkel cell carcinoma, a rare and aggressive neuroectodermal tumor of the skin [27, 28]. HPyV7 has recently been detected in two cases of pruritic rash in lung transplant patients [29]. TSPyV can cause the rare skin disease Trichodysplasia spinulosa in immunosuppressed patients [30]. New Jersey polyomavirus (NJPyV) was recently discovered in a pancreatic transplant recipient with retinal blindness and vasculitic myopathy [18]. To date, HPyV6, HPyV9, HPyV10 (and its variants MWPyV and MXPyV), Saint Louis PyV (STLPyV) and HPyV12 have not been associated with any pathology, and it remains to be determined whether these emerging viruses play a pathogenic role in humans [16, 17, 31–37].

We have performed a retrospective study using DNA extracted from tonsillar biopsies of patients with chronic tonsillitis/tonsillar hyperplasia or with tonsillar carcinoma. The goal of our study was to determine HPyV DNA prevalence and DNA load in benign and malignant tonsillar tissue and compare the data with those found for HR-HPV. This could help to elucidate, whether HPyV infection, besides HR-HPV infection, might contribute to oropharyngeal cancer development.

Materials and methods

Samples

We collected 80 tonsillar biopsies (palatine tonsils) of 80 patients between November 2005 and March 2014. A total of 42 patients had a histologic diagnosis of non-malignant disease (chronic tonsillitis, tonsillar hyperplasia), and 38 patients had tonsillar SCC. DNA was extracted as previously described [38], and the extracted DNA was stored in the bio-bank of Bonn University Hospital at −80 °C until virological analyses were performed. Patients' medical files were

Table 2 Primers and locked nucleic acid probes for the detection and quantification of BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, STLPyV, HPyV12 and NJPyV

Primer name	Primer sequence (5′–3′)	Position in the respective viral genome[a]	LNA probe number, sequence (5′–3′)*
BKPyV LC fw	AAACAGGAGAACCCAGAGAGTG	2493–2514	No. 44, TGGGCAGC
BKPyV LC rev	TCTACCTGGGATTCCATACCAT	2552–2531	
JCPyV UPL3 fw	GCATGTTTACAAACAGGTCTGG	2250–2271	No. 3, CCCAGCAG
JCPyV UPL3 rev	AGCTGCACCTTAAAATATCTGGA	2316–2294	
KIPyV LC fw	TGAGCCCACCCCTCATTAC	1701–1719	No. 53, CTCTGCCA
KIPyV LC rev	TGAACCGCTTTCCTTGTCAT	1761–1742	
WUPyV LC fw	TGCCTCATGTTTATTCCAGTTC	2229–2250	No. 37, CCAGGGCA
WUPyV LC rev	CACCCATGATTCAATGCTGTA	2305–2285	
MCPyV LT3 fw	GCATCTGCACCTTTTCTAGACTC	738–760	No. 6, TTCCTCTG
MCPyV LT3 rev	TTTTGCCTTATAGACTTTTCCATATCT	811–785	
STLPyV VP1 fw	GAAAATCTAGATGCACCTCACAGA	1255–1278	No. 63, AGGAGGAG
STLPyV VP1 rev	TTTGGCACGGATCATATTCA	1338–1319	
HPyV12 UPL108 fw	GGAAAAGGGCTGTAAGAAATCC	2285–2306	No. 108, CTGCTCTC
HPyV12 UPL108 rev	CATCCGGGGCATCATATTAG	2361–2342	
NJPyV-13 LC fw	CCTACTCCAGTGCCTAAATTGC	1137–1158	No. 63, AGGAGGAG
NJPyV-13 LC rev	AGTAGTAGCATCAGGCCCAGTAA	1214–1192	

Primer and probe sequences for MCPyV [41, 63] and for HPyV6, HPyV7, TSPyV, HPyV9 and HPyV10 were recently published [39]

fw forward, *rev* reverse, *LC* light cycler, *LT* Large-T-antigen, *UPL* universal probe library, *VP1* viral protein 1

[a] The respective viral reference genome sequences and their GenBank Accession Numbers are BKPyV strain Dunlop, no V01108; JCPyV, no. J02226, KIPyV Stockholm 60, no. NC_009238; WUPyV, no. NC_009539, MCPyV MCC 350, no. EU375803; STLPyV strain MA138, no. JX463183; HPyV12 strain hu1403, no. JX308829; NJPyV-2013 isolate NJ-PyV-2013, no. KF954417

additionally reviewed for information on smoking habits. The study was performed according to the principles of the Declaration of Helsinki. Human specimens were used after approval by the Ethics Committee of the Medical Faculty of the University Bonn (Approval Number 201/15). Two non-malignant samples had to be excluded from virological analysis due to the lack of cellular input (beta-globin gene PCR negative), leaving 40 non-malignant and 38 cancer samples from 78 patients (Table 1) for virological analysis.

HPyV and HPV detection and DNA load determination

HPyV and HPV DNA analyses were performed as recently described [39, 40]. The MCPyV polymerase chain reaction (PCR) used was established by Sihto et al. [41]. Primer and probe sequences for HPyV6, HPyV7, TSPyV, HPyV9 and HPyV10 PCRs as well as the respective reference materials were recently published [39]. Primer and locked nucleic acid (LNA) probe sequences for the detection and quantification of the remaining eight HPyVs are given in Table 2 (BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, STLPyV, HPyV12, NJPyV). The analytical sensitivity of each assay was 10 copies of cloned or viral synthetic DNA (gBlocks, Integrated DNA Technologies, Leuven, Belgium), respectively. All HPyV quantitative PCRs (q-PCRs) were performed on a LightCycler 480 (Roche, Mannheim, Germany) in 20 µl reactions containing 10 µl LC 480 Probes Master (Roche), 0.2 mM each of the respective forward and reverse primer (TIB Molbiol, Berlin, Germany), 0.1 mM LNA probes (Roche) (Table 2 and [39]) and 2 µl extracted DNA. Cycling conditions were 10 min denaturation at 95 °C, followed by 45 cycles at 95 °C for 10 s, 60 °C for 30 s and 72 °C for 5 s. Negative controls contained human DNA (extracted DNA of RTS3b cells) and water instead of patient samples and never yielded fluorescence signals above the background.

HPV detection and typing were performed by genus alpha-specific PCR (A6/A8-PCR) and bead-based multiplex hybridization using a Luminex Lx200 (Luminex, Austin, TX, USA) as previously described [40, 42, 43]. The Luminex assay can detect 38 different HPV types, 22 high-risk (HR) or probable/possible HR-HPV types and 16 low-risk (LR) types (HR-types: HPV16, 18, 26, 30, 31, 33, 34, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, 82; LR-types: HPV6, 11, 40, 42, 43, 44, 54, 55, 57, 61, 71, 72, 81, 83, 84, 89) [44].

Table 3 HPyV and HPV DNA prevalence in non-malignant and malignant tonsillar tissue

Analyzed virus	Non-malignant tonsillar tissue[a] $n = 40$ (%, 95 % CI)	Tonsillar cancer $n = 38$ (%, 95 % CI)	p value, non-adjusted	OR, non-adjusted (95 % CI)	p value*, adjusted	OR, adjusted* (95 % CI)
BKPyV	0	0	–	–	–	–
JCPyV	1 (2.5, 0.1–13.2)	0	1.000	–	–	–
KIPyV	0	0	–	–	–	–
WUPyV	3 (7.5, 1.6–20.4)	0	0.241	–	–	–
MCPyV	4 (10.0, 2.8–23.7)	8 (21.1, 9.6–37.3)	0.176	2.4 (0.7–8.8)	0.953	0.9 (0.1–18.3)
HPyV6	1 (2.5, 0.1–13.2)	2 (5.3, 0.6–17.7)	0.610	2.2 (0.2–24.9)	0.138	0.1 (0.0–2.5)
HPyV7	0	1 (2.6, 0.1–13.8)	0.487	–	–	–
TSPyV	0	0	–	–	–	–
HPyV9	0	0	–	–	–	–
HPyV10	0	7 (18.4, 7.7–34.3)	**0.005**	–	–	–
STLPyV	0	0	–	–	–	–
HPyV12	0	0	–	–	–	–
NJPyV	0	0	–	–	–	–
Any HPyV	8 (20.0, 9.1–35.6)	16 (42.1, 26.3–59.2)	**0.034**	**2.9 (1.1–8.0)**	0.700	0.6 (0.0–9.7)
HPV16	1 (2.5, 0.1–13.2)	16 (42.1, 26.3–59.2)	**<0.001**	**28.4 (3.5–228.6)**	0.165	89.6 (0.2–51173.0)
HPV18	1 (2.5, 0.1–13.2)	1 (2.6, 0.1–13.8)	1.000	1.1 (0.1–17.5)	0.737	3.7 (0.02–8137.9)
Any HR-HPV	2 (5.0, 0.6–16.9)	17 (44.7, 28.6–61.7)	**<0.001**	**15.4 (3.2–73.1)**	0.159	16.3 (0.3–789.7)

– Incalculable; p values in bold indicate a statistically significant association

* Adjustment was performed for age, sex and smoking

[a] Chronic tonsillitis or tonsillar hyperplasia

In HPV16 or HPV18-positive samples, HPV DNA load was determined by q-PCR with type-specific primers and probes as recently described [40].

The single-copy beta-globin gene was detected and quantified as described previously [45, 46]. HPyV and HPV loads were each calculated as viral DNA copies per beta-globin gene copy [39, 40]. Cumulative HPV or HPyV DNA loads were calculated by considering all HPV or HPyV types found in the samples.

Statistical analysis

The data on quantitative variables were summarized by median (25th–75th percentile), on qualitative variables by count (percentage). Confidence intervals at level 95% for proportions were calculated according to the exact Clopper–Pearson method. Bivariable association was evaluated by cross-tabulation (calculating odds ratios) and Fisher's exact test. Adjustment for potentially confounding variables was done by multiple logistic regression (again calculating odds ratios). Location differences in distributions (e.g., of viral load) were assessed by the non-parametric Mann–Whitney U test. p values below 5% were considered statistically significant. All analyses were done with SPSS Statistics (version 23, IBM Corp., Armonk, NY, USA).

Results

Patient characteristics

A total of 78 tonsillar biopsies comprising 40 with non-malignant disease (chronic tonsillitis, tonsillar hyperplasia) and 38 with malignant disease (squamous cell carcinoma) were included in the analysis. Patient age ranged between 2 and 81 (mean 41.4) years. Sex, age and smoking habits of patients with tonsillar cancer differed from those with non-malignant disease (Table 1). The patients with cancer were significantly older than those with non-malignant disease. 81.6% of the patients with tonsillar cancer were males, compared to 40.0% of the patients with benign disease. 47.4% of all patients were smokers. Significantly more smokers were among the patients with tonsillar cancer than among the individuals with non-malignant disease (73.7 vs. 22.5%, Table 1).

Table 4 Viral DNA loads in non-malignant and malignant tonsillar tissue

Analyzed virus	Median viral DNA load; *n* (IQR; *minimum/maximum*)		*p* value
	Non-malignant tonsillar tissue[a]	Tonsillar cancer	
JCPyV	0.000018; $n = 1$	All biopsies negative	–
WUPyV	0.000499; $n = 3$ (*0.000048/0.001040*)	All biopsies negative	–
MCPyV	0.000004; $n = 4$ (0.000003–0.000016; *0.000003/0.000028*)	0.000064; $n = 8$ (0.000034–0.000166; *0.000026/0.008072*)	**0.008**
HPyV6	0.000092; $n = 1$	0.357149; $n = 2$ (*0.000012/0.714286*)	1.000
HPyV7	All biopsies negative	0.000042; $n = 1$	–
HPyV10	All biopsies negative	0.000075; $n = 7$ (0.000035–0.000157; *0.000017/0.000621*)	–
Cumulative HPyV load	0.000047; $n = 8$ (0.000004-0.000295; *0.000003/0.001040*)	0.000071; $n = 16$ (0.000032–0.000194; *0.000012/0.714286*)	0.320
HPV16	0.000014; $n = 1$	67.469; $n = 16$ (0.025772–403.993; *0.000017/3180.645*)	0.118
HPV18	0.000000; $n = 1$	0.000075; $n = 1$	1.000
Cumulative HPV load	0.000007; $n = 2$ (*0.000000/0.000014*)	11.861; $n = 17$ (0.014915–334.112; *0.000017/3180.645*)	**0.012**

Viral DNA load is expressed as viral DNA copies per beta-globin gene copy

Italic indicates the minimum and maximum viral load values

– Incalculable; *p* values in bold indicate a statistically significant association

[a] Chronic tonsillitis or tonsillar hyperplasia

Human polyomavirus DNA prevalence in benign and in malignant tonsillar biopsies

BKPyV, KIPyV, TSPyV, HPyV9, STLPyV, HPyV12 and NJPyV were not detected in any of the samples. JCPyV (2.5%) and WUPyV (7.5%) were only found in non-malignant tonsillar tissue. HPyV7 and HPyV10 were only detected in one (2.6%) and seven (18.4%) cancer biopsies, respectively, and never in non-malignant tissue. The difference in HPyV DNA prevalence between non-malignant and malignant tissue was statistically significant only for HPyV10 (Table 3). Both MCPyV (10.0 vs. 21.1%) and HPyV6 (2.5 vs. 5.3%) were detected more frequently in cancer samples than in non-malignant tissue, but the differences were not statistically significant, neither unadjusted nor after adjustment for age, sex and smoking. The prevalence of any HPyV was higher in cancer samples compared to non-malignant tissue (42.1 vs. 20%, $p = 0.034$, OR 2.9 (95% CI 1.1–8.0)). However, after adjustment for age, sex and smoking, the *p* value was no longer significant (0.700) and the OR was lower (0.6 (95% CI 0.0–9.7)), indicating a confounding effect of these factors (Table 3). Two different polyomaviruses were found in three (12.5%) of the 24 HPyV-positive samples: JCPyV and WUPyV were found in a non-malignant sample from a 2-year-old girl, and MCPyV plus HPyV10 were detected in two HPV-negative cancer samples from a 52-year-old male and a 70-year-old female. Three or more polyomaviruses were not found concomitantly in any of the samples.

Human papillomavirus DNA prevalence in benign and in malignant tonsillar biopsies

HPV DNA was found in 44.7% of the malignant biopsies, compared to 5.0% of the non-malignant tissues ($p < 0.001$, OR 15.4 (95% CI 3.2–73.1)). Of the 22 HR-HPV types analyzed, only HPV16 and HPV18 were detected (Table 3). HPV16 and HPV18 were found in one non-malignant tonsillar tissue specimen each (2.5%, respectively). Sixteen cancer samples were HPV16-positive (42.1%), and one cancer biopsy (2.6%) was HPV18-positive (Table 3). With the exception of one HPV16-positive cancer that additionally carried HPV57 and HPV84 DNA, LR-HPV types were not found in any of the samples. In contrast to HPyVs, the ORs for HPV-positivity (HPV16 or any HR-HPV, non-malignant compared to cancer samples) were similar or greater after adjustment for age, sex and smoking, which argues against confounding by these variables (Table 3).

Viral DNA loads in benign and in malignant tonsillar biopsies

In all HPyV DNA-positive samples, the respective HPyV DNA loads were determined (Table 4). The HPyV loads

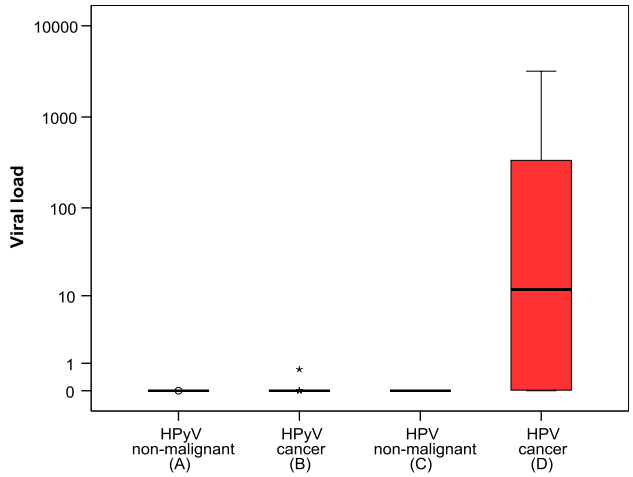

Fig. 1 HPyV and high-risk HPV DNA load distribution in non-malignant tonsillar tissue and in tonsillar cancer. The four *box plots* show the distribution of the following viral DNA loads: cumulative HPyV DNA load of non-malignant tonsillar tissue ($n = 8$) (*A*), cumulative HPyV DNA load of oropharyngeal cancers ($n = 16$) (*B*), cumulative high-risk HPV DNA load of non-malignant tonsillar tissue ($n = 2$) (*C*) and cumulative high-risk HPV DNA load of oropharyngeal cancers ($n = 17$) (*D*). The *bottom* and *top* of the *box* are the first and third quartile, and the band inside the box represents the median. Whiskers are *vertical lines* ending in *horizontal lines* at the largest and smallest observed values that are not statistical outliers, i.e., values more than three IQRs from the end of a box are labeled as extreme, denoted with an asterisk (*), and values more than 1.5 IQRs but less than 3 IQRs from the end of the *box* are labeled as outliers (o)

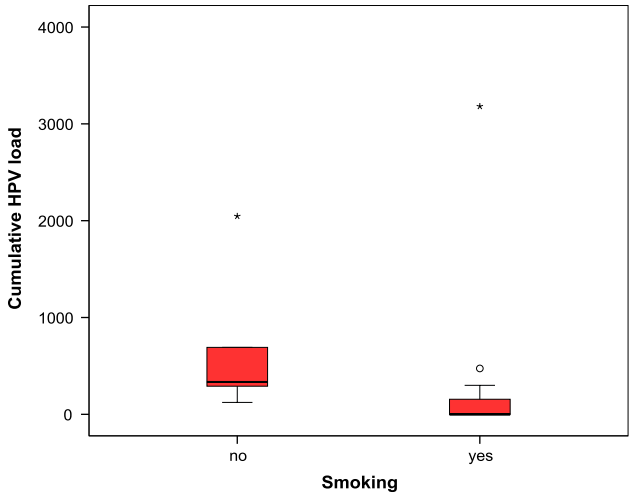

Fig. 2 High-risk HPV DNA load distribution in HPV-positive tonsillar cancers of non-smokers and smokers. The *box plots* show the distribution of the cumulative high-risk HPV DNA loads found in tonsillar cancers of non-smokers ($n = 5$) (*left*) and smokers ($n = 11$) (*right*). *Boxes* represent the interquartile range and the median. Whiskers are *vertical lines* ending in horizontal lines at the largest and smallest observed values that are not statistical outliers, i.e., values more than three IQRs from the end of a *box* are labeled as extreme, denoted with an *asterisk* (*), and values more than 1.5 IQRs but less than 3 IQRs from the end of the box are labeled as outliers (o)

found were very low both in non-malignant and malignant samples (Fig. 1) and did not exceed 0.008 HPyV DNA copies per beta-globin gene copy, with the exception of one cancer sample of a HPV-negative 62-year-old male heavy smoker with a HPyV6 load of close to one (0.7, Table 4). MCPyV loads were significantly higher in malignant than in non-malignant biopsies ($p = 0.008$), but were nonetheless very low in both groups (median 0.000004 vs. 0.000064) and also never exceeded 0.008. Median HPyV10 loads of tonsillar cancers were 0.000075, and the highest HPyV10 load found was 0.0006. Cumulative HPyV loads were not different in non-malignant compared to malignant tissue ($p = 0.320$; Table 4; Fig. 1).

HPV16 and HPV18 DNA loads found in two non-malignant samples were similarly low as HPyV loads (Table 4; Fig. 1). In contrast, HPV16 loads and cumulative HR-HPV loads (HPV16 + 18) were higher in malignant samples compared to non-malignant tissue (median 67.469 vs. 0.000014 and 11.861 vs. 0.000007, respectively) (Table 4, Fig. 1). The difference was statistically significant for the latter ($p = 0.012$). Nine of the sixteen HPV16-positive cancers had a viral load above 10 copies per beta-globin gene copy and thus were most likely HPV induced. When comparing HR-HPV DNA loads of the HPV-positive cancer patients according to their smoking status, the non-smokers had significantly higher viral loads than the smokers ($p = 0.047$) (Fig. 2). All seven cancer patients with very low HPV16 or HPV18 loads (below 0.05 HPV copies per beta-globin gene copy) were smokers.

In seven cancer biopsies (and in none of the non-malignant samples), both HPV and HPyV DNA were detected (HPV16 + MCPyV, $n = 4$; HPV16 + HPyV10, $n = 3$). In these samples, HPV16 DNA loads were significantly higher than HPyV loads ($p = 0.031$).

Discussion

We have analyzed HPyV and HPV DNA prevalence and viral load in 78 tonsillar biopsies comprising 40 non-malignant and 38 SCC samples. Seven of the 13 analyzed HPyVs were not found in any of the biopsies, and JCPyV and WUPyV were only found in a small percentage of the non-malignant samples. The most frequently found HPyV was MCPyV, both in non-malignant and in cancer tissue, but the difference in prevalence between the two sample groups was not significant. HPyV6 was found in 3 biopsies, one non-malignant and two tonsillar cancer biopsies, without a significant difference between the two groups. HPyV7 was only found in one cancer biopsy. HPyV10 was the only

polyomavirus detected significantly more frequently in malignant compared to non-malignant tissue (18.4 vs. 0%), but HPyV10 DNA loads found in the tumor tissue were extremely low (see below). Since the patients with tonsillar cancer were significantly older than those with non-malignant disease, the difference in HPyV10 prevalence could be attributable to age. It has been shown for several HPyVs that HPyV (sero)prevalence increases with age [16]. For HPyV10, Karachaliou et al. [47] report a seroprevalence of 55.6% in 3-year-old children and of 82.3% in 4-year-old children. Gossai et al. [19] found a HPyV10 seroprevalence of 99.1% for adults from 24 to 75 years, without significant differences between age groups, between males and females, and between smokers and non-smokers.

To date, only relatively few studies on HPyVs in tonsils have been published with only one other study, Salakova et al. [48], focusing on the recently discovered HPyVs and tonsillar cancer. BKPyV and JCPyV, two HPyVs discovered 45 years ago, have been detected by PCR in 0.3–6.9% (BKPyV) and in 0–44% (JCPyV) of non-malignant tonsillar tissue in different studies [49–55]. Two earlier studies found JCPyV in 39 and 44% of non-malignant tonsillar tissue with tonsillitis or tonsillar hypertrophy using T-antigen or regulatory region targeting nested PCR protocols [53, 54]. Several more recent single-round PCR-based studies could not detect JCPyV DNA in non-malignant tonsillar tissue [49–52]. Differences in the detection rates of non-integrating polyomaviruses such as JCPyV and BKPyV should not depend on the genomic region targeted by PCR. We could confirm the low prevalence of JCPyV found in our study with a VP1-directed PCR also with a second PCR protocol [56] targeting the N-terminus of the T-antigen (data not shown). We therefore speculate that the high JCPyV prevalence found in earlier studies might be due to either a higher sensitivity or proneness to contamination of the nested PCR. Günel et al. [57] have analyzed specimens of children with recurrent tonsillitis ($n = 26$) or tonsillar hypertrophy ($n = 25$) for the presence of Epstein–Barr virus, Human Bocavirus, WUPyV and KIPyV DNA. While they found Epstein–Barr virus and Bocavirus in 12.0–53.8% of samples, they did not detect KIPyV in any of the biopsies, and WUPyV was detected in 2 of the 26 (7.7%) recurrent tonsillitis samples but in none of the tonsillar hypertrophy samples. This is almost identical to the prevalence rates found in our study for KIPyV (0%) and WUPyV (7.5%) in non-malignant samples. KIPyV and WUPyV have also been detected in previous PCR-based studies of non-malignant tonsillar tissue with prevalence rates reaching from 0 to 13.2% (KIPyV) and 0–12.0% (WUPyV) [49, 50, 52, 58–60]. Sadeghi et al. [22] found TSPyV DNA in 8 of 229 (3.5%) tonsillar tissues of children and adults with chronic tonsillitis or tonsillar hypertrophy. Two of the TSPyV-positive cases were co-infected with KIPyV or WUPyV. In contrast, we have not found TSPyV in any of our samples. However, we have analyzed fewer non-malignant biopsies than Sadeghi et al., and our sample size might have been too small to detect viruses with a low prevalence rate. Peng et al. [61] discovered MWPyV, a variant of HPyV10, and STLPyV in 2% (2/99) of swabs from the surface of palatine tonsils of Chinese children with chronic tonsillar disease. In our study, neither HPyV10 nor STLPyV was detected in non-malignant tonsils. Peng et al., however, studied more samples and did not analyze tonsillar biopsies. Recently, Salakova et al. analyzed MCPyV, HPyV6 and HPyV7 in over 200 non-malignant and malignant tonsillar tissue samples [48]. They found MCPyV, HPyV6 and HPyV7 in 10.2, 4.6 and 0.9% of non-malignant biopsies, compared to 35.7, 5.4 and 1.8% in malignant tissue, respectively [48]. Our results for these three HPyVs are closely in line with the data of Salakova et al., with both studies reporting similar HPyV DNA detection rates. In contrast to our study, the difference in MCPyV prevalence between non-malignant and malignant tissue was statistically significant in the study by Salakova et al. [48]. Kantola et al. [60] also found MCPyV DNA in non-malignant tonsillar tissues, although in only 3.5% of 229 tonsillectomy patients, which is lower than in our study and in the study of Salakova et al. [48]. Altogether, our data and these of the above-mentioned papers on HPyV prevalence suggest that lymphoid tissue such as palatine tonsils could be a possible site of persistence for several HPyVs.

Viral loads of all analyzed HPyVs were very low, below 0.01 viral copies per beta-globin gene copy for all samples, with the exception of one oropharyngeal (HPV-negative) cancer of a 62-year-old smoking male with a HyPV6 load of 0.7 copies per beta-globin gene copy. MCPyV-positive cancers had significantly higher loads than non-malignant tonsillar biopsies, but MCPyV loads were very low in both groups, and the maximum MCPyV load found in a cancer biopsy was 0.008. In contrast, viral loads of MCPyV-associated Merkel cell carcinomas were regularly above 0.1 and reached values of up to 1500 MCPyV copies per beta-globin gene copy in previous studies [62, 63]. Similar to MCPyV, HPyV10 loads were very low in all HPyV10-positive cancers (maximum 0.0006). The low HPyV DNA loads we found are in line with low tonsillar DNA loads found by others for BKPyV, MCPyV, HPyV6 or HPyV7 in non-malignant [48, 51, 55] and malignant tonsillar tissue [48]. Furthermore, Salakova et al. could not detect statistically significant viral load differences for MCPyV, HPyV6 and HPyV7 between non-malignant and malignant tonsillar tissue. It has been shown for HPV that elevated viral loads are found in HPV-induced premalignant lesions and cancers [40, 64–66]. Therefore, the very low HPyV loads found in the tonsillar cancer biopsies are in sharp contrast to both MCPyV loads of Merkel cell carcinomas and HPV

loads in HPV-related lesions, thus making a causal role for MCPyV, HPyV6, HPyV7 or HPyV10 in tonsillar cancer development or maintenance unlikely. Polyomavirus Large-T-antigen is usually expressed in MCPyV-positive Merkel cell carcinomas [28, 67]. Unfortunately, material for Large-T-antigen immunohistochemistry was not available in our study, but we have shown previously that Large-T-antigen is not expressed in MCPyV-positive skin tumors or cutaneous lymphomas with low MCPyV DNA loads [63, 68, 69].

It has been shown that an increasing number of tonsillar and base of the tongue carcinomas are HPV associated [1, 3]. In our study, 44.7% of tonsillar cancers were HPV-positive, which is in line with recent studies of oropharyngeal cancer in central Europe but lower than found for northern Europe or North America [5, 11, 70, 71]. As in our study, HPV16 has consistently been the predominant type, and other HR-HPV types have only been found in a small subset of HPV-positive head and neck cancers [5, 9].

We have detected HR-HPV DNA in 5.0% of non-malignant tonsillar samples, which is in line with a previous study that found HPV16 DNA in 6.3% of 206 biopsies of patients with tonsillitis or tonsillar hypertrophy [72]. HPV loads were very low in the two HPV-positive non-malignant samples of our study. In contrast, cumulative HPV loads found in cancer biopsies were significantly higher, reaching over 3000 HPV DNA copies per beta-globin gene copy (median 12). This is in line with previous studies on HPV DNA loads in HPV-induced oropharyngeal cancers [73–75]. Interestingly, non-smoking cancer patients had significantly higher HPV loads than smokers, and all seven cancer patients with very low HPV DNA loads were smokers, strongly suggesting that these tumors were not HPV induced but rather smoking related.

Prophylactic HPV vaccination of boys is currently only recommended in a few countries (e.g., USA, Canada, Australia, Austria, Switzerland), in contrast to the HPV vaccination of girls [76]. It has not yet been shown that prophylactic HPV vaccination can reduce or prevent HPV-associated oropharyngeal cancer, but it has been demonstrated that it can significantly reduce oral HPV16 infection [77, 78]. Of the 17 HPV-positive cancers found in our study, 16 were from male patients including nine cancers with high HPV16 loads (>10) that were most likely HPV induced. This is in line with the male predominance previously described for HPV-positive oropharyngeal cancers [1]. Hopefully, gender-neutral HPV vaccination before HPV acquisition (sexual debut) will be able to prevent HPV-associated oropharyngeal cancers in the future and reverse the rising incidence described for this cancer entity.

Our study had some limitations: (1) The number of samples included in the analysis was small (<100), and HPyVs with low prevalence rates might have been missed. A larger sample also could have yielded different results concerning HPyV load distribution. (2) The age distribution, gender and smoker status were different in both groups with only a small overlap. (3) Data on lifestyle factors such as sexual preferences (oral sex) and on socioeconomic factors were not available. (4) Since only extracted DNA was available, we could neither perform in situ hybridization to localize the detected viruses within the tonsils nor immunohistochemistry to analyze Large-T-antigen expression in MCPyV DNA-positive samples. (5) We have not performed further diagnostic tests such as p16 immunohistochemistry or HPV E6/E7-mRNA determination to corroborate the attribution of HPV in the HPV DNA-positive cancers [5, 79], but the high HPV16 loads found in a subset of the samples make it likely that these cancers were HPV induced.

In summary, of the 13 HPyVs analyzed, only six have been found in tonsils and only MCPyV, HPyV6, HPyV7 and HPyV10 were detectable in cancer samples, but, with the exception of HPyV10, detection rates were similar in malignant and non-malignant tonsillar tissue. In non-malignant samples, both HPyV and HPV loads were very low with levels below 0.002 viral copies per beta-globin gene. In contrast, tonsillar HPV16 loads of non-smoking cancers patients regularly exceeded one copy per beta-globin gene copy, while HPyV loads found in cancer samples were extremely low, with a median cumulative load of 0.000071, and generally not higher than those found in non-malignant tissue. This argues against a causal role of HPyV in tonsillar carcinogenesis as it has already been discussed for other malignancies [59, 80]. While both HPV and HPyVs may persist in tonsillar tissue, only HR-HPVs seem to play a role in tonsillar carcinogenesis. It has been discussed whether HPyVs are co-factors for cancers induced by other oncoviruses such as anal, cervical, laryngeal, hepatic, gastric or skin cancer, but a link between co-infection and cancer could not yet be established [81]. Our data on HPyV DNA prevalence and load in malignant and non-malignant tonsillar tissue support the notion that HPyVs do not play a role in tonsillar carcinogenesis, neither as independent etiologic agents nor as co-infecting agents of HPV.

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References

1. Gillison ML, Chaturvedi AK, Anderson WF, Fakhry C (2015) Epidemiology of human papillomavirus-positive head and neck squamous cell carcinoma. J Clin Oncol 33(29):3235–3242. doi:10.1200/JCO.2015.61.6995
2. Hübbers CU, Akgül B (2015) HPV and cancer of the oral cavity. Virulence 6(3):244–248. doi:10.1080/21505594.2014.999570

3. Young D, Xiao CC, Murphy B, Moore M, Fakhry C, Day TA (2015) Increase in head and neck cancer in younger patients due to human papillomavirus (HPV). Oral Oncol 51(8):727–730. doi:10.1016/j.oraloncology.2015.03.015
4. Gooi Z, Chan JY, Fakhry C (2016) The epidemiology of the human papillomavirus related to oropharyngeal head and neck cancer. Laryngoscope 126(4):894–900. doi:10.1002/lary.25767
5. Ndiaye C, Mena M, Alemany L, Arbyn M, Castellsague X, Laporte L, Bosch FX, de Sanjose S, Trottier H (2014) HPV DNA, E6/E7 mRNA, and p16INK4a detection in head and neck cancers: a systematic review and meta-analysis. Lancet Oncol 15(12):1319–1331. doi:10.1016/S1470-2045(14)70471-1
6. Deschler DG, Richmon JD, Khariwala SS, Ferris RL, Wang MB (2014) The "new" head and neck cancer patient-young, nonsmoker, nondrinker, and HPV positive: evaluation. Otolaryngol Head Neck Surg 151(3):375–380. doi:10.1177/0194599814538605
7. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127(12):2893–2917. doi:10.1002/ijc.25516
8. RKI (2015) Krebs in Deutschland 2011/2012, 10th edn. Robert Koch Institut, Berlin
9. Fakhry C, Westra WH, Li S, Cmelak A, Ridge JA, Pinto H, Forastiere A, Gillison ML (2008) Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J Natl Cancer Inst 100(4):261–269. doi:10.1093/jnci/djn011
10. Rosenthal DI, Harari PM, Giralt J, Bell D, Raben D, Liu J, Schulten J, Ang KK, Bonner JA (2016) Association of human papillomavirus and p16 status with outcomes in the IMCL-9815 phase III registration trial for patients with locoregionally advanced oropharyngeal squamous cell carcinoma of the head and neck treated with radiotherapy with or without cetuximab. J Clin Oncol 34(12):1300–1308. doi:10.1200/JCO.2015.62.5970
11. Tinhofer I, Johrens K, Keilholz U, Kaufmann A, Lehmann A, Weichert W, Stenzinger A, Stromberger C, Klinghammer K, Becker ET, Dommerich S, Stolzel K, Hofmann VM, Hildebrandt B, Moser L, Ervens J, Bottcher A, Albers A, Stabenow R, Reinecke A, Budach V, Hoffmeister B, Raguse JD (2015) Contribution of human papilloma virus to the incidence of squamous cell carcinoma of the head and neck in a European population with high smoking prevalence. Eur J Cancer 51(4):514–521. doi:10.1016/j.ejca.2014.12.018
12. Sedghizadeh PP, Billington WD, Paxton D, Ebeed R, Mahabady S, Clark GT, Enciso R (2016) Is p16-positive oropharyngeal squamous cell carcinoma associated with favorable prognosis? A systematic review and meta-analysis. Oral Oncol 54:15–27. doi:10.1016/j.oraloncology.2016.01.002
13. Guo T, Eisele DW, Fakhry C (2016) The potential impact of prophylactic human papillomavirus vaccination on oropharyngeal cancer. Cancer. doi:10.1002/cncr.29992
14. Starr P (2015) Encouraging results for pembrolizumab in head and neck cancer. Am Health Drug Benefits 8(Spec Issue):16
15. DeCaprio JA, Garcea RL (2013) A cornucopia of human polyomaviruses. Nat Rev Microbiol 11(4):264–276. doi:10.1038/nrmicro2992
16. Ehlers B, Wieland U (2013) The novel human polyomaviruses HPyV6, 7, 9 and beyond. APMIS 121(8):783–795. doi:10.1111/apm.12104
17. Feltkamp MC, Kazem S, van der Meijden E, Lauber C, Gorbalenya AE (2013) From Stockholm to Malawi: recent developments in studying human polyomaviruses. J Gen Virol 94(Pt 3):482–496. doi:10.1099/vir.0.048462-0
18. Mishra N, Pereira M, Rhodes RH, An P, Pipas JM, Jain K, Kapoor A, Briese T, Faust PL, Lipkin WI (2014) Identification of a novel polyomavirus in a pancreatic transplant recipient with retinal blindness and vasculitic myopathy. J Infect Dis 210(10):1595–1599. doi:10.1093/infdis/jiu250
19. Gossai A, Waterboer T, Nelson HH, Michel A, Willhauck-Fleckenstein M, Farzan SF, Hoen AG, Christensen BC, Kelsey KT, Marsit CJ, Pawlita M, Karagas MR (2016) Seroepidemiology of human polyomaviruses in a US population. Am J Epidemiol 183(1):61–69. doi:10.1093/aje/kwv155
20. Kean JM, Rao S, Wang M, Garcea RL (2009) Seroepidemiology of human polyomaviruses. PLoS Pathog 5(3):e1000363. doi:10.1371/journal.ppat.1000363
21. Nicol JT, Robinot R, Carpentier A, Carandina G, Mazzoni E, Tognon M, Touze A, Coursaget P (2013) Age-specific seroprevalences of merkel cell polyomavirus, human polyomaviruses 6, 7, and 9, and trichodysplasia spinulosa-associated polyomavirus. Clin Vaccine Immunol 20(3):363–368. doi:10.1128/CVI.00438-12
22. Sadeghi M, Aaltonen LM, Hedman L, Chen T, Soderlund-Venermo M, Hedman K (2014) Detection of TS polyomavirus DNA in tonsillar tissues of children and adults: evidence for site of viral latency. J Clin Virol 59(1):55–58. doi:10.1016/j.jcv.2013.11.008
23. Akhgari S, Mohraz M, Azadmanesh K, Vahabpour R, Kazemimanesh M, Aghakhani A, Jozpanahi M, Banifazl M, Bavand A, Ramezani A (2016) Frequency and subtype of BK virus infection in Iranian patients infected with HIV. Med Microbiol Immunol 205(1):57–62. doi:10.1007/s00430-015-0426-x
24. Hirsch HH, Kardas P, Kranz D, Leboeuf C (2013) The human JC polyomavirus (JCPyV): virological background and clinical implications. APMIS 121(8):685–727. doi:10.1111/apm.12128
25. Rinaldo CH, Tylden GD, Sharma BN (2013) The human polyomavirus BK (BKPyV): virological background and clinical implications. APMIS 121(8):728–745. doi:10.1111/apm.12134
26. Teutsch K, Schweitzer F, Knops E, Kaiser R, Pfister H, Verheyen J, Gobel H, Cingoz T, Di Cristanziano V (2015) Early identification of renal transplant recipients with high risk of polyomavirus-associated nephropathy. Med Microbiol Immunol 204(6):657–664. doi:10.1007/s00430-015-0398-x
27. Feng H, Shuda M, Chang Y, Moore PS (2008) Clonal integration of a polyomavirus in human Merkel cell carcinoma. Science 319(5866):1096–1100. doi:10.1126/science.1152586
28. Shuda M, Arora R, Kwun HJ, Feng H, Sarid R, Fernandez-Figueras MT, Tolstov Y, Gjoerup O, Mansukhani MM, Swerdlow SH, Chaudhary PM, Kirkwood JM, Nalesnik MA, Kant JA, Weiss LM, Moore PS, Chang Y (2009) Human Merkel cell polyomavirus infection I. MCV T antigen expression in Merkel cell carcinoma, lymphoid tissues and lymphoid tumors. Int J Cancer 125(6):1243–1249. doi:10.1002/ijc.24510
29. Ho J, Jedrych JJ, Feng H, Natalie AA, Grandinetti L, Mirvish E, Crespo MM, Yadav D, Fasanella KE, Proksell S, Kuan SF, Pastrana DV, Buck CB, Shuda Y, Moore PS, Chang Y (2015) Human polyomavirus 7-associated pruritic rash and viremia in transplant recipients. J Infect Dis 211(10):1560–1565. doi:10.1093/infdis/jiu524
30. Kazem S, van der Meijden E, Feltkamp MC (2013) The trichodysplasia spinulosa-associated polyomavirus: virological background and clinical implications. APMIS 121(8):770–782. doi:10.1111/apm.12092
31. Buck CB, Phan GQ, Raiji MT, Murphy PM, McDermott DH, McBride AA (2012) Complete genome sequence of a tenth human polyomavirus. J Virol 86(19):10887. doi:10.1128/JVI.01690-12
32. Korup S, Rietscher J, Calvignac-Spencer S, Trusch F, Hofmann J, Moens U, Sauer I, Voigt S, Schmuck R, Ehlers B (2013) Identification of a novel human polyomavirus in organs of the

gastrointestinal tract. PLoS ONE 8(3):e58021. doi:10.1371/journal.pone.0058021
33. Lim ES, Reyes A, Antonio M, Saha D, Ikumapayi UN, Adeyemi M, Stine OC, Skelton R, Brennan DC, Mkakosya RS, Manary MJ, Gordon JI, Wang D (2013) Discovery of STL polyomavirus, a polyomavirus of ancestral recombinant origin that encodes a unique T antigen by alternative splicing. Virology 436(2):295–303. doi:10.1016/j.virol.2012.12.005
34. Schowalter RM, Pastrana DV, Pumphrey KA, Moyer AL, Buck CB (2010) Merkel cell polyomavirus and two previously unknown polyomaviruses are chronically shed from human skin. Cell Host Microbe 7(6):509–515. doi:10.1016/j.chom.2010.05.006
35. Scuda N, Hofmann J, Calvignac-Spencer S, Ruprecht K, Liman P, Kuhn J, Hengel H, Ehlers B (2011) A novel human polyomavirus closely related to the african green monkey-derived lymphotropic polyomavirus. J Virol 85(9):4586–4590. doi:10.1128/JVI.02602-10
36. Siebrasse EA, Reyes A, Lim ES, Zhao G, Mkakosya RS, Manary MJ, Gordon JI, Wang D (2012) Identification of MW polyomavirus, a novel polyomavirus in human stool. J Virol 86(19):10321–10326. doi:10.1128/JVI.01210-12
37. Yu G, Greninger AL, Isa P, Phan TG, Martinez MA, de la Luz Sanchez M, Contreras JF, Santos-Preciado JI, Parsonnet J, Miller S, DeRisi JL, Delwart E, Arias CF, Chiu CY (2012) Discovery of a novel polyomavirus in acute diarrheal samples from children. PLoS ONE 7(11):e49449. doi:10.1371/journal.pone.0049449
38. Falcone V, Ridder GJ, Panning M, Bierbaum S, Neumann-Haefelin D, Huzly D (2011) Human bocavirus DNA in paranasal sinus mucosa. Emerg Infect Dis 17(8):1564–1565. doi:10.3201/eid1708.101944
39. Wieland U, Silling S, Hellmich M, Potthoff A, Pfister H, Kreuter A (2014) Human polyomaviruses 6, 7, 9, 10 and Trichodysplasia spinulosa-associated polyomavirus in HIV-infected men. J Gen Virol 95(Pt 4):928–932. doi:10.1099/vir.0.061259-0
40. Wieland U, Hellmich M, Wetendorf J, Potthoff A, Hofler D, Swoboda J, Fuchs W, Brockmeyer N, Pfister H, Kreuter A (2015) Smoking and anal high-risk human papillomavirus DNA loads in HIV-positive men who have sex with men. Int J Med Microbiol 305(7):689–696. doi:10.1016/j.ijmm.2015.08.019
41. Sihto H, Kukko H, Koljonen V, Sankila R, Bohling T, Joensuu H (2009) Clinical factors associated with Merkel cell polyomavirus infection in Merkel cell carcinoma. J Natl Cancer Inst 101(13):938–945. doi:10.1093/jnci/djp139
42. Schmitt M, Bravo IG, Snijders PJ, Gissmann L, Pawlita M, Waterboer T (2006) Bead-based multiplex genotyping of human papillomaviruses. J Clin Microbiol 44(2):504–512. doi:10.1128/JCM.44.2.504-512.2006
43. Silling S, Kreuter A, Hellmich M, Swoboda J, Pfister H, Wieland U (2012) Human papillomavirus oncogene mRNA testing for the detection of anal dysplasia in HIV-positive men who have sex with men. J Clin Virol 53(4):325–331. doi:10.1016/j.jcv.2011.12.029
44. IARC (2012) Monographs on the evaluation of carcinogenic risks to humans. Biological agents. A review of human carcinogens., vol 100B. Lyon
45. van Duin M, Snijders PJ, Schrijnemakers HF, Voorhorst FJ, Rozendaal L, Nobbenhuis MA, van den Brule AJ, Verheijen RH, Helmerhorst TJ, Meijer CJ (2002) Human papillomavirus 16 load in normal and abnormal cervical scrapes: an indicator of CIN II/III and viral clearance. Int J Cancer 98(4):590–595
46. Wieland U, Silling S, Scola N, Potthoff A, Gambichler T, Brockmeyer NH, Pfister H, Kreuter A (2011) Merkel cell polyomavirus infection in HIV-positive men. Arch Dermatol 147(4):401–406. doi:10.1001/archdermatol.2011.42
47. Karachaliou M, Waterboer T, Casabonne D, Chalkiadaki G, Roumeliotaki T, Michel A, Stiakaki E, Chatzi L, Pawlita M, Kogevinas M, de Sanjose S (2016) The natural history of human polyomaviruses and herpesviruses in early life-the Rhea birth cohort in Greece. Am J Epidemiol 183(7):671–679. doi:10.1093/aje/kwv281
48. Salakova M, Koslabova E, Vojtechova Z, Tachezy R, Sroller V (2016) Detection of human polyomaviruses MCPyV, HPyV6, and HPyV7 in malignant and non-malignant tonsillar tissues. J Med Virol 88(4):695–702. doi:10.1002/jmv.24385
49. Astegiano S, Terlizzi ME, Elia M, Cavallo GP, Costa C, Cavallo R, Bergallo M (2010) Prevalence of polyomaviruses BK, JC, SV40, KI, and WU in non-malignant tonsil specimens. Minerva Med 101(6):385–389
50. Babakir-Mina M, Ciccozzi M, Bonifacio D, Bergallo M, Costa C, Cavallo R, Di Bonito L, Perno CF, Ciotti M (2009) Identification of the novel KI and WU polyomaviruses in human tonsils. J Clin Virol 46(1):75–79. doi:10.1016/j.jcv.2009.06.009
51. Comar M, Zanotta N, Bovenzi M, Campello C (2010) JCV/BKV and SV40 viral load in lymphoid tissues of young immunocompetent children from an area of north-east Italy. J Med Virol 82(7):1236–1240. doi:10.1002/jmv.21786
52. Comar M, Zanotta N, Rossi T, Pelos G, D'Agaro P (2011) Secondary lymphoid tissue as an important site for WU polyomavirus infection in immunocompetent children. J Med Virol 83(8):1446–1450. doi:10.1002/jmv.22124
53. Monaco MC, Jensen PN, Hou J, Durham LC, Major EO (1998) Detection of JC virus DNA in human tonsil tissue: evidence for site of initial viral infection. J Virol 72(12):9918–9923
54. Kato A, Kitamura T, Takasaka T, Tominaga T, Ishikawa A, Zheng HY, Yogo Y (2004) Detection of the archetypal regulatory region of JC virus from the tonsil tissue of patients with tonsillitis and tonsilar hypertrophy. J Neurovirol 10(4):244–249. doi:10.1080/13550280490468663
55. Sahiner F, Gumral R, Yildizoglu U, Babayigit MA, Durmaz A, Yigit N, Saracli MA, Kubar A (2014) Coexistence of Epstein–Barr virus and Parvovirus B19 in tonsillar tissue samples: quantitative measurement by real-time PCR. Int J Pediatr Otorhinolaryngol 78(8):1288–1293. doi:10.1016/j.ijporl.2014.05.012
56. Ryschkewitsch C, Jensen P, Hou J, Fahle G, Fischer S, Major EO (2004) Comparison of PCR-southern hybridization and quantitative real-time PCR for the detection of JC and BK viral nucleotide sequences in urine and cerebrospinal fluid. J Virol Methods 121(2):217–221. doi:10.1016/j.jviromet.2004.06.021
57. Günel C, Kirdar S, Omurlu IK, Agdas F (2015) Detection of the Epstein–Barr virus, human bocavirus and novel KI and KU polyomaviruses in adenotonsillar tissues. Int J Pediatr Otorhinolaryngol 79(3):423–427. doi:10.1016/j.ijporl.2015.01.007
58. Bergallo M, Terlizzi ME, Astegiano S, Ciotti M, Babakir-Mina M, Perno CF, Cavallo R, Costa C (2009) Real time PCR TaqMan assays for detection of polyomaviruses KIV and WUV in clinical samples. J Virol Methods 162(1–2):69–74. doi:10.1016/j.jviromet.2009.07.016
59. Papadimitriou JC, Randhawa P, Rinaldo CH, Drachenberg CB, Alexiev B, Hirsch HH (2016) BK polyomavirus infection and renourinary tumorigenesis. Am J Transpl 16(2):398–406. doi:10.1111/ajt.13550
60. Kantola K, Sadeghi M, Lahtinen A, Koskenvuo M, Aaltonen LM, Mottonen M, Rahiala J, Saarinen-Pihkala U, Riikonen P, Jartti T, Ruuskanen O, Soderlund-Venermo M, Hedman K (2009) Merkel cell polyomavirus DNA in tumor-free tonsillar tissues and upper respiratory tract samples: implications for respiratory transmission and latency. J Clin Virol 45(4):292–295. doi:10.1016/j.jcv.2009.04.008

61. Peng J, Li K, Zhang C, Jin Q (2016) MW polyomavirus and STL polyomavirus present in tonsillar tissues from children with chronic tonsillar disease. Clin Microbiol Infect 22(1):97.e1–3. doi:10.1016/j.cmi.2015.08.028
62. Schmitt M, Wieland U, Kreuter A, Pawlita M (2012) C-terminal deletions of Merkel cell polyomavirus large T-antigen, a highly specific surrogate marker for virally induced malignancy. Int J Cancer 131(12):2863–2868. doi:10.1002/ijc.27607
63. Wieland U, Scola N, Stolte B, Stucker M, Silling S, Kreuter A (2012) No evidence for a causal role of Merkel cell polyomavirus in keratoacanthoma. J Am Acad Dermatol 67(1):41–46. doi:10.1016/j.jaad.2011.07.026
64. Josefsson AM, Magnusson PK, Ylitalo N, Sorensen P, Qwarforth-Tubbin P, Andersen PK, Melbye M, Adami HO, Gyllensten UB (2000) Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study. Lancet 355(9222):2189–2193. doi:10.1016/S0140-6736(00)02401-6
65. Sundström K, Ploner A, Dahlstrom LA, Palmgren J, Dillner J, Adami HO, Ylitalo N, Sparen P (2013) Prospective study of HPV16 viral load and risk of in situ and invasive squamous cervical cancer. Cancer Epidemiol Biomarkers Prev 22(1):150–158. doi:10.1158/1055-9965.EPI-12-0953-T
66. Rödel F, Wieland U, Fraunholz I, Kitz J, Rave-Frank M, Wolff HA, Weiss C, Wirtz R, Balermpas P, Fokas E, Rodel C (2015) Human papillomavirus DNA load and p16INK4a expression predict for local control in patients with anal squamous cell carcinoma treated with chemoradiotherapy. Int J Cancer 136(2):278–288. doi:10.1002/ijc.28979
67. Nakamura T, Sato Y, Watanabe D, Ito H, Shimonohara N, Tsuji T, Nakajima N, Suzuki Y, Matsuo K, Nakagawa H, Sata T, Katano H (2010) Nuclear localization of Merkel cell polyomavirus large T antigen in Merkel cell carcinoma. Virology 398(2):273–279. doi:10.1016/j.virol.2009.12.024
68. Kreuter A, Silling S, Dewan M, Stucker M, Wieland U (2011) Evaluation of 4 recently discovered human polyomaviruses in primary cutaneous B-cell and T-cell lymphoma. Arch Dermatol 147(12):1449–1451. doi:10.1001/archdermatol.2011.330
69. Scola N, Wieland U, Silling S, Altmeyer P, Stucker M, Kreuter A (2012) Prevalence of human polyomaviruses in common and rare types of non-Merkel cell carcinoma skin cancer. Br J Dermatol 167(6):1315–1320. doi:10.1111/j.1365-2133.2012.11141.x
70. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, Westra WH, Gillison ML (2007) Case-control study of human papillomavirus and oropharyngeal cancer. N Engl J Med 356(19):1944–1956. doi:10.1056/NEJMoa065497
71. Rietbergen MM, Leemans CR, Bloemena E, Heideman DA, Braakhuis BJ, Hesselink AT, Witte BI, Baatenburg de Jong RJ, Meijer CJ, Snijders PJ, Brakenhoff RH (2013) Increasing prevalence rates of HPV attributable oropharyngeal squamous cell carcinomas in the Netherlands as assessed by a validated test algorithm. Int J Cancer 132(7):1565–1571. doi:10.1002/ijc.27821
72. Chen R, Sehr P, Waterboer T, Leivo I, Pawlita M, Vaheri A, Aaltonen LM (2005) Presence of DNA of human papillomavirus 16 but no other types in tumor-free tonsillar tissue. J Clin Microbiol 43(3):1408–1410. doi:10.1128/JCM.43.3.1408-1410.2005
73. Klussmann JP, Weissenborn SJ, Wieland U, Dries V, Kolligs J, Jungehuelsing M, Eckel HE, Dienes HP, Pfister HJ, Fuchs PG (2001) Prevalence, distribution, and viral load of human papillomavirus 16 DNA in tonsillar carcinomas. Cancer 92(11):2875–2884
74. Zheng Y, Xia P, Zheng HC, Takahashi H, Masuda S, Takano Y (2010) The screening of viral risk factors in tongue and pharyngolaryngeal squamous carcinoma. Anticancer Res 30(4):1233–1238
75. Mooren JJ, Gultekin SE, Straetmans JM, Haesevoets A, Peutz-Kootstra CJ, Huebbers CU, Dienes HP, Wieland U, Ramaekers FC, Kremer B, Speel EJ, Klussmann JP (2014) P16(INK4A) immunostaining is a strong indicator for high-risk-HPV-associated oropharyngeal carcinomas and dysplasias, but is unreliable to predict low-risk-HPV-infection in head and neck papillomas and laryngeal dysplasias. Int J Cancer 134(9):2108–2117. doi:10.1002/ijc.28534
76. Stanley M (2014) HPV vaccination in boys and men. Hum Vaccin Immunother 10(7):2109–2111. doi:10.4161/hv.29137
77. Herrero R, Quint W, Hildesheim A, Gonzalez P, Struijk L, Katki HA, Porras C, Schiffman M, Rodriguez AC, Solomon D, Jimenez S, Schiller JT, Lowy DR, van Doorn LJ, Wacholder S, Kreimer AR, Group CVTV (2013) Reduced prevalence of oral human papillomavirus (HPV) 4 years after bivalent HPV vaccination in a randomized clinical trial in Costa Rica. PLoS ONE 8(7):e68329. doi:10.1371/journal.pone.0068329
78. Grün N, Ahrlund-Richter A, Franzen J, Mirzaie L, Marions L, Ramqvist T, Dalianis T (2015) Oral human papillomavirus (HPV) prevalence in youth and cervical HPV prevalence in women attending a youth clinic in Sweden, a follow up-study 2013-2014 after gradual introduction of public HPV vaccination. Infect Dis (Lond) 47(1):57–61. doi:10.3109/00365548.2014.964764
79. Boscolo-Rizzo P, Pawlita M, Holzinger D (2016) From HPV-positive towards HPV-driven oropharyngeal squamous cell carcinomas. Cancer Treat Rev 42:24–29. doi:10.1016/j.ctrv.2015.10.009
80. Dalianis T, Hirsch HH (2013) Human polyomaviruses in disease and cancer. Virology 437(2):63–72. doi:10.1016/j.virol.2012.12.015
81. Moens U, Van Ghelue M, Ehlers B (2014) Are human polyomaviruses co-factors for cancers induced by other oncoviruses? Rev Med Virol 24(5):343–360. doi:10.1002/rmv.1798