

Prevention and Epidemiology

See commentary by Méndez, p. 4896

Viral RNA Patterns and High Viral Load Reliably Define Oropharynx Carcinomas with Active HPV16 InvolvementDana Holzinger^{1,2}, Markus Schmitt², Gerhard Dyckhoff¹, Axel Benner³, Michael Pawlita², and Franz X. Bosch¹**Abstract**

Oropharyngeal squamous cell carcinomas (OPSCC) that are associated with human papilloma virus (HPV) infection carry a more favorable prognosis than those that are HPV-negative. However, it remains unclear which biomarker(s) can reliably determine which OPSCC specimens are truly driven by HPV infection. In this study, we analyzed 199 fresh-frozen OPSCC specimens for HPV DNA, viral load, RNA expression patterns typical for cervical carcinomas (CxCaRNA⁺), and the HPV-targeted tumor suppressor protein p16^{INK4a} as markers for HPV infection. In this set of specimens, there was a 49% prevalence of DNA for the cancer-associated HPV type 16 (HPV⁺). However, there was only a 16% prevalence of high viral load and only a 20% prevalence of CxCaRNA⁺, a marker of HPV16 carcinogenic activity. Among the CxCaRNA⁺ tumors, 78% of the specimens exhibited overexpression of p16^{INK4a}, which also occurred in 14% of the HPV-negative tumors. Using a multivariate survival analysis with HPV negativity as the reference group, CxCaRNA⁺ as a single marker conferred the lowest risk of death [HR = 0.28, 95% confidence interval (CI), 0.13–0.61] from oropharyngeal cancer, closely followed by high viral load (HR = 0.32, 95% CI, 0.14–0.73). In contrast, a weaker inverse association was found for OPSCC that were HPV⁺ and p16^{INK4a} high (HR = 0.55, 95% CI, 0.29–1.08). In summary, our findings argued that viral load or RNA pattern analysis is better suited than p16^{INK4a} expression to identify HPV16-driven tumors in OPSCC patient populations. *Cancer Res*; 72(19); 4993–5003. ©2012 AACR.

Introduction

Specific oncogenic types of human papilloma viruses (HPV), most frequently HPV type 16, are causally associated with a subset of oropharyngeal squamous cell carcinomas (OPSCC) (1–4). There are some distinct differences between HPV-negative and HPV-positive OPSCC regarding gender, age, tumor histology, size and lymph node involvement, and also in lifestyle and sexual behavior of the patients (2, 5–11). Of interest, patients with HPV-positive OPSCC have an improved survival compared with patients with HPV-negative OPSCC (1, 2, 5, 10, 12–14).

In several studies, HPV DNA-positive OPSCC were found to be heterogeneous in both biological (e.g., in tumor histology, genetic status of p53, extent of cytogenetic alterations, expression of cell-cycle-related proteins) and clinical behavior (dis-

ease progression and patient survival; refs. 1, 3, 9, 15–21). Thus, the markers or marker combinations best suited to identify OPSCC with an active HPV involvement (HPV-driven OPSCC) are still unclear.

HPV DNA detection by *in situ* hybridization (ISH) theoretically should be best suited to identify HPV-driven tumors. However, ISH was found to lack sufficient sensitivity (22). In contrast, HPV DNA detection by quantitative PCR (qPCR) may lack disease specificity, but it allows precise determination of viral load. Viral load in turn can directly be compared with tumor biology and clinical outcome (8, 9). Overexpression of p16^{INK4a}, a consequence of the inactivation of the cellular retinoblastoma protein (pRb) by the viral oncoprotein E7, was proposed to be a surrogate marker for active HPV involvement in OPSCC carcinogenesis (8, 22, 23). However, high p16^{INK4a} alone also has insufficient sensitivity (13, 24–26) and specificity (22, 25–27). It was therefore suggested to use HPV DNA positivity in conjunction with high p16^{INK4a} to detect HPV-driven OPSCC with increased specificity (14, 26, 27). Because the oncogenic potential of high-risk HPV is mediated through continuous expression of the 2 major viral oncogenes E6 and E7 (28, 29), E6 and/or E7 RNA expression (in the lack of suitable protein reagents) indeed has shown good value as marker for active HPV (13, 16, 20, 21).

In HPV16-positive cervical carcinomas (CxCa) and cervical high-grade intraepithelial lesions, 2 characteristic RNA patterns have been found: upregulated E6*II (226^526) and/or E6*I (226^409) transcripts with a concurrently low or absent expression of the E1^E4 (880^3358) transcript (high E6*/E1^E4) and/or upregulated E1C (880^2582) transcript with a

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concurrently low or absent L1 (3632^5639) transcript (high E1C/L1; ref. 30). Whether HPV-positive OPSCC express viral RNA patterns as in CxCa is unclear. Also, viral load and expression of viral RNA patterns have not been directly compared with regard to clinical behavior and patient survival.

In this large German OPSCC case series ($n = 199$), the HPV DNA-positive tumors were analyzed in detail for viral load, viral oncogene RNA (E6*II and/or E6*I), and CxCa-like viral RNA patterns (CxCaRNA). We hypothesized that CxCa-like viral RNA patterns should be the best marker to identify truly HPV-driven OPSCC and, therefore, these patterns should be associated with the most favorable patient survival. It was further of interest to examine whether high p16^{INK4a} expression is concordant with these CxCa-like viral RNA patterns.

Materials and Methods

Patients, tissue samples

Of the patients diagnosed with primary OPSCC and treated at the ENT Department of the University Hospital Heidelberg, Germany, between 1990 and 2008, 199 gave informed consent and provided fresh-frozen tumor biopsies. The study was approved by the Ethics Committee of the Medical Faculty of the University of Heidelberg, study code 176/2002. Biopsies were directly snap-frozen in precooled isopentane/liquid nitrogen and stored at -80°C until further processing. Patient and tumor characteristics were obtained from clinical records. Management decisions for the patients were not guided by knowledge of HPV status or p16^{INK4a} expression. Information on first-line treatment modality was available for 184 patients. In 125 patients, surgery was applied, and in 59 patients with poor performance status or with nonresectable tumors, radiation and/or chemotherapy were applied first.

For nucleic acid extractions, cryosections of 16- μm thickness yielding between 2 and 10 mg of tissue were cut, homogenized in liquid nitrogen, and stored at -80°C .

After each biopsy, the cryostat was cleaned with acetone and ethanol and new gloves, forceps, and blades were used. For each homogenization, a fresh pistil was used. As negative controls, 2 mucosal biopsies from healthy patients were processed in the same way. To verify the presence and to estimate the content of tumor cells in cryosections, adjacent sections were stained with hematoxylin and eosin. The mean tumor cell content was 55% (range, 25%–90%).

Multiplex HPV genotyping and real-time qPCR for viral load determination

DNA was isolated using Qiagen's QIAamp DNA Mini Kit. Twenty-seven mucosal HPV types were analyzed simultaneously by BSGP5+/6+-PCR/MPG as described (31–33). The BSGP5+/6+-PCR/MPG assay comprises the BSGP5+/6+-PCR, which homogeneously amplifies all known genital HPV types generating biotinylated amplicons of approximately 150 bp from the L1 region (32, 33) and a multiplex HPV genotyping (MPG) assay with bead-based xMAP Luminex suspension array technology, which is able to simultaneously identify 27 HPV types and the β -globin gene (31, 32). The assay coamplifies cellular β -globin sequences as internal DNA quality control.

Quantification of HPV16 signals was accomplished as follows: to yield a relative HPV16 signal (in%), each measured HPV16 signal was first divided by the highest HPV16 signal observed among all tumor samples; the resulting relative HPV16 signal was then divided by the β -globin signal of the same sample to give a normalized viral load value (%HPV signal/ β -globin signal). A cutoff between low and high viral load of 0.0007 units was chosen. This high viral load cutoff has been developed for high-risk HPV types and optimized to distinguish cervical smears with normal cytology from those with abnormal cytology (manuscript in preparation).

Tumors positive for HPV16 DNA by BSGP5+/6+-PCR/MPG (HPV⁺) were further analyzed using real-time qPCR targeting HPV16-specific sequences of the E6 gene to obtain quantitative viral load values and an objective high viral load cutoff. qPCR for β -globin was used to verify the quality of DNA in the sample and to measure the amount of input DNA. Viral load in each specimen was expressed as the number of HPV genome copies per cell. Because on average the specimens contained approximately 50% tumor cells and at least 1 genome copy per cell is expected in HPV-transformed cells, we defined 0.5 copies per cell as cutoff for a high viral load (HPV_{high}) and OPSCC below this cutoff had a low viral load (HPV_{low}). Primer sequences used for real-time qPCR are available upon request (manuscript in preparation).

HPV16 RNA analysis

HPV16 DNA-positive OPSCC determined by BSGP5+/6+-PCR/MPG were analyzed for viral RNA expression. RNA was isolated using Qiagen's RNeasy Mini Kit. DNase I digestion (Qiagen) was included to ensure an exclusive amplification of RNA. Nucleic acid sequence-based amplification and hybridization to splice-specific probes on Luminex beads were carried out as described (30). Any E6*II and/or E6*I signal above background was considered positive (RNA⁺). For quantification of E6*II RNA, a calibrator RNA was competitively coamplified in the same reaction and used to normalize the E6*II signals. E6*I copy numbers in E6*II-negative samples were estimated by the same calibrator RNA. Tumors that were positive for E6*I and/or E6*II (RNA⁺) were further examined quantitatively for viral transcripts E1C (880^2582), E1^E4 (880^3358), and L1 (3632^5639) using their respective calibrators. For absolute quantification, each experiment included external standard dilution series of *in vitro* RNA. Transcript ratios were calculated for E6*II or E6*I over E1^E4 and for E1C over L1. Tumors with either an E6*/E1^E4 ratio of >1.5 (high E6*/E1^E4) and/or an E1C/L1 ratio of >0.003 (high E1C/L1) defined the CxCa-like viral RNA pattern-positive OPSCC (CxCaRNA⁺). OPSCC below both of these cutoffs defined the tumors without viral RNA patterns (CxCaRNA⁻). RNA of 1 healthy mucosa and of 1 HPV⁻ tumor served as negative controls. All RNA⁻ samples were positive for the ubiquitin C transcript and the controls were negative for all viral transcripts.

p16^{INK4a} immunohistochemistry on tissue microarray

OPSCCs (188 of 199) of this study were assembled on a tissue microarray (TMA) and were stained for p16^{INK4a} using 2

monoclonal antibodies, MTM-E6H4 (MTM Laboratories AG) and DSC-106 (Progen Biotech). For a detailed description of TMA preparation and immunohistochemistry (IHC), see refs. 34, 35. Tissue cores were evaluated by D. Holzinger and F.X. Bosch without knowledge of patient identities, clinical parameters, and HPV status. For tissue cores with discordant primary reading, a final decision was reached after joint re-evaluation and discussion. Scoring p16-high was in accordance with the literature (14, 22, 27, 36) and required strong nuclear and cytoplasmic staining in the proliferating tumor cells, guided, if necessary, by Ki-67 positivity. Patchy and negative staining was recorded as p16-low.

Statistical analysis

Patient and tumor characteristics were analyzed in relation to their HPV16 DNA and RNA status. Overall survival (OS) was measured as the time from the date of primary tumor diagnosis to the date of OPSCC-related death. One-hundred and eleven patients died during this study, out of which only 9 patients died unrelated to OPSCC. Survival times of patients who were alive at the date of last follow-up or were dead because of causes other than OPSCC were censored. Progression-free survival (PFS) time was calculated from the date of primary tumor diagnosis to the date of the first local recurrence, lymph node or distant metastasis, second primary carcinoma or date of OPSCC-related death (events), or to the date of OPSCC-unrelated death or last follow-up without progression (censored). Follow-up data after diagnosis were obtained from electronic health records of the University Hospital. In case of incomplete records, the resident physicians were contacted to obtain the follow-up data. The cutoff date for follow-up was 31 December, 2009. Thirty-five of 199 (18%) patients were not completely followed. However, the baseline characteristics, for example, age, gender, clinical stage, therapy status, and the distribution of HPV status of these patients were similar to the patients with complete follow-up. The method of Kaplan and Meier was used to estimate survival distributions. Median duration of follow-up was calculated according to Korn (37). Multivariate Cox proportional hazard models were used to analyze the effects of single HPV markers (DNA, viral load, viral E6*II and/or E6*I RNA, and viral RNA patterns) and of HPV marker combinations (HPV_{transf}⁻/HPV_{transf}⁺) as well as of p16^{INK4a} expression on OS and PFS together with covariates age, gender, clinical stage (I–III vs. IV), alcohol and tobacco consumption (never vs. current vs. former user), and first-line therapy (surgery vs. radiation/chemotherapy). By combining the HPV_{high} group ($n = 33$) with the CxCaRNA⁺ cases among the HPV_{low} group ($n = 9$), we formed the HPV_{transf}⁺ group ($n = 42$), whereas HPV_{low} tumors without viral RNA transcript expression formed the HPV_{transf}⁻ group ($n = 54$). For all HPV markers or marker combinations, the HPV DNA negative (HPV⁻) group was the reference category. For the IHC marker, tumors with a low p16^{INK4a} expression represented the reference category. Cox models were built using missing value imputation for all covariates by application of bootstrap resampling (38). The proportional hazards assumption, required for valid inference in Cox proportional hazards regression, was tested

according to Grambsch and Therneau (39) and was met for all covariates. The ability of the prediction models to classify risk for death and disease progression was assessed by computing the area under the time-dependent receiver operating characteristic (ROC) curves (40). To summarize and compare the prediction accuracy of the different Cox models, we calculated the integrated Brier score as a summary of the prediction error curve over the time interval of 5 years from date of primary tumor diagnosis (41). The prediction error was computed by the 0.632+ bootstrap estimate (42).

In all statistical tests a P value of 0.05 or below was considered as statistically significant. The statistical analyses were carried out using SAS, version 9.2, as well as the statistical software environment R, version 2.12.2.

Results

HPV-type prevalence and viral load

All 199 OPSCC biopsies yielded DNA of good quality with the β -globin gene coamplified in each sample. Of 100 OPSCC (50%) positive for HPV DNA analyzed by BSGP5+/6+-PCR/MPG, 97 contained HPV16 DNA (HPV⁺, Table 1, Fig. 1). Three tumors contained a single HPV genotype, HPV18, 33, or 35; these were not further analyzed here. No multiple infections were detected. To confirm the quantitative BSGP5+/6+-PCR/MPG results, all HPV⁺ tumors were again analyzed by qPCR. Eighty-six tumors were HPV16 DNA positive, resulting in 89% concordance ($r = 0.93$, $P < 0.01$; Spearman's rank correlation). Eleven tumors with low viral load by BSGP5+/6+-PCR/MPG were below detection level by qPCR but remained in the low viral load group for the further analyses. Thus, of the 97 HPV⁺ tumors, 64 (66%) had a low (HPV_{low}) and 33 (34%) had a high viral load (HPV_{high}; Fig. 1).

Quantitative assessment of HPV16 E6*I and E6*II RNA expression

Of 96 HPV⁺ tumors (one HPV_{low} sample was excluded because of insufficient biopsy material), 48 (50%, RNA⁺) expressed E6*I and 38 of them additionally expressed E6*II. Of 33 HPV_{high} tumors, 32 (97%) were RNA⁺ and all but 1 positive for both E6*I and E6*II. Of 64 HPV_{low} tumors, 16 (25%) were RNA⁺ (Fig. 1) and only 5 positive for both E6*I and E6*II.

HPV16 RNA patterns

Of 48 RNA⁺ tumors, 40 (83%) displayed CxCa-like viral RNA patterns (CxCaRNA⁺, Fig. 1). Of these, 12 (25%) displayed high E6*/E1^E4, 25 (52%) displayed high E1C/L1, and 3 (6%) displayed both patterns (Fig. 2). The 8 tumors not displaying CxCa-like viral RNA patterns (CxCaRNA⁻) exhibited very low signals for all viral transcripts.

HPV16 marker combinations

The groups HPV_{high} and CxCaRNA⁺ were in good agreement as 31 of 33 (94%) HPV_{high} tumors were CxCaRNA⁺. However, 9 CxCaRNA⁺ tumors were not detected by the marker HPV_{high} (Fig. 3). Assuming that CxCaRNA⁺ was a critical parameter for tissues transformed by HPV16 (HPV_{transf}), we combined the

Table 1. Patient and tumor characteristics in relation to HPV16 status studied on OPSCC collected between 1990 and 2008 in Heidelberg, Germany

Characteristics	Total N = 196 n (%)	HPV16 status			
		HPV ⁻ N = 99 n (%)	HPV ⁺ N = 97 n (%)	HPV _{transf} ⁻ N = 54 n (%)	HPV _{transf} ⁺ N = 42 n (%)
Gender					
Male	146 (74)	79 (80)	67 (69)	39 (72)	27 (64)
Female	50 (26)	20 (20)	30 (31)	15 (28)	15 (36)
Age, y					
Median	57.0	56.7	57.4	56.1	62.1
Oropharynx subsite					
Tonsils	84 (43)	33 (33)	51 (53)	29 (54)	21 (50)
Base of tongue	45 (23)	27 (27)	18 (19)	7 (13)	11 (26)
Other ^a	67 (34)	39 (40)	28 (28)	18 (33)	10 (24)
Tumor size					
T1–T2	87 (45)	44 (45)	43 (45)	20 (37)	23 (56)
T3–T4	107 (55)	54 (55)	53 (55)	34 (63)	18 (44)
Missing	2	1	1		1
Lymph nodes					
N0	47 (24)	24 (24)	23 (24)	18 (33)	5 (12)
N ⁺	147 (76)	74 (76)	73 (76)	36 (67)	36 (88)
Missing	2	1	1		1
Distant metastasis					
M0	179 (95)	93 (95)	86 (96)	47 (92)	38 (100)
M1	9 (5)	5 (5)	4 (4)	4 (8)	0 (0)
Missing	8	1	7	3	4
Clinical stage					
I–III	60 (31)	33 (34)	27 (28)	20 (37)	7 (17)
IV	134 (69)	65 (66)	69 (72)	34 (63)	34 (83)
Missing	2	1	1		1
First-line therapy					
Surgery	125 (68)	59 (64)	66 (72)	32 (63)	33 (83)
Radiation/chemotherapy	59 (32)	33 (36)	26 (28)	19 (37)	7 (17)
Missing	12	7	5	3	2
Tobacco					
Never	21 (11)	6 (6)	15 (16)	0 (0)	15 (37)
Former	20 (10)	9 (9)	11 (11)	6 (11)	5 (12)
Current	153 (79)	83 (85)	70 (73)	48 (92)	21 (51)
Missing	2	1	1		1
Alcohol					
Never	15 (8)	9 (9)	6 (6)	2 (4)	4 (10)
Former	23 (12)	18 (18)	5 (5)	4 (8)	1 (2)
Current	155 (80)	71 (73)	84 (89)	47 (92)	36 (88)
Missing	3	1	2	1	1

Abbreviations: HPV⁻, HPV16 DNA negative analyzed by BSGP5+/6+-PCR/MPG; HPV⁺, HPV16 DNA positive analyzed by BSGP5+/6+-PCR/MPG; HPV_{transf}⁻, HPV⁺ with low viral load analyzed by qPCR, but without CxCa-like viral RNA patterns; HPV_{transf}⁺, HPV⁺ with high viral load analyzed by qPCR and/or CxCa-like viral RNA patterns.

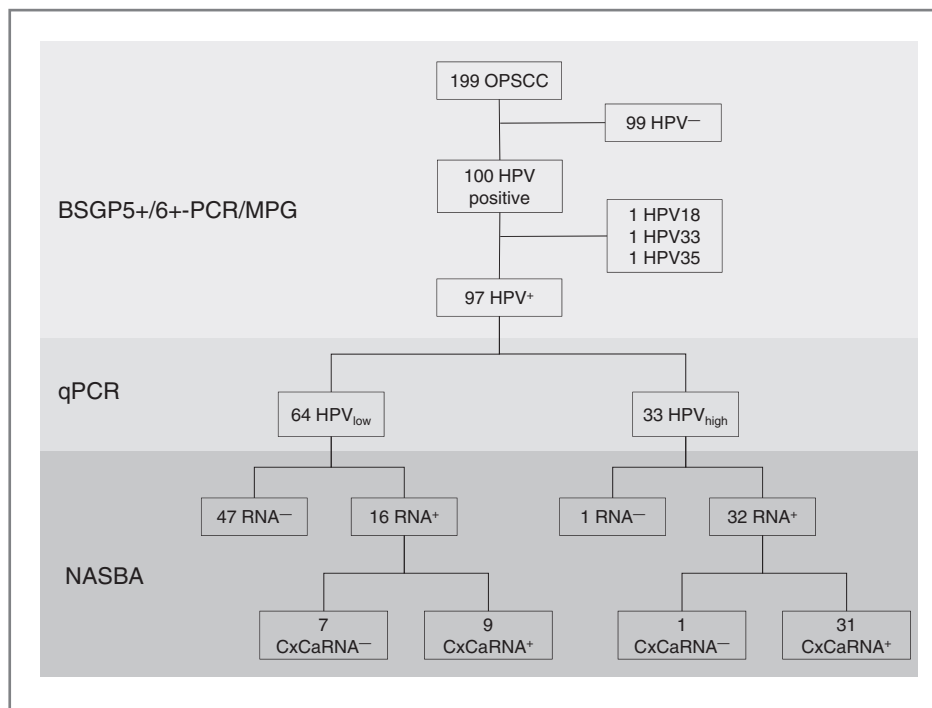
^aNineteen palate, 18 extending outside oropharynx (hypopharynx, nasopharynx, and/or larynx), 11 uvula, 19 unspecified.

HPV_{high} group ($n = 33$) with the CxCarNA⁺ cases among the HPV_{low} group ($n = 9$) to form the HPV_{transf}⁺ group ($n = 42$), whereas HPV_{low} tumors without CxCa-like viral RNA formed the HPV_{transf}⁻ group ($n = 54$).

Patient and tumor characteristics in relation to HPV16 DNA and RNA status

HPV⁻ and HPV⁺ tumors showed similar clinical characteristics except that the tonsillar subsite and alcohol

Figure 1. Overall flow chart showing the grouping of OPSCC in relation to DNA, viral load, and RNA status. HPV⁻, OPSCC negative for HPV DNA; HPV⁺, OPSCC positive for HPV16 DNA determined by multiplex HPV genotyping (BSGP5+/6+-PCR/MPG); HPV_{low} or HPV_{high}, OPSCC with low or high viral load determined by real-time qPCR; RNA⁻, OPSCC positive for HPV16 DNA, but negative for HPV16 E6⁺II and E6⁺I RNA; RNA⁺, OPSCC positive for HPV16 DNA and positive for HPV16 E6⁺II and/or E6⁺I RNA; CxCaRNA⁻, OPSCC positive for HPV16 DNA and for HPV16 E6⁺II and E6⁺I RNA, but without CxCa-like viral RNA patterns; CxCaRNA⁺, OPSCC positive for HPV16 DNA and for HPV16 E6⁺II and/or E6⁺I RNA with CxCa-like viral RNA patterns. NASBA, nucleic acid sequence-based amplification. Note that 1 of 64 HPV_{low} OPSCC was not included in RNA analyses because of insufficient tissue material.



consumption were associated with the HPV⁺ group. The HPV_{transf⁺} group in comparison to the HPV_{transf⁻} group was associated with older age, more lymph node involvement, advanced clinical stages, less receipt of radiation/chemother-

apy for first-line treatment, and it contained all never-smokers of the HPV⁺ group (Table 1).

Prevalence of HPV and high p16^{INK4a} expression in the tumors grouped according to HPV16 DNA and RNA status

HPV16 DNA prevalence increased from 37% in tumors collected between 1990 and 1999 to 63% in the tumors collected from 1999 to 2008. In contrast, the prevalence of HPV_{transf⁺} or CxCaRNA⁺ tumors did not change over time (Table 2). One-hundred and seventy eight of the 188 OPSCC included on the TMA could be evaluated. Fifty-four (30%) showed high p16^{INK4a} expression (Table 2, Supplementary Fig. S1). As expected, high p16^{INK4a} expression was significantly associated with the presence of HPV16. However, even in the HPV_{transf⁺} group, there was a sizeable fraction of discordant cases with 9 of 41 (22%) not showing high p16^{INK4a} levels. In the HPV⁺ group (HPV DNA positivity alone), only 42 out of 92 (46%) of the cases showed high p16^{INK4a} levels, whereas the HPV_{transf⁻} group showed a prevalence of high p16^{INK4a} expression (10 of 50; 20%) similar to the HPV⁻ reference group (12 of 85; 14%, Table 2).

OS and PFS in relation to HPV DNA, RNA, and p16^{INK4a}

The median follow-up time for this study was 103 months. In the HPV⁺ group, both OS and PFS were better than in the HPV⁻ group (median survival times 61 and 32 months in HPV⁺ patients vs. 26 and 12 months in HPV⁻ patients, respectively; Fig. 4A and B). However, stratifying the patients by viral load or RNA status revealed larger differences, and the strongest differences between survival curves was seen in the CxCaRNA⁺ or the HPV_{transf⁺} group (Fig. 4C-F). High p16^{INK4a} expression was also associated with better OS (median survival

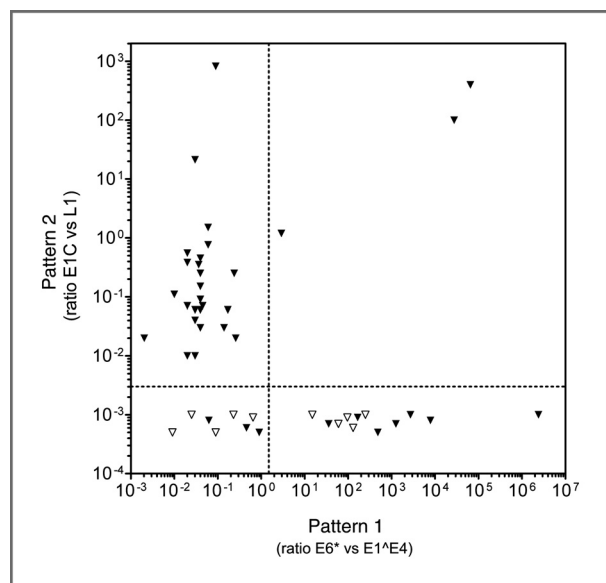


Figure 2. Viral RNA patterns analogous to CxCa in HPV16 E6⁺II and/or E6⁺I positive OPSCC ($n = 48$). Tumors with high E6⁺/E1^{E4} are located in the lower right quadrant ($n = 12$), tumors with high E1C/L1 in the upper left ($n = 25$), tumors with both patterns in the upper right ($n = 3$), and tumors without CxCa-like viral RNA patterns in the lower left quadrant ($n = 8$). Closed triangles, OPSCC positive for E6⁺II and E6⁺I; open triangles, OPSCC positive for E6⁺I only. Dashed lines represent thresholds for transcript ratios.

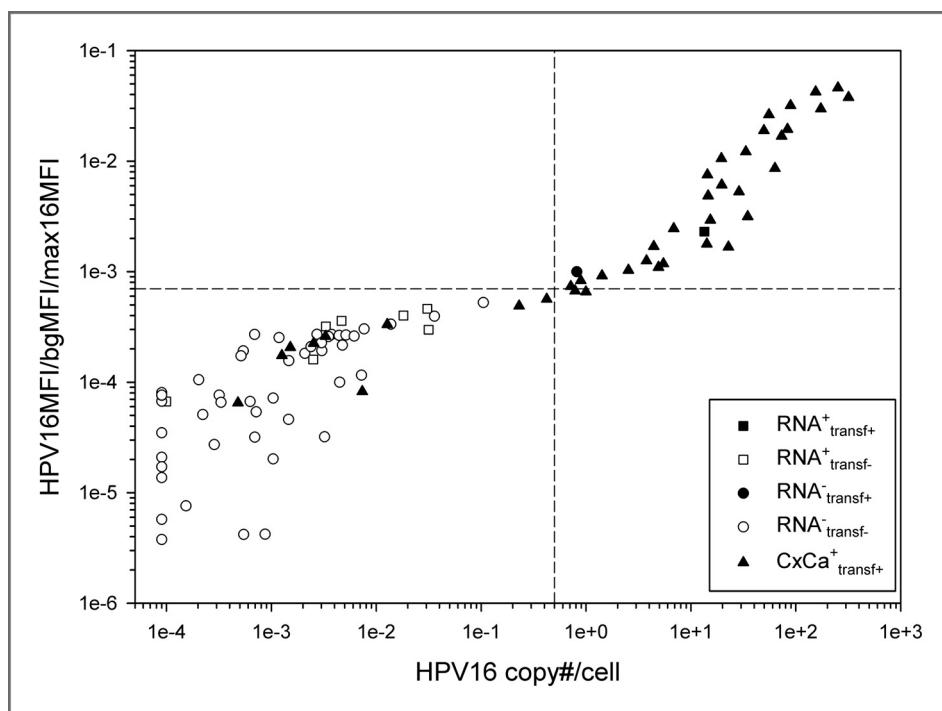


Figure 3. Correlation between HPV16 DNA copy numbers determined by qPCR and estimated HPV16 viral load using BSGP5+/6+-PCR/MPG in OPSCC ($n = 97$). Each symbol depicts 1 tumor; HPV_{transf}⁻ and HPV_{transf}⁺ tumors are displayed as open and closed symbols, respectively. Tumors were either positive for CxCa-like viral RNA patterns (CxCa⁺_{transf}⁺, closed triangles) or positive for viral E6*II and/or E6*I only (RNA⁺, squares). Circles denote E6*II- and E6*I-negative tumors (RNA⁻). Dashed lines represent cutoffs between low viral load (HPV_{low}) and high viral load (HPV_{high}) groups for each analysis ($CO_{BSGP5+/6+-PCR/MPG} 7 \times 10^{-4}$; $CO_{copy\#/cell} 0.5$). Note that 9 of 64 HPV_{low} tumors had CxCa-like viral RNA patterns; 1 HPV_{high} tumor was RNA negative. Viral load values (y-axis) analyzed by BSGP5+/6+-PCR/MPG correlated significantly with viral DNA copy numbers (x-axis), resulting from qPCR analysis ($r = 0.93$, $P < 0.001$; Spearman's rank correlation).

time 122 months in patients with p16-high vs. 34 months in patients with p16-low) and PFS times (median survival time 67 months in patients with p16-high vs. 20 months in

patients with p16-low; Fig. 4G and H), but performed much weaker compared with high viral load or RNA patterns. In addition, the combination p16-high and HPV⁺ did not

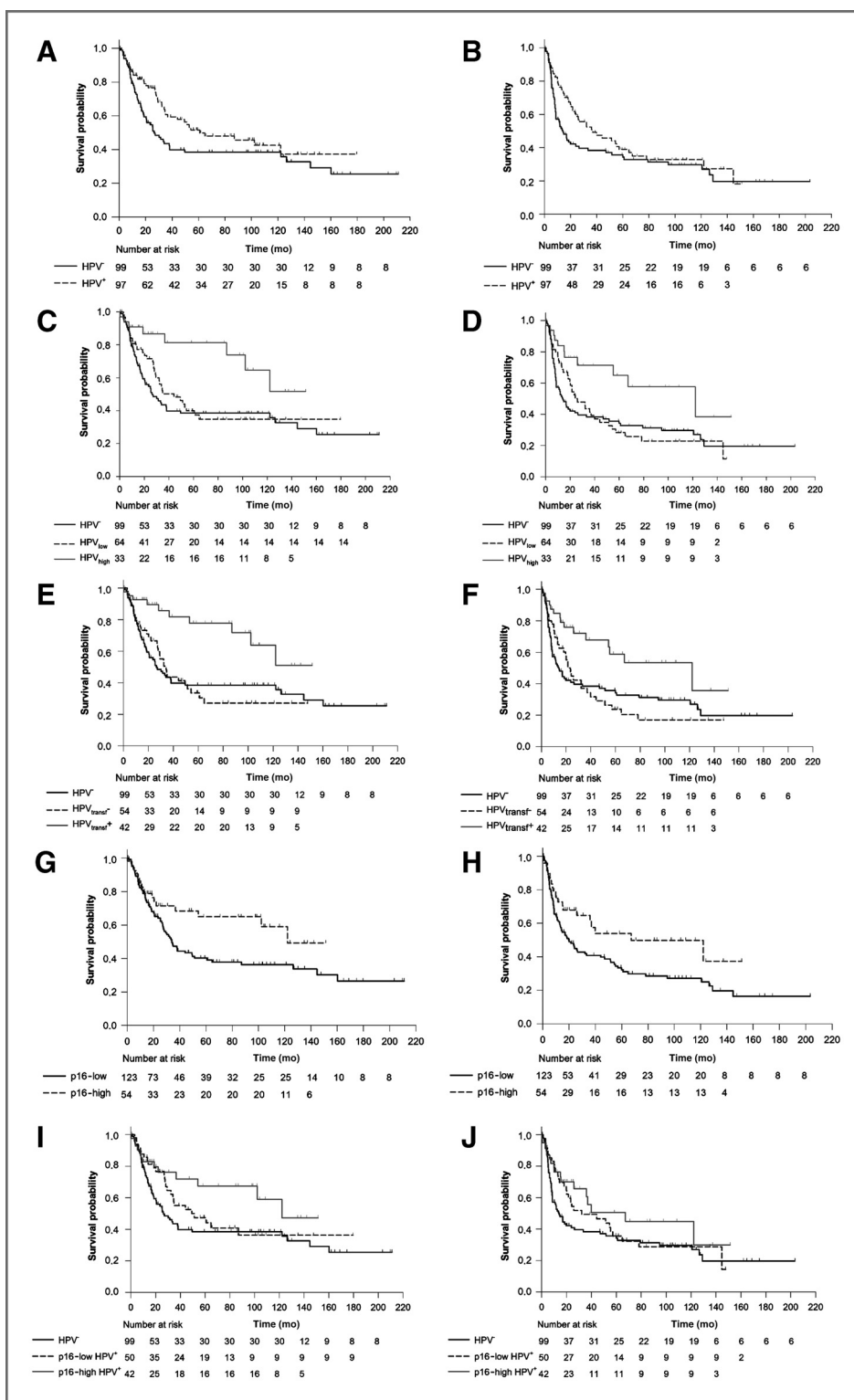
Table 2. Prevalence of HPV16 DNA and RNA and p16^{INK4a} expression according to HPV DNA and RNA status in a German OPSCC cohort, collected between 1990 and 2008 in Heidelberg

	HPV16 status									
	Total (N = 196) n (%)	HPV ⁻ (N = 99) n (%)	HPV ⁺ (N = 97) n (%)	P	RNA ⁻ (N = 56) n (%)	CxCaRNA ⁺ (N = 40) n (%)	P	HPV _{transf} ⁻ (N = 54) n (%)	HPV _{transf} ⁺ (N = 42) n (%)	P
Diagnosis date										
5/1990-7/1999	98 (50)	62 (63)	36 (37)		20 (36)	15 (38)		19 (35)	16 (38)	
8/1999-7/2008	98 (50)	37 (37)	61 (63)	<0.001	36 (64)	25 (62)	0.9	35 (65)	26 (62)	0.8
p16 ^{INK4a} , ^a										
Low	124 (70)	73 (86)	50 (54)		41 (79)	8 (21)		40 (80)	9 (22)	
High	54 (30)	12 (14)	42 (46)	<0.001	11 (21)	31 (79)	<0.001	10 (20)	32 (78)	<0.001
Missing	19	14	5		4	1		4	1	

Abbreviations: HPV⁻, HPV16 DNA-negative analyzed by BSGP5+/6+-PCR/MPG; HPV⁺, HPV16 DNA-positive analyzed by BSGP5+/6+-PCR/MPG; RNA⁻, HPV⁺ OPSCC without viral RNA patterns (CxCaRNA⁻) and without viral oncogene expression (E6*II, E6*I transcripts); CxCaRNA⁺, OPSCC with viral RNA patterns; HPV_{transf}⁻, HPV⁺ with low viral load analyzed by qPCR, but without CxCa-like viral RNA patterns; HPV_{transf}⁺, HPV⁺ with high viral load analyzed by qPCR and/or CxCa-like viral RNA patterns

^aBased on immunohistochemical analysis.

Figure 4. OS (A, C, E, G, I) and PFS (B, D, F, H, J) in relation to HPV16 status and p16^{INK4a} expression. Tumors were grouped by HPV16 DNA status (A, B), viral load (C, D), viral marker combinations (HPV_{transf}⁺, HPV_{high} tumors combined with HPV_{low} tumors positive for CxCa-like viral RNA patterns; HPV_{transf}⁻, HPV_{low} tumors without CxCa-like viral RNA patterns; E, F), p16^{INK4a} expression (p16-high, p16-low; G, H), and combination of p16^{INK4a} with HPV16 DNA (p16-low+HPV⁺, p16-high+HPV⁺; I, J). Note that survival was not significantly different if only HPV16 DNA status was assessed. Grouping by viral load status or by marker combinations revealed significant differences in OS and PFS. HPV⁻ or HPV⁺, OPSCC negative or positive for HPV16 DNA; HPV_{low} or HPV_{high}, OPSCC with low or high viral load.



provide improved OS or PFS over p16-high alone (Fig. 4I and J).

Multivariate Cox regression using only demographic, clinical, and behavioral factors showed a statistically significant

association of clinical stage IV (OS only), current tobacco consumption (PFS only), and the first-line therapy with a shorter survival (Table 3). Most notable, Cox proportional hazard models of HPV markers adjusting for demographic,

Table 3. Multivariate analysis of OS and PFS for OPSCC patients collected between 1990 and 2008 in Heidelberg, Germany

Parameter	OS		PFS	
	HR (95% CI)	P value	HR (95% CI)	P value
Demographic, clinical, and behavioral factors				
Age (10 y)	1.01 (0.82–1.26)	0.9	1.02 (0.84–1.24)	0.8
Gender (female vs. male)	1.09 (0.67–1.79)	0.7	0.94 (0.60–1.46)	0.8
Clinical stage (IV vs. I–III)	2.10 (1.25–3.54)	0.005	1.45 (0.94–2.24)	0.09
Therapy status (Radiation/chemotherapy vs. surgery)	1.95 (1.26–3.03)	0.003	1.71 (1.13–2.59)	0.01
Tobacco				
Current vs. never	2.13 (0.84–5.38)	0.1	2.56 (1.09–6.00)	0.03
Former vs. never	1.24 (0.39–3.92)	0.7	1.39 (0.50–3.88)	0.5
Alcohol				
Current vs. never	1.27 (0.50–3.22)	0.6	1.12 (0.48–2.63)	0.8
Former vs. never	1.41 (0.49–4.06)	0.5	1.30 (0.50–3.38)	0.6
Single HPV markers				
	n			
HPV ⁻ (reference)	99	1.00	1.00	
HPV ⁺	97	0.67 (0.44–1.03)	0.77 (0.53–1.12)	0.2
HPV ⁻ (reference)	99	1.00	1.00	
HPV _{low}	64	0.82 (0.53–1.26)	0.89 (0.60–1.31)	0.5
HPV _{high}	33	0.32 (0.14–0.73)	0.45 (0.23–0.90)	0.02
HPV ⁻ (reference)	99	1.00	1.00	
RNA ⁻	48	0.91 (0.56–1.46)	0.92 (0.60–1.43)	0.7
RNA ⁺	48	0.44 (0.24–0.80)	0.61 (0.37–1.02)	0.06
HPV ⁻ (reference)	99	1.00	1.00	
RNA ⁻ plus CxCaRNA ⁻	56	0.95 (0.61–1.49)	0.94 (0.62–1.42)	0.8
CxCaRNA ⁺	40	0.28 (0.13–0.61)	0.50 (0.27–0.92)	0.02
HPV marker combinations				
HPV ⁻ (reference)	99	1.00	1.00	
HPV _{transf} ⁻	54	0.96 (0.62–1.51)	0.96 (0.63–1.45)	0.8
HPV _{transf} ⁺	42	0.30 (0.14–0.62)	0.50 (0.28–0.90)	0.02
p16 ^{INK4a} Immunohistochemistry				
p16-low (reference)	123	1.00	1.00	
p16-high	54	0.90 (0.55–1.49)	1.07 (0.69–1.66)	0.8
p16 ^{INK4a} HPV DNA combination				
HPV ⁻ (reference)	99	1.00	1.00	
p16-low+HPV ⁺	50	0.70 (0.43–1.13)	0.74 (0.48–1.14)	0.2
p16-high+HPV ⁺	42	0.55 (0.29–1.08)	0.83 (0.47–1.44)	0.5

NOTE: HR for age, gender, clinical stage, therapy status, and alcohol and tobacco consumption were calculated using a Cox model including only these factors. HR for single HPV markers were computed using 4 different models: (i) DNA model (HPV⁺, HPV16 DNA-positive OPSCC), (ii) viral load model (HPV_{low}, OPSCC with low viral load; HPV_{high}, OPSCC with high viral load), (iii) RNA models (RNA⁻, DNA-positive and RNA-negative OPSCC; RNA⁺, DNA-positive and RNA-positive OPSCC, or (iv) RNA pattern model (RNA⁻ plus CxCaRNA⁻, DNA-positive and RNA-negative OPSCC plus DNA-positive and RNA-positive OPSCC without CxCa-like viral RNA patterns; CxCaRNA⁺, DNA-positive and RNA-positive with CxCa-like viral RNA patterns). HRs were also calculated for HPV marker combinations: HPV_{transf}⁻, OPSCC with low viral load, but without CxCa-like viral RNA patterns; HPV_{transf}⁺, OPSCC with high viral load and/or CxCa-like viral RNA patterns. HPV⁻ group (HPV16 DNA-negative OPSCC) resulting from BSGP5+/6+-PCR/MPG analysis was reference category for all models. p16-low or p16-high, OPSCC with low or high p16^{INK4a} expression level analyzed by IHC. Models were adjusted by age, gender, clinical stage, therapy status, and alcohol/tobacco consumption. Missing values in covariates were imputed. Statistically significant values are in bold. P values were calculated for each marker model separately.

clinical, and behavioral factors revealed a statistically significant association of HPV viral load and viral RNA patterns with both OS and PFS. Although DNA positivity alone was not related with a strong inverse association with outcomes,

HPV_{high} patients had a 68% and CxCaRNA⁺ patients a 72% lower risk of death from OPSCC compared with HPV⁻ patients. The HRs with respect to PFS were HR = 0.45 for HPV_{high} patients and HR = 0.50 for CxCaRNA⁺ patients compared with

HPV⁻ patients. The HPV_{transf}⁺ group (HPV_{high} and/or CxCarNA⁺) had a comparable survival advantage as the single marker groups HPV_{high} and CxCarNA⁺ (Table 3). Importantly, tumors without transcriptionally active HPV16 represented by the HPV_{transf}⁻ group conferred similar risks for OPSCC cancer death and tumor relapse as patients with HPV⁻ tumors (Table 3). Of interest, the combination p16-high and HPV⁺ conferred a decreased risk of OS but did not perform as well as CxCarNA⁺ or high viral load in the multivariate analysis. In contrast, p16^{INK4a} alone did not confer a decreased risk of OS. Both, p16-high and the combination of p16-high and HPV⁺ had no effect on PFS, in contrast to CxCarNA⁺ and high viral load (Table 3). Compared with the Cox model of demographic, clinical, and behavioral factors alone, prediction accuracy of OS was always improved by including the single HPV markers or marker combinations into the model, but not by including p16^{INK4a} alone. The strongest improvement resulted from using the HPV16 marker combination and CxCa-like RNA patterns. For OS, the cumulative prediction error decreased from 0.192 for the clinical factors model to 0.183 using additional CxCa-like RNA patterns and to 0.182 using additional HPV marker combination. On the contrary, no improvement in prediction accuracy was observed for PFS by adding HPV markers, p16^{INK4a}, or marker combinations to clinical factors (Supplementary Table S1). Similar results were obtained with respect to the area under the time-dependent ROC curves (Supplementary Fig. S2).

Discussion

We analyzed 199 OPSCC for the active involvement of HPV16 using viral load, viral oncogene RNA, CxCa-like viral RNA patterns, and p16^{INK4a} as markers. For the first time, we assessed whether the HPV16 DNA-positive OPSCC displayed similar viral RNA patterns as seen in HPV16-positive CxCa. By HPV DNA status only, HPV⁺ and HPV⁻ tumors were marginally distinct entities, since they showed similar survival curves, and in multivariate Cox proportional hazard models, patients with HPV⁺ tumors had no statistically significantly better outcome in OS and PFS compared with patients with HPV⁻ tumors (Table 3).

We are aware that some of the HPV_{low} tumors in our cohort likely in respect to HPV-driven OPSCC were false positive because of the presence of low amounts of HPV DNA in the tissues resulting from past infection that had not progressed to malignancy or from recent oral exposure to the virus. However, the use of high sensitivity detection technology with high viral load quantitation was justified since we clearly detected HPV_{low} tumors in which HPV16 was transcriptionally active (see below). Compared with HPV DNA status, expression of the viral E6*I and/or E6*II transcripts (RNA⁺ group) was superior in defining patients with better survival, a finding in full agreement with a recent French study (17). However, both high viral load and the expression of CxCa-like viral RNA patterns exhibited similar, and stronger, inverse associations with survival. Although high viral load identified only 33 patients, the CxCarNA⁺ group consisted of 40 patients, suggesting that the HPV RNA pattern analysis in comparison to

viral load analysis has higher sensitivity for identifying patients with improved survival.

The analysis of CxCa-like viral RNA patterns is complex and not feasible for every clinical laboratory. A feasible and precise algorithm to identify truly HPV-driven OPSCC was provided by taking the HPV_{high} tumors, which showed a very high overlap with CxCarNA⁺ tumors (Fig. 1), and combining them with HPV_{low} tumors positive for CxCa-like RNA patterns. With this algorithm, analyses of RNA patterns can be limited to HPV_{low} tumors, without losing sensitivity. The HR of the HPV_{transf}⁺ group was comparable to the best single marker CxCarNA⁺ (Table 3).

Importantly, the CxCa-like viral RNA patterns as well as high viral load were more strongly associated with improved survival [CxCarNA⁺, HR = 0.28, 95% confidence interval (CI), 0.13–0.61 and HPV_{high}, HR = 0.32, 95% CI, 0.14–0.73] than overexpression of p16^{INK4a} (HR = 0.90, 95% CI, 0.55–1.49) because in our study cohort by far, not all CxCarNA⁺ and HPV_{high} tumors showed increased p16^{INK4a} expression, but a few HPV-negative tumors did (Table 2, Supplementary Fig. S1). We believe that the large number of discordant cases in our study population was not because of some technical problems, but reflects true differences in the biology of HPV16-associated OPSCC and HPV16-positive CxCa, because in a parallel study on CxCa, only 1 of 58 cervical tumors lacked p16^{INK4a} overexpression (43). Thus, our results are in conflict with some reports in the literature in which only very few HPV-positive OPSCC or HNSCC were not showing high p16^{INK4a} expression and hence p16^{INK4a} was proposed to provide a cellular surrogate marker for HPV involvement and predictor of improved survival (8, 22, 23, 44). Our results are in agreement with other studies in which the combination of high p16^{INK4a} with positive HPV DNA typing was proposed to better correlate with active HPV involvement and improved patient survival than high p16^{INK4a} alone (14, 26, 27). However, the combination p16-high and HPV⁺ did not match high viral load or viral RNA pattern analysis (p16-high and HPV⁺ vs. HPV⁻, HR = 0.55; 95% CI, 0.29–1.08; Table 3). Surprisingly, both p16-high and the combination p16-high and HPV⁺ had some beneficial effect on OS but not on PFS (Table 3). The reason for this is unclear. However, our data did not reveal any differential impact of p16^{INK4a} overexpression in HPV DNA-positive versus HPV DNA-negative OPSCC as was observed by Smith and colleagues (26).

A limitation of this study is that we could not confirm improved prediction accuracy of the CxCa-like RNA patterns over RNA⁺ by statistical analysis, probably because of the small number of RNA⁺ tumors without CxCa-like RNA patterns (*n* = 8). However, because the HR for the 8 CxCarNA⁻ tumors was similar to the HR of the tumors without transcriptionally active HPV16 (RNA⁻ and HPV⁻ group), and since they also showed similar histology, tumor subsite distribution, and patient outcome (data not shown), we conclude that CxCarNA⁻ tumors behave like HPV⁻ tumors. It may be helpful for future studies to develop a quantitative cutoff for viral RNA copy numbers per OPSCC tumor cell.

The prevalence in OPSCC of both HPV16 DNA (49%) and, in particular, the CxCa-like RNA patterns (20%; the latter reflecting biological activity of HPV16) in this study are in line with

several previous Western European studies (16, 17, 20, 21, 23, 44), but are distinctly lower than reported in the major Scandinavian and North American studies (5, 7, 13, 15, 45). In these, the reported HPV prevalence ranged from 65% to 80% and absent HPV RNA expression was exceptional, indicating that nearly all of these tumors harbored active virus. The reasons for these relative prevalence differences in active HPV16 involvement between the North American/Scandinavian and Western European study populations are unclear, at present.

In conclusion, by using highly sensitive HPV DNA and RNA detection technologies, we not only detected OPSCC with high viral load and with CxCa-like viral RNA patterns but also a fraction of tumors with low viral load in which the transcriptional activity of the virus indicated a carcinogenic role. These tumors represent a clinically distinct subgroup with significantly better OS and PFS. The viral markers were superior over p16^{INK4a} expression as diagnostic and prognostic biomarkers. Patients with HPV-driven OPSCC have been shown to have better survival even with radiochemotherapy as primary treatment modality. In situations like in Germany, with heterogeneity among the HPV DNA-positive tumor patients, precise definition of HPV activity in the tumor as suggested here could help in selection of the primary treatment modality.

Disclosure of Potential Conflicts of Interest

M. Schmitt and M. Pawlita received research funding from Qiagen and Roche, respectively. No potential conflicts of interest were disclosed by the other authors.

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Viral RNA Patterns and High Viral Load Reliably Define Oropharynx Carcinomas with Active HPV16 Involvement

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