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Original article

Comparison of detection methods for HPV status as a prognostic marker for loco-regional control after radiochemotherapy in patients with HNSCC

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ABSTRACT

Objective: To compare six HPV detection methods in pre-treatment FFPE tumour samples from patients with locally advanced head and neck squamous cell carcinoma (HNSCC) who received postoperative ($N = 175$) or primary ($N = 90$) radiochemotherapy.

Materials and methods: HPV analyses included detection of (i) HPV16 E6/E7 RNA, (ii) HPV16 DNA (PCR-based arrays, A-PCR), (iii) HPV DNA (GP5+/GP6+ qPCR, (GP-PCR)), (iv) p16 (immunohistochemistry, p16 IHC), (v) combining p16 IHC and the A-PCR result and (vi) combining p16 IHC and the GP-PCR result. Differences between HPV positive and negative subgroups were evaluated for the primary endpoint loco-regional control (LRC) using Cox regression.

Results: Correlation between the HPV detection methods was high (chi-squared test, $p < 0.001$). While p16 IHC analysis resulted in several false positive classifications, A-PCR, GP-PCR and the combination

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Loco-regional control
p16
Radiochemotherapy

of p16 IHC and A-PCR or GP-PCR led to results comparable to RNA analysis. In both cohorts, Cox regression analyses revealed significantly prolonged LRC for patients with HPV positive tumours irrespective of the detection method.

Conclusions: The most stringent classification was obtained by detection of HPV16 RNA, or combining p16 IHC with A-PCR or GP-PCR. This approach revealed the lowest rate of recurrence in patients with tumours classified as HPV positive and therefore appears most suited for patient stratification in HPV-based clinical studies.

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Head and neck squamous cell carcinoma (HNSCC) is the 6th leading cancer worldwide [1]. While the number of cases related to tobacco smoking history and alcohol consumption is remaining stable or is even decreasing [2], the incidence of human papilloma virus (HPV)-driven tumours, especially in the subgroup of oropharyngeal squamous cell carcinomas (OPSCC), is steadily increasing [3]. Previous studies showed that patients with HPV positive HNSCC have a better overall survival (OS) compared to patients with HPV negative tumours [4]. In preclinical studies, HPV-infected cell lines derived from primary HNSCC displayed a higher degree of radiosensitivity [5] which appears to be related to impairments in DNA repair [6]. Furthermore, the HPV status has also been identified as a robust prognostic factor of loco-regional control (LRC) in patients with HNSCC who received primary [7–11] as well as postoperative radio(chemo)therapy [12,13]. Due to the better outcome of patients with HPV positive HNSCC compared to patients with HPV negative HNSCC, the HPV status is considered as one of the biomarkers, which allows for risk stratification in personalized radiation oncology (reviewed in [14,15]). Better outcome has mainly been reported for patients with HPV positive OPSCC [12,16,17]. In this regard, a number of clinical trials have been initiated in order to assess whether patients with HPV-driven OPSCC may benefit from radiation dose de-escalation with the aim of reducing adverse effects while maintaining comparable clinical outcome (www.clinicaltrials.gov; e.g. NCT01088802, NCT01530997, NCT01687413, NCT02281955, NCT03396718).

HPV infection typically appears in small defects of the basal epithelium. Only if the target cell is mitotically active, new HPV virions can be synthesized. In contrast to most viral infections, HPV DNA synthesis is fully dependent on the DNA replication machinery of the host cells [18]. The HPV E7 oncoprotein is of particular relevance for HNSCC pathogenesis via a deregulation cell cycle control. HPV E7 targets members of the retinoblastoma (Rb) family and mediates their subsequent degradation. In turn, E2 transcription factors are activated resulting in expression of S-phase-related genes and thus cell cycle progression [19]. This functional inactivation of the Rb-pathway finally leads to p16 over-expression – a phenomenon which is commonly used as surrogate marker for HPV infection in HNSCC (reviewed in [20]). In addition, the HPV E6 oncoprotein initiates ubiquitylation and subsequent proteasomal degradation of p53, impaired activation of p21, and finally uncontrolled cell cycle progression. Accordingly, it is of similar relevance for HNSCC pathogenesis as HPV E7 [20,21], and detection of HPV E6 and/or E7 is frequently used for diagnostic purposes [22].

To date, various HPV detection strategies guided by the biology of HPV-induced carcinogenesis are available, including detection of (i) HPV DNA, (ii) viral E6/E7 mRNA (from transcriptionally active HPV infection), and (iii) p16 over-expression as a result of altered cell cycle control [23,24]. The accepted gold standard for the identification of clinically relevant HPV infections is the detection of viral E6/E7 mRNA in fresh or frozen tumour material using polymerase-chain reaction (PCR) and more recently, fluorescence *in situ* hybridization (FISH) analysis [23,25]. Since fresh or frozen tumour material is not routinely available, and mRNA isolation from formalin-fixed and paraffin-embedded (FFPE) tissues and the subsequent PCR are technically challenging [23], detection of p16 over-expression by IHC is being used as a surrogate marker for HPV infection alone or in combination with assessment of HPV DNA using different PCR-based approaches in a number of clinical studies and meanwhile also in clinical routine (reviewed in [23,24]). So far, it has not been systematically analysed which of the methods is the most reliable one for patient stratification with particular focus on the response to radio(chemo)therapy, i.e. in a field where an increasing number of trials are currently undertaken and critically require precise means of HPV status detection.

Therefore, the overall aim of this study was to compare six methods, which are available for HPV status analysis in pre-treatment FFPE samples of HNSCC and to evaluate the results in connection with the clinical outcome of radio(chemo)therapy.

Material and methods

Patients

In this retrospective multicentre study of the German Cancer Consortium Radiation Oncology Group (DKTK-ROG), two previously published patient cohorts were examined [11,12]. All patients had been diagnosed with locally advanced HNSCC and were treated with state-of-the-art contemporary treatment schedules, either by curatively intended primary radiochemotherapy (RCTx) or surgery followed by postoperative RCTx (PORT-C) at one of the eight DKTK partner sites. Treatments and patient inclusion followed narrow stratification criteria [11,12] with a minimum follow-up of 24 months. Staging was based on the UICC TNM classification, 7th edition (2009). Data and biomaterial collection have been previously described in detail [11,12]. Briefly, patients of the postoperative cohort presented with a tumour stage pT4 and/or >3 positive lymph nodes and/or positive microscopic resection margins and/or extracapsular spread. In the primary cohort, patients usually presented with inoperable HNSCC. In this analysis, patients from both cohorts were included if sufficient biomaterial was available to perform all six HPV detection methods (postoperative: 175 out of 221 patients, primary: 90 out of 158 patients). Patients' characteristics are summarized in Table 1. Characteristics of all patients, including those who did not meet the inclusion criteria because of insufficient biomaterial for the completion of all six methods, are summarized in Supplementary Table 1.

Preparation of biomaterials for HPV analysis

Formalin-fixed and paraffin-embedded (FFPE) material of the primary tumour collected prior to radiotherapy was subjected to staining with haematoxylin and eosin, followed by histological evaluation and confirmation for the presence of squamous cell carcinoma and a minimum tumour content of 5%. Afterwards, the FFPE blocks were processed for biomaterial preparation under standardized conditions.

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Table 1
Patient characteristics of both cohorts, including patients for whom all HPV analyses were performed. LN = lymph nodes.

		Postoperative cohort		Primary cohort	
Variable		of 175	Fraction (%)	of 90	Fraction (%)
Sex	Male	141	80.6	72	80.0
	Female	34	19.4	18	20.0
Tumour localization	Oral cavity	49	28.0	15	16.7
	Oropharynx	100	57.1	40	44.4
	Hypopharynx	26	14.9	35	38.9
T stage	1	31	17.7		
	2	79	45.1	10	11.1
	3	41	23.4	25	27.8
	4	24	13.7	55	61.1
N stage	0	17	9.7	18	20.0
	1	23	13.1	4	4.4
	2	112	64.0	65	72.2
	3	23	13.1	3	3.3
UICC stage	II	6	3.4		
	III	26	14.9	9	10.0
	IVa, b	143	81.7	81	90.0
HPV16 RNA	Negative	126	72.0	81	90.0
	Positive	49	28.0	9	10.0
A-PCR	Negative	118	67.4	78	86.7
	Positive	57	32.6	12	13.3
GP-PCR	Negative	124	70.9	77	85.6
	Positive	51	29.1	13	14.4
p16	Negative	112	64.0	77	85.6
	Positive	63	36.0	13	14.4
p16 and A-PCR	One or both negative	127	72.6	81	90.0
	Both positive	48	27.4	9	10.0
p16 and GP-PCR	One or both negative	126	72.0	80	88.9
	Both positive	49	28.0	10	11.1
Variable		Median (Range)		Median (Range)	
Age		56.0 (24.0–75.0)		60.3 (41.9–81.9)	
Volume Tumour (ccm)		–		27.6 (4.4–175.8)	
Volume LN (ccm)		–		4.8 (0.0–135.0)	
Volume total (ccm)		–		36.8 (5.6–220.0)	
Dose (Gy)		64.0 (56.0–68.4)		72.0 (68.4–74.0)	
Treatment time (days)		44.0 (32.0–57.0)		48.0 (39.0–69.0)	

p16 immunohistochemistry (p16 IHC)

Immunohistochemistry staining for p16 was performed using the CINtec Histology Kit (Roche mtm laboratories AG, Basel, CH) as described previously [12]. Tumours with a strong and diffuse nuclear and cytoplasmic staining intensity in ≥70% of the tumour cells were defined as p16 positive tumours [4]. All samples were evaluated by two independent observers (AL and CvN) with an inter-observer variability of <5%.

Extraction of genomic DNA and RNA

Extraction of genomic DNA (gDNA) and total RNA from FFPE tumour material has been described previously [12]. Briefly, gDNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen GmbH, Hilden, DE) according to the instructions of the manufacturer, aliquoted and stored at −20 °C until further use. Total RNA was extracted using the fully automated Tissue Preparation System (TPS; Siemens Healthcare Diagnostics, Tarrytown, NY) following the instructions of the manufacturer, aliquoted and stored at −80 °C until required. DNA and RNA quantities were estimated using the Qubit fluorometer (Life Technologies GmbH, Darmstadt, DE).

HPV DNA assessment using PCR-based array (A-PCR)

HPV DNA analyses were carried out using the LCD-Array HPV 3.5 kit (CHIPRON GmbH, Berlin, DE) as described previously [12]. Briefly, PCR was carried out using Primer Mix A (My 11/09) and B ('125') provided with the kit, and the HotStarTaq Plus Master Mix (Qiagen GmbH). Hybridization mix including 5 μl of each amplified PCR products A and B as well as a positive control (UT-SCC-45 xenografted tumour, HPV33 positive) and a negative control (RNase-free water; Qiagen GmbH) was added to the respective field of the LCD-Array. The hybridization spots were scanned and analysed using the SlideReader Software (CHIPRON). For internal quality control purposes, input DNA control was performed using 10 ng of the respective gDNA and 0.8 μM of β-globin specific primers (forward: 5′-TTGGACCCAGAGGTTCTTTG-3′, reverse: 5′-CACT CAGTGTGGCAAAGGTG-3′) in 25 μl final volume of HotStarTaq Plus Master Mix (Qiagen GmbH). The PCR conditions were as follows: 95 °C for 5 min followed by 38 cycles (60 s at 94 °C, 60 s at 58 °C and 60 s sat 71 °C). In order to exclude any inhibition of the HPV-specific PCR, spike-in controls (10 ng gDNA of UT-SCC-45 xenografted tumour) were performed using the Primer Mix B as described above, and PCR-products were evaluated semi-quantitatively on agarose gels.

HPV DNA assessment using GP5+/GP6+ qPCR (GP-PCR)

GP-PCR was performed with 1.85–10.6 ng gDNA extracted from FFPE samples and 900 nM primers as previously described [26,27] in 20 μl final volume of 1x Maxima SYBR Green (Thermo Fisher, Heidelberg, Germany) and a cycling protocol of initial 10 s at 95 °C followed by 45 cycles (15 s 95 °C, 30 s 54 °C, 15 s 72 °C) on an LC480 Real-time PCR platform (Roche Applied Science, Penzberg, Germany). HeLa cell gDNA (positive for HPV 18) served as positive control. Input DNA control was carried out using 1.85–10.6 ng gDNA and 75 nM of 18S rDNA specific primers (forward: 5′-CGGC TACCACATCCAAGGAA-3′, reverse: 5′-GCTGGAATTACCGCGGCT-3′) in 20 μl final volume of 1x Maxima SYBR Green and a cycling protocol of 95 °C for 10 s, and 45 cycles (15 s at 95 °C, 30 s at 60 °C).

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To exclude the presence of PCR inhibitors in the extracts, spike-in controls were performed: 1 ng of HeLa DNA was added to each sample, and the difference in Cp values compared to the spike-in control alone was calculated ($\Delta Cp = Cp_{spike\text{-}in\ sample} - Cp_{mean\ spike\text{-}in\ controls}$). Only samples with positive 18S rDNA signal were further evaluated using GP5+/GP6+ qPCR (Supplementary Fig. 1A). Samples with positive HPV qPCR signal were considered HPV gDNA positive. Samples with negative HPV qPCR signal were further subjected to the spike-in control PCR, and samples with ΔCp values >5 were excluded from the analyses due to poor sample quality (Supplementary Fig. 1B).

HPV16 RNA analysis using nanoString technology (HPV16 E6/E7)

To determine HPV16 E6/E7 RNA expression levels, total RNA was subjected to nanoString analysis (nanoString Technologies, Seattle, WA, USA). nanoString analysis has been performed as described previously [28]. Raw counts were logarithmized followed by normalization to the mean of the reference genes *ACTR3*, *B2M*, *GNB2L1*, *NDFIP1*, *POLR2A*, *RPL11* and *RPL37A*. RNA expression of HPV16 E6/E7 revealed two separated clusters of tumours, see Supplementary Fig. 2. These clusters were labelled as HPV16 E6/E7 RNA negative and HPV16 E6/E7 RNA positive, respectively. Tumours positive for both, HPV E6 and HPV E7 RNA were classified as HPV16 RNA positive.

Clinical endpoints and statistical analyses

The primary endpoint of this study was LRC. Freedom from distant metastases (FDM) and OS were considered as secondary endpoints. All endpoints were calculated from the first day of radiotherapy until occurrence of the event of interest or censoring. The corresponding survival curves were estimated by the Kaplan–Meier method and compared by Log-rank tests. In addition, the impact of the HPV status on the endpoints was evaluated using univariable and multivariable Cox-regression. In multivariable regression, one HPV parameter was included together with extracapsular extension (ECE) status and tumour localization (oropharynx vs oral cavity and hypopharynx) for the postoperative cohort and with N stage (0,1 vs 2,3) and the natural logarithm of the gross tumour volume (GTV) for the primary cohort. These parameters were previously shown to be associated with outcome for the patient cohorts reported here [11,12]. Correlations between the different methods for analysis of HPV status were illustrated by contingency tables and analysed by chi-squared tests. Statistical analyses were performed using IBM SPSS Statistics 24 (IBM Corporation, Armonk, NY). For all analyses, two-sided tests were performed and *p*-values <0.05 were considered statistically significant.

Results

In this retrospective study, patients with locally advanced HNSCC from two previously published cohorts treated by state-of-the-art approaches were included [11,12]. The first cohort received PORT-C ($N = 175$) while the second cohort was treated using primary RCTx ($N = 90$).

The postoperative cohort included 100 patients (57.1%) with OPSCC, 49 (28%) with oral cavity carcinomas and 26 (14.9%) with hypopharyngeal carcinomas (Table 1).

Immunohistochemical analysis showed p16 over-expression in 63 cases (36.0%). By using the HPV DNA PCR-based array method (A-PCR), HPV DNA was detected in 59 cases including 57 HPV16 DNA (32.6%) cases as well as 1 HPV18 DNA and 1 HPV33 DNA positive case, whereas the HPV DNA GP5+/GP6+ qPCR method (GP-PCR) revealed HPV positivity in 51 cases (29.1%). The HPV16 RNA method showed 49 positive cases (28.0%). In the primary cohort, 40 patients (44.4%) with OPSCC, 15 (16.7%) with oral cavity carcinomas and 35 (38.9%) with hypopharyngeal carcinomas were included (Table 1). Assessment of p16 over-expression revealed 13 positive cases (14.4%). The A-PCR method showed 14 HPV DNA positive cases including 12 HPV16 DNA (13.3%) and 2 HPV33 DNA positive cases. The GP-PCR method revealed 13 positive cases (14.4%). HPV16 RNA positivity was detected in 9 cases (10.0%).

Table 2
Correlation of different methods for the determination of HPV status for the postoperative cohort ($p < 0.001$ for all).

		A-PCR		GP-PCR		p16		p16 and A-PCR		p16 and GP-PCR	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
HPV16 RNA	Negative	115	11	123	3	110	16	123	3	123	3
	Positive	3	46	1	48	2	47	4	45	3	46
A-PCR	Negative			113	5	103	15	118	0	114	4
	Positive			11	46	9	48	9	48	12	45
GP-PCR	Negative					110	14	121	3	124	0
	Positive					2	49	6	45	2	49
p16	Negative							112	0	112	0
	Positive							15	48	14	49
p16 and A-PCR	Negative									123	4
	Positive									3	45

Table 3
Correlation of different methods for the determination of HPV status for the primary cohort ($p < 0.001$ for all).

		A-PCR		GP-PCR		p16		p16 and A-PCR		p16 and GP-PCR	
		Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive
HPV16 RNA	Negative	78	3	77	4	77	4	81	0	80	1
	Positive	0	9	0	9	0	9	0	9	0	9
A-PCR	Negative			75	3	74	4	78	0	77	1
	Positive			2	10	3	9	3	9	3	9
GP-PCR	Negative					74	3	77	0	77	0
	Positive					3	10	4	9	3	10
p16	Negative							77	0	77	0
	Positive							4	9	3	10
p16 and A-PCR	Negative									80	1
	Positive									0	9

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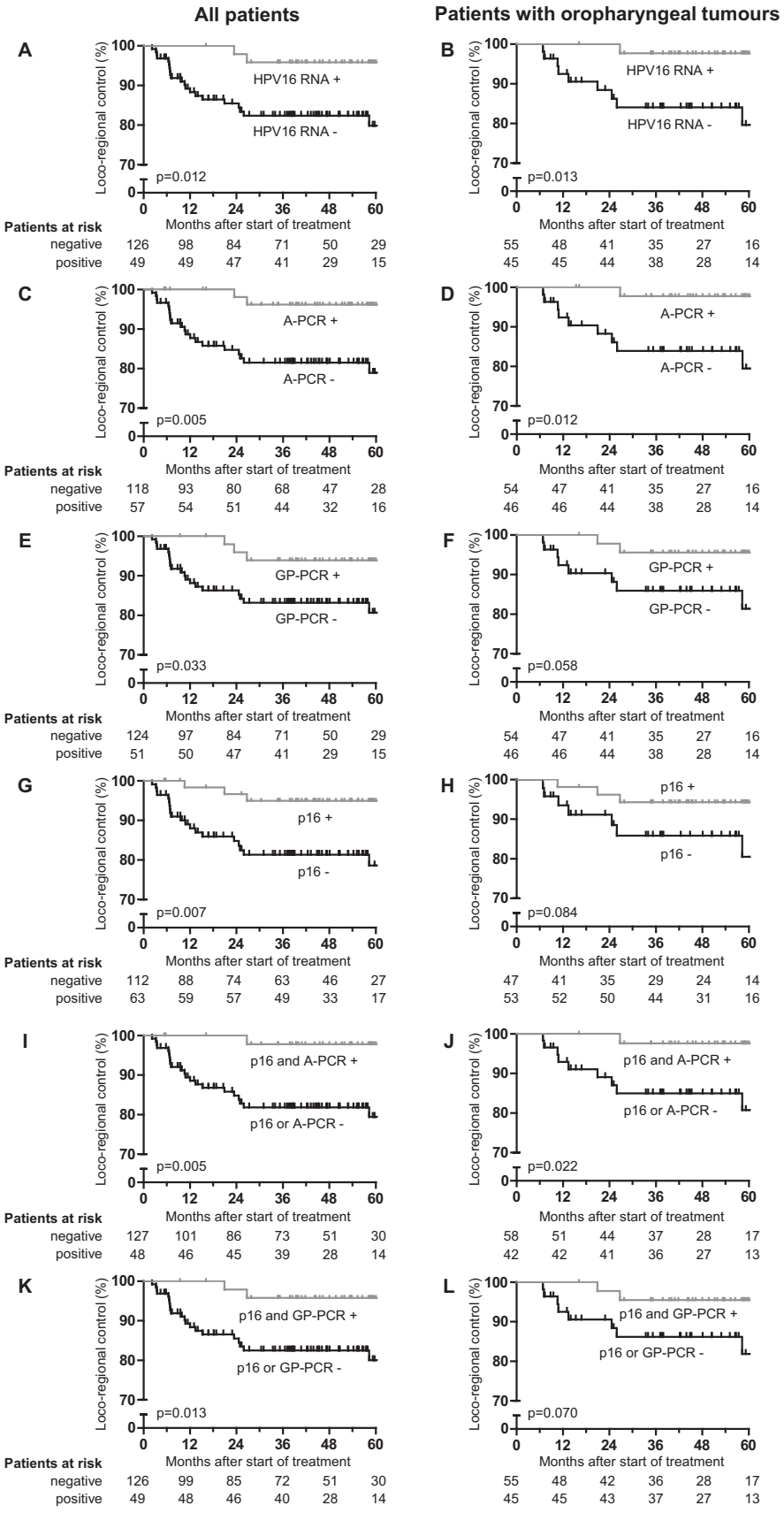

Fig. 1. Kaplan–Meier estimates of loco-regional control (LRC) of all patients with locally advanced HNSCC (left column) and of patients with oropharyngeal tumours only (right column), who received PORT-C. (A,B) HPV16 RNA analysis, (C,D) HPV16 DNA using A-PCR, (E,F) HPV DNA analysis using GP-PCR, (G,H) p16, (I,J) both p16 and A-PCR and (K,L) both p16 and GP-PCR. Patient groups were compared by log-rank tests.

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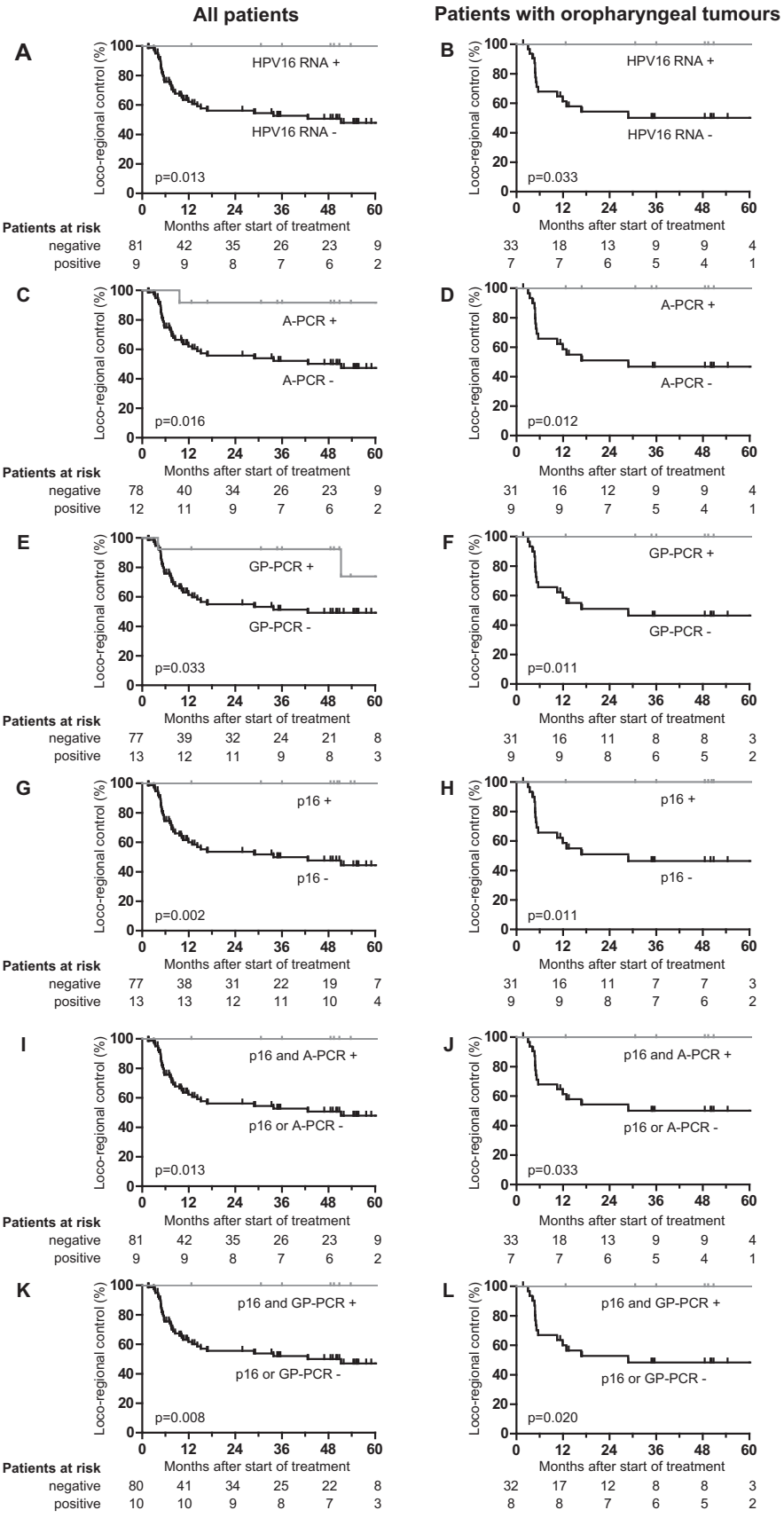

Fig. 2. Kaplan–Meier estimates of loco-regional control (LRC) of all patients with locally advanced HNSCC (left column) and of patients with oropharyngeal tumours only (right column), who received primary RCTx. (A,B) HPV16 RNA analysis, (C,D) HPV16 DNA using A-PCR, (E,F) HPV DNA analysis using GP-PCR, (G,H) p16, (I,J) both p16 and A-PCR and (K,L) both p16 and GP-PCR. Patient groups were compared by log-rank tests.

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Table 4
Multivariable Cox regression of loco-regional control, freedom from distant metastases and overall survival for the postoperative and primary cohort. In each model one HPV parameter was combined with tumour localization (oropharynx vs oral cavity and hypopharynx) and ECE status for the postoperative cohort and with N stage (0,1 vs 2,3) and the logarithm of tumour volume for the primary cohort. Only the result for the HPV parameter is reported. HR = hazard ratio; 95% CI = 95 percentage confidence interval; ECE = extracapsular extension.

	Loco-regional control		Freedom from distant metastases		Overall survival	
Variable	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value	HR (95% CI)	*p*-Value
Postoperative cohort						
Tumour localization, ECE status and						
HPV16 RNA	0.23 (0.05–1.06)	0.059	0.17 (0.04–0.73)	**0.018**	0.24 (0.10–0.59)	**0.002**
A-PCR	0.19 (0.04–0.82)	**0.027**	0.41 (0.15–1.13)	0.086	0.35 (0.17–0.74)	**0.006**
GP-PCR	0.37 (0.10–1.35)	0.13	0.26 (0.07–0.90)	**0.034**	0.34 (0.15–0.75)	**0.008**
p16	0.26 (0.07–0.91)	**0.036**	0.27 (0.09–0.81)	**0.019**	0.40 (0.20–0.80)	**0.010**
p16 and A-PCR	0.12 (0.02–0.89)	**0.039**	0.18 (0.04–0.82)	**0.026**	0.34 (0.15–0.79)	**0.012**
p16 and GP-PCR	0.24 (0.05–1.11)	0.068	0.18 (0.04–0.78)	**0.022**	0.38 (0.17–0.85)	**0.018**
Primary cohort						
N stage, tumour volume and						
HPV16 RNA		*	0.38 (0.05–2.86)	0.35	0.26 (0.06–1.06)	0.061
A-PCR	0.12 (0.02–0.87)	**0.036**	0.61 (0.14–2.70)	0.52	0.41 (0.15–1.15)	0.089
GP-PCR	0.24 (0.06–0.98)	**0.047**	0.27 (0.04–2.02)	0.20	0.27 (0.08–0.86)	**0.028**
p16		*	0.25 (0.03–1.93)	0.19	0.16 (0.04–0.67)	**0.012**
p16 and A-PCR		*	0.38 (0.05–2.86)	0.35	0.26 (0.06–1.06)	0.061
p16 and GP-PCR		*	0.35 (0.05–2.69)	0.32	0.23 (0.06–0.95)	**0.043**

* Since there were no events in the HPV positive group, the Cox model did not converge.

The results of the different methods correlated significantly ($p < 0.001$ for all comparisons; Tables 2 and 3). For the postoperative cohort, out of the 49 HPV16 RNA positive cases, 48 were also positive using GP-PCR and 46 were positive using A-PCR (Table 2). In the primary cohort, only 9 HPV16 RNA positive cases were observed. All of these cases were also positive for HPV DNA as assessed by A-PCR or GP-PCR. However, in the A-PCR or GP-PCR subgroups, additional 3 or 4 positive cases were detected, respectively (Table 3).

Out of the 49 HPV16 RNA positive cases in the postoperative cohort, 47 showed also p16 over-expression. However, 16 out of 63 p16 positive cases were found to be negative for HPV16 RNA, including 9 OPSCC and 7 tumours in the oral cavity. Out of 49 cases that were positive for HPV16 RNA, 45 were positive for p16 and HPV16 DNA (A-PCR) and 46 were positive for p16 and HPV DNA (GP-PCR) simultaneously (Table 2). In the primary cohort, all HPV16 RNA positive tumours were p16 positive, while over-expression of p16 was seen in additional 4 cases. When combining p16 and HPV DNA (A-PCR or GP-PCR), tumours were classified almost identically to HPV16 RNA (Table 3). p16 protein over-expression was further analysed together with the keratinization of those tumours [29]. In the postoperative cohort, 57.1% of the p16 positive tumours and 76.8% of the p16 negative tumours were keratinized ($p = 0.007$). As expected, keratinization was found to be negatively associated with HPV positivity. Out of the 27 p16 positive, non-keratinizing tumours, 24 were found to be positive for HPV DNA (A-PCR or GP-PCR). Similar results were obtained for HPV16 RNA (23 tumours). However, these 23 tumours represent only 47% of all HPV16 RNA positive tumours. In the primary cohort, keratinization was found in 30.9% of the p16 positive and 53.2% of the p16 negative tumours ($p = 0.13$). Here, 8 out of the 9 p16 positive, non-keratinizing tumours were found to be positive for HPV DNA (A-PCR or GP-PCR) and 7 for HPV RNA. The HPV classification of the subgroup of patients with OPSCC was also assessed and revealed a similar agreement between the six methods (Supplementary Tables 2 and 3), since the majority of the HPV positive tumours were OPSCC (postoperative cohort: ~80%, primary cohort: ~75%).

Concerning the correlation to outcome, all of the four methods and the two combinations of p16 and HPV DNA status revealed statistically significant associations of the HPV status with LRC (Fig. 1) but also with OS and FDM in the postoperative cohort (Supplementary Figs. 3 and 5) for all patients (left columns). In the primary cohort, HPV DNA status was significantly associated with LRC ($p = 0.016$ for A-PCR; $p = 0.033$ for GP-PCR; Log-rank test; Fig. 2). In the subgroups positive for p16, HPV16 RNA or combined p16 and HPV DNA (both, A-PCR or GP-PCR-based methods), no loco-regional recurrence (LRR) occurred. Results for OS and FDM are shown in Supplementary Figs. 4 and 6. For both cohorts different methods for the determination of the HPV status led to similar patient stratifications regarding LRC (Figs. 1 and 2) and OS (Supplementary Figs. 3 and 4), respectively. This is also summarized in Supplementary Fig. 7, which shows the 5-year LRC for the patients with HPV positive tumours vs the number of HPV positive tumours for each method. For the secondary endpoint FDM similar results were achieved by all six methods, while significance was only reached in the postoperative cohort (Supplementary Fig. 5). This may be due to the lower number of HPV positive tumours and the low incidence of distant metastases in the primary cohort (Supplementary Fig. 6). Limiting the cohorts to patients with OPSCC led to similar results (right columns of Figs. 1, 2 and Supplementary Figs. 3–6). The results of univariable and multivariable Cox regression are presented in Supplementary Table 4 and Table 4, respectively. Multivariable regression revealed that HPV status is an important prognostic factor independent of tumour localization and ECE status on the postoperative cohort and of N stage and tumour volume on the primary cohort.

Discussion

This is the first study investigating the relative power of different HPV biomarkers for the outcome of postoperative or primary radio(chemo)therapy using patient cohorts that followed narrow inclusion criteria and were treated with state-of-the-art contemporary treatment schedules. Clinically, this kind of methodological comparison is urgently needed, because there are several ongoing prospective trials which aim at personalizing radio(chemo)therapy in patients with OPSCC based on the HPV status and employ different means of detection.

This multicentre contemporary study compared six approaches for the determination of the HPV status and its association with LRC, FDM and OS after PORT-C or primary RCTx in patients with locally advanced HNSCC including OPSCC but also oral cavity and hypopharyngeal squamous cell carcinomas, using pre-treatment FFPE material of the primary tumour. It showed that all six meth-

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ods yield similar results and thus apparently are able to stratify patients into those with HPV positive and HPV negative HNSCC. In the subgroup of patients with OPSCC, 80% of p16 positive tumours were also positive for HPV16 DNA, whereas other tumour locations revealed HPV16 DNA positivity in only 50% of the p16 positive cases, showing that the technology may not be equally suited for all tumour locations.

HPV E6/E7 mRNA assessment meanwhile has emerged as the gold standard. It is recommended to be performed by FISH analysis [25], since PCR turned out to be considerably more error-prone. However, both methods are not routinely being used for HPV detection in the clinical routine. To the best of our knowledge, this is the first study using another hybridization-based technique (nanoString technology) for HPV RNA assessment on FFPE samples, which are routinely obtained for diagnostic purposes. In our cohorts, cases positive for HPV E6/E7 RNA revealed a better LRC with only two LRR within the postoperative cohort and no LRR in the primary cohort within the HPV positive subgroup. In clinical routine, p16 over-expression is widely being used as a surrogate marker for HPV infection due to its easy feasibility and a rather robust sensitivity of 95-100%. However, since p16 is also involved in other biological processes like cellular senescence, the specificity of this method for the detection of HPV-driven tumours is limited to 85–95% [22,30–32]. The erroneous classification of false-positive tumours undoubtedly needs to be avoided – particularly in the context of HPV-based patient stratification for de-escalation trials. In an attempt to identify false-positive HPV tumours based on p16 over-expression, it has been suggested to include the keratinization of the respective tumours [29]. However, we could not observe an additional benefit. Another drawback is that so far no standardized cut-off for the identification of p16 over-expressing tumours is available, although 70% positivity is widely being accepted (reviewed in [32]). To improve the accuracy of the HPV detection, p16 over-expressing tumours are often being subjected to HPV DNA analysis by PCR-based methods [22,24] or HPV DNA *in situ* -hybridization methods [33]. In our study, the application of this combination led to similar results as HPV RNA analysis alone. In the postoperative cohort, three LRR were detected in p16 positive cases, which were reduced to one LRR when combined with the HPV16 DNA status (A-PCR). However, by combining p16 with HPV16 DNA status, also the number of positive cases was reduced from 63 to 48 such that the relative fraction of LRR in the positive groups decreased less clearly (from 4.8% to 4.2%). In the primary cohort, no LRR occurred in HPV RNA positive tumours or in p16 and HPV DNA positive tumours (A-PCR and GP-PCR based methods). Similar results were also observed for the secondary endpoint OS [4].

In terms of PCR-based HPV DNA analysis, we compared two methods that are being used in the daily routine. Although the HPV DNA array-based method is capable of detecting 32 genotypes simultaneously, most clinical evidence is currently available for the HPV16 DNA genotype. To the best of our knowledge this is also the only genotype that has been described as a causative agent in the development of HNSCC to date [34]. Therefore, the classification related to the HPV16 DNA status was included in this study. The GP5+/GP6+ qPCR-based method enables the concomitant detection of 11 genotypes [26,27] and revealed as expected, similar results comparable results to the HPV DNA array-based method. Concerning patient stratification with respect to LRC and OS both analyses led to similar results in both patient cohorts, with the majority of them reaching statistical significance. However, A-PCR based detection identified more positive cases than the GP-PCR based method on the postoperative cohort, while their results were similar on the primary cohort. In contrast, HPV RNA analysis revealed less HPV positive cases in both cohorts. Concerning patient stratification by means of carrying the transcriptionally active virus (HPV RNA or combinations of p16 IHC with A-PCR or GP-PCR) versus no or inactive virus (p16 IHC or A-PCR or GP-PCR), the methods show variations in their detection power (see Supplementary Fig. 7). Although DNA-PCR based methods and p16 IHC are capable of detecting above 90% of HPV RNA positive tumours, there is an additional subset of RNA-negative cases detected as being HPV positive and this subset can only be minimized by combining the two methods. Therefore, only the HPV RNA hybridization-based method or the two combinations of A-PCR or GP-PCR with p16 IHC can be recommended for stratification purposes of patients regarding HPV-driven tumours. The determination of patients with HPV-driven rather than HPV-positive tumours seems to be especially relevant for oropharyngeal tumours, since OPSCC with sole p16 over-expression have been reported to show a genetic pattern that is similar to that of HPV negative OPSCC [35]. In general, the reported HPV positivity of OPSCC in the primary cohort is rather low compared to the OPSCC in the postoperative cohort (22.5% vs 73%), which may be due to the correlation of the HPV status and the T stage: In the primary cohort the T stage of OPSCC is significantly higher than in the postoperative cohort ($p < 0.001$). At the same time HPV positive tumours have a significantly lower T stage ($p = 0.002$), which is in line with the literature [4]. Hence, patients with HPV positive tumours are more likely to receive surgery followed by postoperative radiotherapy, whereas larger tumours are often functionally inoperable.

For further patient stratification beyond the HPV status, additional biomarker analyses are currently being carried out within this DKTK-ROG study covering multiple radiobiological aspects [11,28,36–40].

Taken together, the determination of the HPV status by HPV RNA or by the combination of p16 IHC and HPV DNA analysis led to comparable results regarding HPV classification. However for clinical routine, the combined analyses (p16 IHC followed by PCR-based method) seem to be advantageous with respect to its feasibility in routine laboratories. To validate the small differences between the methods, larger patient cohorts are required.

Conflict of interest

Volker Gudziol is a member of the advisory board of Bristol-Myers Squibb and received speaking fees from Roche Company. Ute Ganswindt received speaking fees from MERCK Serono Travel. The other authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.radonc.2017.12.007.

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