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HPV in anal cancer

Influence of human papillomavirus and p16^{INK4a} on treatment outcome of patients with anal cancer

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ABSTRACT

Background: The purpose of this study was to evaluate HPV-DNA and p16^{INK4a} (p16) expression as prognostic markers for outcome in patients with anal cancer.
Methods: From January 2000 to December 2011 a cohort of 105 anal cancer patients was treated with definitive chemoradiation at our institution. Tumor biopsies from 90 patients were analyzed for HPV-DNA by polymerase chain reaction and for p16 expression by immunohistochemistry.
Results: Median follow-up was 48.6 months (range 2.8–169.1 months). HPV-DNA or p16-expression was found in 75 anal cancers each (83.3%), concordance was detectable in 70 tumors (77.8%). Significantly improved overall survival (OS) [77.1% vs. 51.4%, $p = 0.005$], progression-free survival (PFS) [64.0% vs. 35.0%, $p < 0.001$] and improved local control [81.0% vs. 55.9%, $p = 0.023$] was found for concomitant HPV- and p16-positive anal carcinomas (cHPPAC) in univariate analysis. Multivariate analysis showed better OS [$p = 0.015$] and PFS [$p = 0.002$] for cHPPAC.
Conclusion: The combination of HPV-DNA and p16 can be used as an independent prognostic parameter in anal cancer patients.

Anal squamous cell carcinoma (SCC) represents with an overall incidence rate of 1.08/100,000 an uncommon malignancy, but incidence rates of 70/100,000 in HIV-positive men and an overall increasing incidence over the past years highlight its medical importance [1,2]. Therefore several studies are focusing on new treatment approaches for patients with anal SCC, such as a possible reduction of side effects by using vaginal dilators or intensity-modulated radiotherapy (IMRT) technology [3–6]. In this regard the identification of prognostic markers will be important.

Anal SCC display interesting etiological similarities to genital and some oropharyngeal malignancies: several studies have shown associations between anal cancer with human immunodeficiency virus (HIV) and human papillomavirus (HPV) [7,8].

The contribution of HPV to cancers of various anatomic locations has attracted increasing interest with more data implying an important clinical significance. For oropharyngeal cancer patients, HPV infection is a strong and independent prognostic factor for survival. Ang et al. have demonstrated that 3-year overall survival (OS) was significantly better (82.4% vs. 57.1%; $p < 0.001$) for patients with HPV-positive oropharyngeal tumors than for those without a detection of HPV [9]. The sole detection of HPV-DNA bears the risk of misclassifying cancers as HPV-associated, since it does not prove overexpression of the viral oncogenes and consequently HPV-induced transformation. Therefore additional biomarkers are studied to refine the identification of HPV-associated tumors with the goal of achieving a clinically acceptable accuracy. In this regard, the cyclin-dependent kinase inhibitor 2A (CDKN2A), better known as p16^{INK4a} (p16), is considered as an important marker. Controlled by the retinoblastoma gene product pRB, p16 acts as a tumor suppressor protein by inhibiting the G1-checkpoint regulatory cyclin-dependent kinases (CDK) -4 and -6 [10–13]. In HPV-associated cancers however the viral oncoprotein E7 indirectly substantially increases transcription of

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p16 in proliferating cells, resulting in a strong overexpression without tumor suppressive action.

Several studies demonstrate that overexpression of p16 allows for precise identification of HPV-associated transformation [14,15]. Expression of p16 in squamous cell carcinoma of the pharynx and supraglottic larynx was significantly correlated with improved 5-year locoregional tumor control (LC), disease-specific survival (DSS) and OS (all: $p < 0.001$) [16]. Furthermore, there are studies demonstrating a better prognostic stratification of oropharyngeal cancers by p16 and combined HPV/p16 testing compared to HPV DNA alone [10,17].

The shared HPV-transforming phenotype of anal squamous cell cancer (SCC) and oropharyngeal cancer suggests similar clinical implications. Accordingly, patients with HPV-associated anal SCC appear to have a better outcome than HPV-negative anal SCC [18,19] – similar to what has been shown for head and neck cancers. However, the number of studies addressing the prognostic relevance of HPV in anal SCC is still limited. Thus, the basis for prospective trials adapting treatment protocols stratified by HPV status is not sufficiently established yet. Particularly, the role of HPV and p16 and combined detection in anal SCC is still under discussion [20].

With the present study, we aimed to investigate the impact of tumor HPV status and p16 expression on clinical outcome of patients with anal cancer treated with definitive chemoradiation. Retrospective analysis was performed using a patient cohort undergoing chemoradiation at our institution.

Methods

Patients' characteristics

From January 2000 to December 2011, 105 patients with histologically proven anal cancer were treated with chemoradiation at the Department of Radiation Oncology, University Hospital Heidelberg or at the German Cancer Research Center, Heidelberg. Tumor tissue biopsies from 90 patients taken at the time of diagnosis were available and could be included in the study. The biopsies were retrieved as formalin-fixed, paraffin-embedded tissue from local pathologists and the Institute of Pathology of the University Hospital Heidelberg (Tissue Bank of the National Center for Tumor Disease (NCT), Heidelberg). Patients with palliative treatment or recurrent disease were excluded. Tumor stage, details of chemoradiation, follow-up exams and patient characteristics were obtained from medical records. The study was granted ethical approval by the local ethics committee Heidelberg.

The cohort of 90 patients with a median age of 55 years (range 22–94 years) was treated with radiochemotherapy or radiotherapy alone. 77 (85.6%) patients were female, 13 (14.4%) were male. A majority of patients (53.3%) showed tumor size from 2 to 5 cm, typically without nodal involvement (75.5%). Four carcinomas were located at the anal margin.

Treatment and follow-up

Patients were treated with 3D-conformal radiotherapy (35.6%) or intensity-modulated radiotherapy (IMRT), either as step-and-shoot-IMRT or helical tomotherapy (63.3%). One patient (1.1%) received only electrons because of a small, superficial lesion. The median radiotherapy dose was 45.0 Gray (Gy) (range 36.0–50.4 Gy) including the macroscopic tumor, bilateral iliac, inguinal and pararectal lymph nodes. A sequential or simultaneous boost on macroscopic tumor or involved lymph nodes was performed up to a median total dose of 54.0 Gy (range 45.0 Gy–63.2 Gy). 76.7% of all patients received chemotherapy with 5-fluorouracil (5-FU) 1000 mg/m^2 body surface (typically days 1–5 and 29–33) plus mitomycin (MMC) 10 mg/m^2 body surface (days 1 and 29). A discontinuation of chemotherapy after the first cycle was necessary because of fulminant side effects (e.g. thrombocytopenia) for 3 patients (3.3%). 18 patients (20.0%) with relevant comorbidities (e.g. cardiac conditions) and/or poor Karnofsky performance score received only radiotherapy, 3 patients (3.3%) received other types of chemotherapy (Cisplatin + Etoposide, Cisplatin + 5-FU, 5-FU + Carboplatin). Patient and treatment characteristics are listed in Table 1.

Follow-up examinations were performed every 3–6 months for a minimum of 3 years and then annually including physical examination, imaging (MRI or CT), rectoscopy and biopsy for abnormal findings.

HPV-DNA detection and genotyping

DNA extraction was performed using QIAGEN (Venlo, Netherlands) DNeasy© Blood and Tissue Kit from the available, formalin-fixed and paraffin-embedded tissue sections. For polymerase chain reaction (PCR) consensus HPV primers-sets (modified GP5+6+) were used. PCR was performed with two positive controls ("CaSki" for HPV16, "HeLa" for HPV18) and one negative control (water). After 40 cycles of nucleic acid amplification, 10 µL of amplified DNA were stained with GelRed™ and analyzed by agarose gel electrophoresis. Samples with bands of about 150 base pairs corresponding to the length of positive control amplicons were considered positive.

We performed genotyping with positive samples using the Multiplex HPV Genotyping Kit© from Multimetrix GmbH (Regensburg, Germany, now DiaMex, Heidelberg, Germany) for subtypes HPV6, HPV11, HPV16, HPV18, HPV26, HPV31, HPV33, HPV35, HPV39, HPV42, HPV43, HPV44, HPV45, HPV51, HPV52, HPV53, HPV56, HPV58, HPV59, HPV66, HPV68, HPV70, HPV73 and HPV82. All procedural steps and evaluation of the results were done according to the manufacturer's instructions. Samples were considered positive – in accordance with the literature [21,22] – if median intensity of fluorescence in the Luminex Analyzer was 10 or higher and equal or more than 70 beads could be counted.

p16 immunohistochemistry

p16-immunohistochemistry was performed on 2 µm tissue sections using the CINtec p16^{INK4a}-histology kit (mtm Laboratories,

Table 1
Patient and treatment characteristics according to HPV-DNA status.

	HPV-DNA positive ($n = 75$)	HPV-DNA negative ($n = 15$)
Age – median (range)	55 years (28–94 years)	56 years (22–86 years)
Sex – % (n)		
Female	92.0% (69)	53.3% (8)
Male	8.0% (6)	46.7% (7)
T stage – % (n)		
T1	17.3% (13)	20.0% (3)
T2	56.0% (42)	40.0% (6)
T3	18.7% (14)	20.0% (3)
T4	8.0% (6)	20.0% (3)
N stage – % (n)		
N0	74.7% (56)	80.0% (12)
N1	6.6% (5)	13.3% (2)
N2	10.7% (8)	6.7% (1)
N3	8.0% (6)	0
Radiotherapy – median (range)	54.0 Gy (45.0–63.0 Gy)	54.0 Gy (50.4–63.2 Gy)
Single fraction dose – median (range)	2.0 Gy (1.8–2.2 Gy)	2.1 Gy (1.8–2.2 Gy)
Overall treatment time – median	36 days	36 days
Treatment break > 3 days (n)	8% (6)	6.7% (1)

Heidelberg, Germany) according to the manufacturer's instructions. p16 expression was detected with a monoclonal anti-human p16INK4a antibody (E6H4™) and a secondary antibody conjugated to horseradish-peroxidase allowing for 3,3′-Diaminobenzidine (DAB)-based visualization. Tissue sections with a diffuse tumoral p16-staining variably reaching the entire tumor area as previously published [23,24] were considered positive. Lesions showing either a sporadic or focal staining were described as negative.

Statistical analysis

All survival end-points were calculated starting from the first diagnosis date. OS was then defined as the time to death from any cause. LC was defined as the time to locally progressive disease of the primary tumor or regional lymph nodes. PFS and CFS were defined respectively as the time to progressive disease and the time to colostomy or death. All patients who did not experience an event at the last follow-up date were censored. In detail events were death for OS, progressive disease or death for PFS, progressive disease for LC, colostomy or death for CFS. To explore possible differences between the HPV-positive group versus the HPV-negative group, the p16-positive group versus the p16-negative group as well as the group with concomitant HPV- and p16-positive anal carcinomas (cHPPAC) versus the rest of the patients, the Fischer's exact test was applied. The Kaplan–Meier method was used to estimate OS, PFS, CFS, and LC for various group partitions. Univariate survival time comparisons were performed using the log-rank test. Multivariate analyses were performed using Cox regression. The statistical analysis was performed using R version 3.0.2.

Results

After a median follow-up of 48.6 months (range 2.8–169.1 months) for the entire cohort, 20 patients (22.2%) had a local relapse and 10 patients (11.1%) developed a systemic recurrence. The 3-year progression-free survival (PFS), colostomy-free survival (CFS) and overall survival (OS) rates were 63.3% (95% CI 54.1–74.1%), 85.5% (95% CI 77.1–94.8%) and 77.3% (95% CI 69.0–86.6%), respectively. The local control rate at 3 years was 76.4% (95% CI 67.8–86.0%).

75 patients (83.3%) had detectable HPV-DNA in the tumor specimens. On immunohistochemistry, p16-positivity was found in 75 tumors (83.3%). 77.8% (70) tumors were positive for both, HPV-DNA and p16. HPV-16 was the most frequently (92.0%) detected genotype in our cohort (Table 2). Explorative analyses revealed that gender was the only parameter significantly associated with both HPV- and/or p16-positivity ($p < 0.001$). Among female patients, 90% had HPV-DNA-positive tumors whereas only 46% of the male patients had evidence of HPV-DNA in the tumor specimen ($p < 0.001$; explorative analysis). Patients with p16-positive tumors had less advanced nodal disease ($p = 0.035$; explorative analysis).

Patients with HPV-DNA-positive tumors and with cHPPAC had a significantly better overall survival whereas p16-expression alone conferred no significant survival benefit (Fig. 1). The actuarial overall survival rates for patients with HPV-DNA-positive and -negative cancers were 75.8% (95% CI 65.7–87.5%) and 48% (95% CI 26.3–87.7%) ($p = 0.007$). For patients with cHPPAC, actuarial OS was 77.1% (95% CI 66.7–89.0%) compared to 51.4% (95% CI 32.2–82.2%) for patients with one or two negative parameters ($p = 0.005$). For progression-free survival, similar results were found (Fig. 2), although p16-expression was associated with a significant benefit for this survival endpoint ($p = 0.002$). On univariate analyzes also tumor stage and patient age (cut-off 65 years) were related to significantly better PFS and OS. In terms of local recurrence, patients with cHPPAC had a significantly higher local control than patients with one or both parameters being negative ($p = 0.023$). In patients with HPV-DNA-positive tumors this finding was only of borderline significance ($p = 0.051$). Patients with HPV-DNA-positive cancers had a significantly lower risk for distant recurrence ($p = 0.005$) while there was a strong trend toward better distant control for patients with cHPPAC ($p = 0.051$). These results were comparable when performing univariate analyzes only with patients treated with chemoradiation ($n = 72$). PFS and LC were significantly better for cHPPAC patients ($p = 0.005/0.02$). There was a trend toward better OS in patients with cHPPAC, although this difference was not statistically significant ($p = 0.07$). Regarding systemic relapse only for HPV-DNA a statistically significant difference was found ($p = 0.03$). On the other hand large tumor volumes (T3, T4) had a significantly worse OS ($p = 0.001$), PFS ($p = 0.03$) and local control ($p = 0.08$) compared to small tumor volumes (T1, T2). Systemic relapse showed no statistically significant difference between both subgroups.

To identify independent prognostic factors for PFS and OS, multivariate analyses were performed. In a multivariate model consisting of patient age (with two patient groups, $\leqslant$ or >65 years respectively), concomitant HPV-DNA and p16-expression and tumor stage (with T1– T2 vs. T3–T4 patient groups), both concomitant HPV-DNA and p16-expression ($p = 0.002$, HR 0.32) and patient age ($p = 0.026$, HR 0.46) were proved to be independent prognostic factors for PFS. Regarding OS the multivariate analyses revealed that parameters "tumor stage" ($p = 0.003$, HR 4.03) and "coexistence of HPV-DNA and p16-expression" ($p = 0.015$, HR 0.33) emerged as significant predictors. Finally, our multivariate analysis for OS showed age to be a time-dependent covariate, whose effect decreases with time. The results of univariate and multivariate analyses for OS and PFS are summarized in Table 3.

Table 2
HPV DNA genotype status of 90 tissue slides.

HPV genotype	% (*n*)
HPV 16	92.0 (69)
HPV 18	4.0 (3)
HPV 33	1.3 (1)
HPV 45	1.3 (1)
HPV 51	1.3 (1)

Discussion

The influence of tumor HPV-DNA and p16 status on outcome of patients with anal SCC undergoing chemoradiation is still forming the focus of several studies and was also discussed in the current ESMO-ESSO-ESTRO clinical practice guidelines for anal cancer [25]. A couple of analyses suggested a potential benefit of both parameters regarding OS and PFS [18,19,26], while others called into question the prognostic role of HPV-DNA and p16 status [27,28,20]. This inconsistency necessitates further work.

In the current study we analyzed HPV-DNA and p16-status in a cohort of 90 patients with anal carcinoma treated with chemoradiation at our institution and assessed the influence of HPV-DNA, p16 and combined detection on patient's outcome. Our results on sole HPV-DNA positivity showed significantly improved OS (75.8% vs. 48%, $p = 0.007$) and PFS (63.5% vs. 26.7%, $p < 0.001$), confirming other studies: Serup-Hansen et al. reported better OS (74% vs. 52%, $p = 0.036$) in HPV positive tumors of the anal canal compared with malignancies without HPV-DNA detection [19]. These results are in accordance with findings from head and neck squamous cell carcinomas (HNSCC), where a difference in OS of 48% (79% vs. 31%, $p < 0.001$) could be identified in a cohort of 111 patients with oropharyngeal carcinoma [29]. In a retrospective analysis of 361 oropharyngeal cancer patients treated with

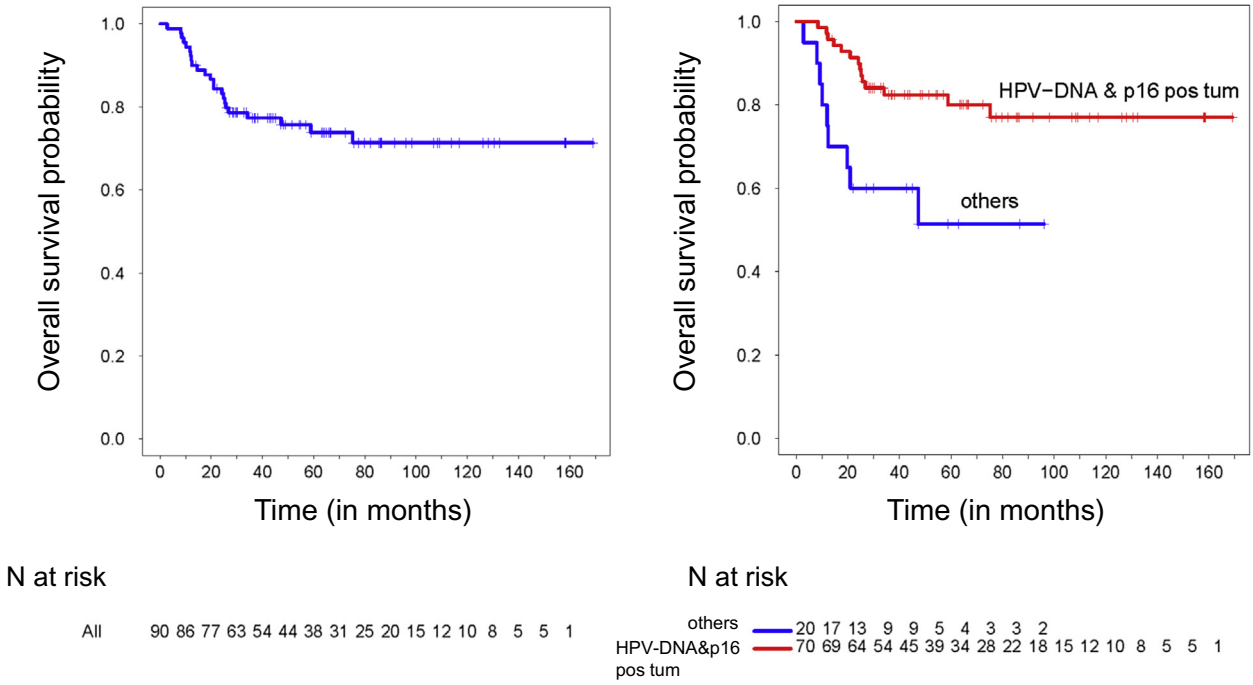

Fig. 1. Overall survival for the entire cohort (left) and for anal carcinomas with both HPV-DNA and p16-positivity (right).

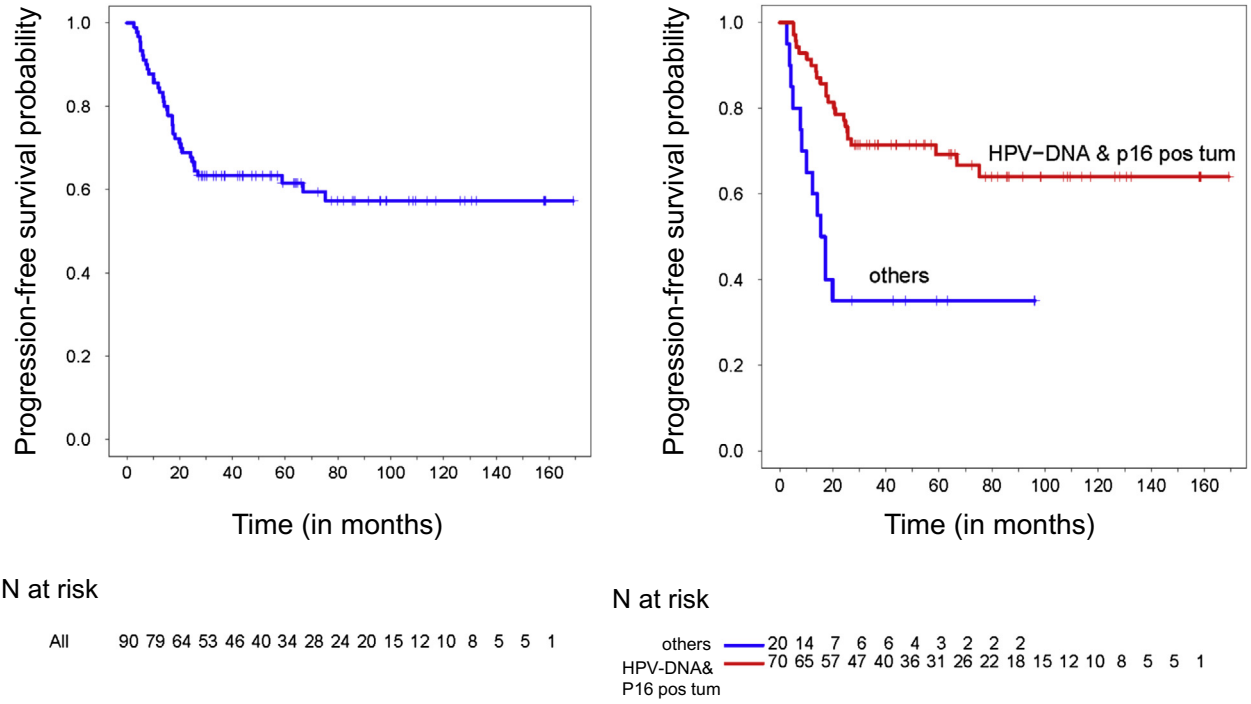

Fig. 2. Progression-free survival for the entire cohort (left) and for anal carcinomas with both HPV-DNA- and p16-positivity (right).

chemoradiation the authors observed a significantly improved 3-year OS for HPV-positive tumors (82.4% vs. 57.1%, $p < 0.001$) [9].

p16-expression is used as a marker indicating the transforming nature of HPV infections, by reflecting overexpression of the viral oncogenes and not only presence of viral genomes (HPV DNA). Although also the direct detection of viral oncogene transcripts has been discussed to provide promising results in HNSCC [30,31], immunohistochemical detection of p16 is technically easy and widely used and its prognostic relevance in anal SCC should be evaluated. Currently, there is only little data regarding p16 and anal SCC. In a cohort of 47 patients 4-year PFS was significantly better ($p = 0.014$) for patients with p16 positive tumors [18]. Similar results were reported by Gilbert et al. with a significantly higher number of relapses and a worse OS ($p < 0.001$) for p16-negative anal cancer patients [26]. p16-expression conferred no statistically significant survival benefit in our cohort ($p = 0.06$) whereas we found improved PFS for p16 positive anal cancers ($p = 0.001$). Since there was a trend favoring p16 positivity regarding OS in our study, the number of analyzed biopsies (90 in our study, 47 in [18] vs. 153 in [26]) could explain the lack of significance. Both

Table 3
Univariate and multivariate analyzes for OS and PFS.

Risk factor	Univariate analysis (KM estimators)			Multivariate analysis (Cox PH model)		
	%	95% CI	*P*	HR	95% CI	*P*
OS						
Sex						
Female	73.4	(63.1; 85.3)	0.1012			
Male	61.5	(40.0; 94.6)				
Age, years						
⩾65	38.0	(18.0; 80.4)	0.0072	1		0.7296*
<65	81.0	(71.7; 91.4)		1.329*	(0.265, 6.658)*	
HPV-status						
Positive	75.8	(65.7; 87.5)	0.0069			
Negative	48.0	(26.3; 87.7)				
p16 status						
Positive	74.1	(63.8; 86.1)	0.0610			
Negative	60.0	(39.7; 90.7)				
HPV & p16						
Both positive	77.1	(66.7; 89.0)	0.0050	0.327	(0.133; 0.806)	0.01
Other	51.4	(32.2; 82.2)		1		51
T stage						
T1–T2	80.1	(69.7; 91.9)	0.0013	1		0.0029
T3–T4	49.5	(31.6; 77.3)		4.027	(1.612; 10.057)	
PFS						
Sex						
Female	59.8	(49.1; 72.8)	0.0814			
Male	46.2	(25.7; 83.0)				
Age, years						
⩾65	30.0	(13.2; 68.1)	0.0424	1		0.0263
<65	65.5	(54.5; 78.6)		0.463	(0.235; 0.913)	
HPV-status						
Positive	63.5	(52.6; 76.8)	<0.001			
Negative	26.7	(11.5; 61.7)				
p16 status						
Positive	62.3	(51.4; 75.6)	0.0017			
Negative	33.3	(16.3; 68.2)				
HPV & p16						
Both positive	64.0	(52.7; 77.7)	< 0.001	0.323	(0.158; 0.659)	0.0019
Other	35.0	(19.3; 63.6)		1		
T stage						
T1–T2	62.6	(50.9; 77.0)	0.0283	1		0.1010
T3–T4	46.2	(30.5; 69.9)		1.8	(0.892; 3.633)	

* *Remark:* These values correspond to the age effect as assumed to be time-independent. However, further analysis shows that age is in fact a time-dependent covariate, with negative coefficient for the time-age interaction (=−0.0674). This interaction is statistically significant with $p = 0.0422$. Our computations prove that the effect of age declines with time: given that initially age has a positive effect on hazard (with the corresponding age-coefficient = 0.2842), this effect decreases with time (at rate = −0.0674 per month) and becomes negative after about 5 months.

the current and other mentioned studies used CINtec kits for p16 detection, which makes an influence of technical differences less probable. However non-uniform interpretation of p16 expression patterns as "positive" and different HPV prevalence may impact the results.

Considering that p16 overexpression is not entirely restricted to HPV-associated transformation, it may be important to analyze the combined HPV-DNA-/p16-status in order to more specifically identify HPV-associated tumors. There is less data assessing the co-detection of HPV-DNA and p16 expression regarding the outcome of patients with anal carcinoma. However, that could be of interest because several studies showed a clear benefit by using a concomitant testing of HPV-DNA and p16 for HNSCC prognosis [32,33]. A study conducted by Rödel et al. reported on decreased local failure ($p = 0.019$) and improved cancer-specific ($p = 0.04$) and overall survival ($p = 0.031$) for anal cancer patients with high HPV16 DNA load and p16 expression [34]. These results are in concordance with our observations demonstrating improved OS ($p = 0.005$), PFS ($p < 0.001$) and decreased local relapse rates ($p = 0.023$) for patients with cHPPAC. In both studies there was no significant relationship of concomitant HPV-DNA positivity and p16 expression with the risk of developing distant metastases. This indicates that the lack of local control could play an important role regarding treatment of the other anal SCC subgroups.

A comparison of all combined HPV-DNA- and p16- subgroups in our cohort is limited due to the small numbers of patients with HPV-DNA-negative p16-positive and HPV-DNA-positive p16-negative tumors. Nevertheless it is interesting to note that patients with HPV-DNA-negative and p16-positive anal cancers tended to have worse OS and PFS than the remaining three subgroups (data not shown). If this finding can be confirmed in larger studies, this would be in conformity with findings from studies on oropharyngeal cancer [35,36]. However, other clinical analyses concluded that patients with p16-positive, HPV-negative oropharyngeal tumors are at similar risk as patients with p16-positive/HPV-positive tumors [37]. Anal SCC patients with detection of HPV-DNA and no expression of p16 exhibited a worse OS but similar PFS compared to HPV-DNA- and p16-positive tumors in our cohort (data not shown). One might speculate that these tumors have no overexpression of viral oncogenes and are therefore not attributable to HPV.

The major limitation of our analysis is its retrospective nature. We included a relatively high number of patients ($n = 105$) with a long follow-up period (median 48.6 months) but prospective data are necessary to confirm our results.

In summary, concomitant detection of HPV-DNA and p16 expression represents a prognostic marker in patients with anal carcinoma. Escalating treatment options for HPV- und

p16-negative tumors and de-escalating therapy for anal cancers with HPV-DNA and p16 positivity could be considered with the purpose of generating better outcome and less treatment-related side effects. In this regard prospective trials are mandatory to further determine the predictive role of HPV-DNA and p16 expression in patients with anal cancer.

Competing interests

The authors declare no potential conflict of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.radonc.2014.11.013.

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