Prognostic value of high-risk HPV DNA and p16^{INK4a} immunohistochemistry in anal cancer patients: an individual patient data meta-analysis

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Abstract

Background:

High-risk human papillomavirus (hrHPV) types represent the etiological agents in a major proportion of anal squamous cell carcinomas (ASCC). Several studies have suggested a prognostic relevance of HPV-related markers, particularly hrHPV DNA and p16^{INK4a} (p16) protein expression, in ASCC patients. However, broader evaluation of these prognostic marker candidates has been hampered by small cohort sizes and heterogeneous survival data among the individual studies. We conducted an individual patient data (IPD) meta-analysis to determine the prognostic value of hrHPV DNA and p16 in ASCC patients while controlling for major clinical and tumor covariates.

Patients and Methods:

A systematic literature search was conducted to identify all published studies analyzing p16 alone or in combination with hrHPV DNA and reporting survival data in ASCC patients. Clinical and tumor-related IPD were requested from authors of potentially eligible studies. Survival analyses were performed with a proportional hazard Cox model stratified by study and adjusted for relevant covariates. The study-specific hazard ratios for the exposures were pooled using a random-effects model. Kaplan-Meier curves from different studies were pooled per exposure group and weighted by the study's total sample size.

Results:

Seven studies providing IPD from 693 ASCC patients could be included in the meta-analysis. 76% were hrHPV DNA+/p16+, whereas 11% were negative for both markers. A discordant marker status was observed in 13% of cases. Patients with hrHPV DNA+/p16+ ASCC showed significantly superior overall survival (OS) compared to patients with hrHPV DNA-/p16- tumors (pooled adjusted HR=0.26 (95% CI, 0.14-0.50)) with pooled three-year OS rates of 86% (95% CI, 82-90%) versus 39% (95% CI, 24-54%). Patients with discordant hrHPV DNA and p16 status showed intermediate three-year OS rates (75% (95% CI, 56-86%) for p16+/hrHPV DNA- and 55% (95% CI, 35-71%) for p16-/hrHPV DNA+ ASCC).

Conclusion:

This first IPD meta-analysis controlling for confounding variables shows that patients with hrHPV DNA+/p16+ ASCC have a significantly better survival than patients with hrHPV DNA-/p16- tumors.

Keywords

ASCC; anal squamous cell carcinoma; HPV; p16; Meta-analysis; IPD

Introduction

Anal cancer represents about 2.5% of all gastrointestinal malignancies (1). Among these cases, squamous cell carcinoma of the anus (ASCC) is by far the most common histological subtype, accounting for more than 80% of anal cancer cases (2, 3). Notably, the incidence of anal cancer has significantly increased worldwide in the past decades (4, 5), with more than 48,000 new cases estimated for 2018 (6). It has been suggested that this rising incidence is related to altered sexual behavior associated with an increased risk of acquiring an infection with oncogenic human papillomaviruses (HPV) (5). Oncogenic, i.e. high-risk (hr), HPV types represent the major etiological factor of anal cancer development. A recent study by Martel and colleagues estimated HPV to account for 88% of all anal cancer cases (7). Further well-known risk factors of anal cancer development such as receptive anal intercourse, iatrogenic or human immunodeficiency virus (HIV)-induced immune suppression, smoking and genital dysplasia in women, most likely promote carcinogenesis by increasing the probability of hrHPV persistence and subsequent hrHPV-induced transformation (8-10).

HrHPV can cause premalignant and malignant lesions in other anogenital sites, including the uterine cervix, vulva, vagina and penis, as well as a proportion of cancers in the oropharynx (7, 11, 12). Comprehensive studies of the past decades have unveiled common molecular events that govern HPV-induced carcinogenesis in those sites. It is well established to date that both initiation and maintenance of hrHPV-induced transformation critically depend on sustained expression of the hrHPV E6 and E7 oncoproteins that deregulate numerous physiological processes in the cell (13, 14). Consequently, a tumor is considered to be causally driven by hrHPV, if E6/E7 overexpression can be demonstrated. However, detection of hrHPV E6/E7 gene products (i.e. mRNA or proteins) from tumor tissue is not easily feasible in all routine diagnostic laboratories. Thus, surrogate markers of a transforming hrHPV infection, e.g. presence of hrHPV DNA or overexpression of $p16^{INK4a}$, represent viable alternatives in this setting, albeit coming along with some individual drawbacks. As such, hrHPV DNA may principally also be detected in non-transforming HPV infections (reviewed in (15) and (16)) and therefore overestimate the proportion of truly HPV-driven tumors. $p16^{INK4a}$ is a cellular protein that becomes considerably overexpressed by hrHPV E7 oncoprotein signaling, thus indicating transforming activity of hrHPV (17). Detection of $p16^{INK4a}$ overexpression by immunochemistry is now commonly used in the diagnosis of HPV-transformed lesions at the uterine cervix and in the triage of screen-positive women (18, 19). However, $p16^{INK4a}$ overexpression also occurs in the absence of HPV infection in about 5-15% of tumors (20-22). To increase the specificity with minimal loss in sensitivity, it has been suggested to combine hrHPV DNA and $p16^{INK4a}$ testing for the reliable identification of HPV-induced tumors in different sites of the genital and head and neck region (20, 23, 24).

Patients with hrHPV-related cancers generally show improved survival compared to patients with HPV-negative cancers in the same locations. This observation has been confirmed in several meta-analyses for cancers arising from the vulva, penis and the oropharynx (25-29). Classification of the HPV status mostly relied upon the detection of HPV DNA and/or $p16^{INK4a}$ overexpression in those studies. Two recent meta-analyses have also demonstrated improved survival for HPV-driven compared to HPV-negative anal cancer patients (30, 31). However, those meta-analyses were based on aggregated study data where control of confounding effects is often problematic due to insufficient and/or heterogeneous documentation. This precludes the assessment of important variables, such as age, gender or tumor status, that are known to impact survival.

We conducted for the first time an individual patient data (IPD) meta-analysis on the prognostic value of HPV in anal cancer patients, taking into consideration multiple clinical and tumor covariates. To account for the limited individual accuracy of hrHPV DNA and $p16^{INK4a}$ to identify HPV-induced cancers, we compared the prognostic impact of these surrogate markers alone and in combination. We further determined survival of patients with discordant results on hrHPV DNA and p16^{INK4a} testing (i.e. hrHPV DNA-positive/p16^{INK4a}-negative or hrHPV DNA-negative/p16^{INK4a}-positive cases) as these subgroups are hypothesized to represent biologically and clinically distinct tumor entities compared to truly HPV-driven or HPV-negative anal cancers.

Methods

Clinical question

The aim of this IPD meta-analysis was to investigate whether p16^{INK4a} overexpression (abbreviated in this paper as p16) assessed by immunohistochemistry (IHC) can serve as a prognostic marker of overall survival (OS) and how it compares to hrHPV DNA detection and a combination of p16 IHC and hrHPV DNA. The clinical question was disentangled in the following PICOS (Population - Index test - Comparator tests - Outcomes - Study design) scheme (Table 1).

Table 1: PICOS scheme

Component	Specification
Population	men or women with a diagnosis of anal cancer confounding variables: gender, age, TNM stage, HIV status, therapy
Intervention	p16 IHC staining of anal cancer tissue sections
Comparator	C1: hrHPV DNA C2: hrHPV DNA and p16 IHC combined
Outcomes	differences in overall survival after distinct time intervals (e.g. after 36 months, 60 months etc.) between test-positive (+) and test-negative (-) groups
Study design	cohort studies (prospective or retrospective) or randomized controlled trials providing data on p16 IHC expression status at diagnosis and patient survival

Data

A broad search string was defined to conduct a systematic search on the NCBI Pubmed database: “(p16*) AND (Anal OR Anus OR ASCC OR AIN OR A-SCC OR ASIL)”. The following inclusion criteria were adopted: provision of original data, conduction of p16 IHC on anal cancer specimens, analyzed sample number ≥10, and availability of patient survival data. No restrictions regarding language were applied. In case study data from a distinct patient cohort were presented in more than one report, only the one providing the most comprehensive study data (according to the inclusion criteria) was included in the meta-analysis. The authors of all studies found to be potentially eligible during the screening process were contacted with specifically designed data forms to obtain the following IPD: gender, age at diagnosis, TNM-stage, p16 status, hrHPV DNA status, HIV status, localization of the tumor, therapy administered and disease-progression and vital status according to calendar date. At least two attempts were done to establish contact with authors or co-authors of eligible published reports. If authors did not respond or could not provide the required data, the respective studies were excluded from further analyses. In addition, authors were asked to provide definitions of p16 positivity, Overall Survival (OS) and Progression-Free Survival (PFS), if these had not been specifically explained in the manuscript. For the current paper, we assessed only Overall Survival (summarized hereafter as “survival”).

Study selection

The selection process of eligible studies is illustrated in the PRISMA flow diagram (Fig. 1). Overall, 207 manuscripts were identified with the designed search string during the last search on June 13, 2020. Nineteen of those studies met all inclusion criteria and were deemed potentially eligible for inclusion. Authors from eight studies who published between 2011 and 2017 provided the requested IPD (32-40). One study represented an update of a smaller study (38). Therefore, the smaller cohort study was excluded (40). One study had to be excluded because the authors could only provide data from p16-positive patients precluding comparison with p16-negative patients (39). In the final IPD meta-analysis, 693 anal cancer patients of seven eligible studies could be included (32-38). Only studies with a predetermined cut-off for hrHPV DNA positivity and clearly defined p16 positivity status were included in the main analysis, one study (37) only had data on the p16 status and was not included in any hrHPV DNA or p16/hrHPV DNA analysis.

Fig. 1: PRISMA flow diagram

Statistical Analysis

The assessed exposure variables were defined in terms of p16 status, hrHPV DNA status and the interaction between both. A proportional hazard Cox model was fitted that was stratified by study to assess differences in survival by exposure and was adjusted or not for the following covariates: age, gender, N-stage and T-stage. Age was entered in the model as a continuous variable, whereas the other covariates were entered as binary variables. Tumor stages were grouped as T1/T2 vs. T3/T4 and nodal stages were grouped as N0 vs. N1/N2/N3. We assumed a common effect for age, gender, N- and T-stage over all the studies, while we allowed the effect of the exposure variables to differ among studies. A random-effects (RE) model was applied for pooling the hazard ratios (HR) using the DerSimonian-Laird estimator with the metafor-package in R (41). The analysis was performed on the log(HR) scale with log-log based standard errors. The percentage of total variation due to inter-study heterogeneity was assessed by the I^2 index (42). The log(HR) values were eventually back-transformed to HRs and shown in forest plots.

Kaplan Meier (KM) curves from different studies were pooled per exposure group, weighted by the study's total sample size (43). The maximum follow-up time was different between exposure groups and studies. Therefore, in the KM the maximum time analyzed for each exposure group was determined by the study with the smallest follow-up time for that group. From these survival curves we obtained the survival rate at three years and the median survival time, the minimum time for which the overall survival is at least 50%. To compare the distribution of categorical variables between groups a chi-square test with Monte Carlo simulated p values was used and when continuous variables between groups were compared ANOVA was applied.

Results

Study and patient characteristics

Six of the seven included studies were conducted in Europe (three in Germany (32, 33, 38), one in the UK (36), one in Italy (37) and one in the Netherlands (34)), whereas one study was conducted in Asia (South Korea) (35). A retrospective cohort study design was applied in all seven studies. The maximum observation time varied between 6 (37) and 27 years (38). In the original studies, patients had been excluded for the following reasons: patients receiving palliative treatment or having recurrent disease (32), no curative intent of treatment or occurrence of previous malignancies (38), metastatic disease (36), metastatic disease or prior pelvic radiotherapy (37), no paraffin-embedded tumor tissue available (34, 35), and patients with missing data (33) . In all studies, patients were treated with radio-chemotherapy, radio-chemotherapy and surgery or radiotherapy alone. Almost two-thirds of the patients were female (n=436, 63%). The median age at the time of diagnosis ranged from 55 to 65 years. Forty-eight among 445 patients (11%) with available information on HIV status were known to be HIV-positive. A summary of patient characteristics is shown in Table 2. In all studies, formalin-fixed paraffin-embedded (FFPE) tissue samples were prepared and used for p16 and HPV DNA analyses. For one study, the cut-off for hrHPV DNA positivity was not pre-defined but based on the median of obtained test results for hrHPV 16 in the original paper (38). The reviewing authors therefore defined a cut-off for this test *a posteriori*. Consequently, this study was included in the main analysis for the results of the prognostic value of p16 but not for hrHPV DNA. However, in a sensitivity analysis, this study was included for the prognostic value of hrHPV DNA and the combination with p16 considering the *a posteriori* HPV DNA cut-off (see Supplementary Materials). More detailed information of the characteristics of the included studies is provided in Supplementary Table 1.

p16 overexpression in anal cancer patients

p16 IHC results were available from 687/693 (99%) patients. Seventy seven percent (526/687) of the patients with anal cancer were found to be p16+, 161 (23%) were p16- (Table 3). Of note, definitions of a positive p16 IHC test result were heterogeneous among the included studies. Some authors considered the staining intensity (36), while others incorporated the staining pattern (32, 33) or composite scores of staining intensity and percentage of positive cells (34, 38) or of staining intensity, pattern and percentage of positive tumor cells (35) into the definition. A common definition was found among two studies only (32, 33). No information on the definition of a positive test result was provided by one study (37). Therefore, this study was excluded from the main analysis. The detailed definitions used within the included studies are provided in Supplementary Table 2. For the six studies with p16 results included in the main analysis, the average age in p16+ patients was 60.2 years and did not significantly differ from that of p16- patients with 61.2 years ($p=0.3864$). In females, the pooled p16-positivity was 83%, significantly differing from the pooled prevalence in men (65%; $p<0.0001$). The distribution of covariates among p16+ and p16- patients is illustrated in Supplementary Fig. 1 and 4.

hrHPV DNA in anal cancer patients

Six of the seven included studies performed HPV DNA testing involving 601 tested patients (32-36, 38). All of them applied PCR-based methods that were able to detect hrHPV DNA types. While some of the six studies also detected low-risk (lr)HPV DNA types, our meta-analysis exclusively focused on hrHPV DNA types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 (44)). The detectable hrHPV DNA types as well as the applied techniques and cut-offs for a positive test result differed among the included studies (Supplementary Table 7). Notably, one study used a rather analytical cut-off for defining HPV DNA-positivity by determining the median of HPV 16 DNA copies of the cohort and was therefore excluded from the main analyses (38). An a posteriori cut-off was determined in this meta-analysis, defining a positive test result by a viral load of ≥1 HPV 16 DNA copy/beta-globin copy. This threshold was based on the rationale that truly HPV-induced tumors should harbor at least one viral copy per tumor cell (45). The HPV 16 DNA data after cut-off adjustment of the respective study was included in the sensitivity analysis (Supplementary Tables 9 and 10).

hrHPV DNA was detected in 376/459 (82%) patients with known hrHPV DNA status of the five studies included in the main analysis (Table 3). The most frequently observed hrHPV DNA type was HPV 16 (312 (85%) with a single infection and 29 (8%) with multiple infections). The second most common hrHPV type was HPV 18, present as single infection in 8 patients (2%) and as co-infection with other types in 11 patients (3%). Further details on hrHPV type distribution are provided in Supplementary Table 8. The percentage of hrHPV DNA+ anal cancers varied from 67% to 87% (see Table 3). The average age at diagnosis was 60.4 years and 59.6 years, in hrHPV DNA positive and negative cases, respectively (p=0.5888). In females, hrHPV DNA-positivity was 89%, whereas it was only 71% in males (p<0.0001). The distribution of covariates among hrHPV DNA+ and hrHPV DNA- patients is illustrated in Supplementary Fig. 2.

Table 2: Patient characteristics among the seven studies included in the meta-analysis

Characteristics	**Yhim, 2011**	**Gilbert, 2013**	**Koerber, 2014**	**Mai, 2015**	**Meulendijks, 2015**	**Balermpas, 2017**	**Belgioia, 2015**	**Total**
Period of patient inclusion	1998-2009	2004-2009	2000-2011	1990-2012	2003-2011	1989-2016	2009-2014	
Follow-up duration: median and range (months)	39 (2-111)	28 (1-87)	49 (3-169)	47 (1-205)	32 (3-96)	40 (1-325)	26 (1-71)	35 (1-325)
Cohort size (n)	47	153	90	106	106	150	41	693
Age: median and range (years)	65 (44-90)	62 (34-93)	55 (22-94)	60 (31-86)	60 (34-86)	59 (30-84)	63 (32-84)	61 (22-94)
Gender								
female	25 (53%)	93 (61%)	77 (86%)	63 (59%)	56 (53%)	84 (56%)	38 (93%)	436 (63%)
male	22 (47%)	60 (39%)	13 (14%)	43 (41%)	50 (47%)	66 (44%)	3 (7%)	257 (37%)
T-stage								
T1/T2	31 (66%)	59 (39%)	64 (71%)	79 (75%)	56 (53%)	106 (71%)	22 (54%)	417 (60%)
T3/T4	16 (34%)	65 (42%)	26 (29%)	27 (25%)	50 (47%)	44 (29%)	19 (46%)	247 (36%)
N/A		29 (19%)						29 (4%)
N-stage								
N0	29 (62%)	80 (52%)	68 (76%)	70 (66%)	48 (45%)	99 (66%)	16 (39%)	410 (59%)
N1-3	18 (38%)	53 (35%)	22 (24%)	36 (34%)	57 (54%)	51 (34%)	25 (61%)	262 (38%)
N/A		20 (13%)			1 (1%)			21 (3%)
UICC-stage (7th ed.)								
stage 1		9 (6%)	15 (17%)	16 (15%)	4 (4%)		2 (5%)	56 (8%)
stage 2		52 (34%)	46 (51%)	45 (43%)	36 (34%)		12 (29%)	191 (28%)
stage 3A		24 (16%)	11 (12%)	11 (10%)	34 (32%)		12 (29%)	92 (13%)
stage 3B		37 (24%)	16 (18%)	20 (19%)	31 (29%)		14 (34%)	118 (17%)
stage 4		3 (2%)	2 (2%)	13 (12%)				18 (3%)

N/A	47 (100%)	28 (18%)		1 (1%)	1 (1%)	150 (100%)	1 (2%)	228 (33%)
HIV status								
positive		9 (6%)	1 (1%)	2 (2%)	10 (9%)	24 (16%)	2 (5%)	48 (7%)
negative	47 (100%)	70 (46%)	81 (90%)		34 (32%)	126 (84%)	39 (95%)	397 (57%)
N/A		74 (48%)	8 (9%)	104 (98%)	62 (59%)			248 (36%)

Table 3: Prevalence of p16 overexpression and of hrHPV DNA among all anal cancer patients

		p16 Status			hrHPV DNA Status		
Study	**Year**	**Positive**	**Negative**	**N/A**	**Positive**	**Negative**	**N/A**
Yhim	2011	39 (83%)	8 (17%)	0 (0%)	35 (74%)	12 (26%)	0 (0%)
Gilbert	2013	137 (90%)	16 (10%)	0 (0%)	102 (67%)	8 (5%)	43 (28%)
Koerber	2014	75 (83%)	15 (17%)	0 (0%)	75 (83%)	15 (17%)	0 (0%)
Mai	2015	74 (70%)	32 (30%)	0 (0%)	72 (68%)	34 (32%)	0 (0%)
Meulendijks	2015	96 (91%)	10 (9%)	0 (0%)	92 (87%)	14 (13%)	0 (0%)
Balermpas*	2017	76 (51%)	74 (49%)	0 (0%)	94* (63%)	48* (32%)	8* (5%)
Belgioia**	2015	29** (71%)	6** (15%)	6** (15%)	-	-	41 (100%)

*excluded from the main meta-analysis for hrHPV since no clinically relevant cut-off was defined by the authors. The prevalence of hrHPV DNA is based on an *a posteriori* cut-off for HPV 16 DNA applied by the reviewers. The meta-analysis with this *a posteriori* cut-off for Balermpas et al., 2017, was included in a sensitivity analysis presented in the Supplementary Materials. The eight cases considered as N/A contained other non-HPV16 types, not classifiable with the a posteriori HPV16 cut-off.

**excluded from the main meta-analysis for p16 since no information on the definition of a positive p16 test result was provided by the authors. A meta-analysis including this study (Belgioia et al., 2015) was included in a sensitivity analysis presented in the Supplementary Materials.

Prognostic value of the p16 status

The three-year survival rate was 84% (95% CI, 81-88%) for patients with a p16+ ASCC and 49% (95% CI, 40-58%) for patients with a p16- ASCC. A median survival time was not reached in p16+ tumor patients during the observation period of 87 months, while p16- patients showed a median survival of 35 months (Fig. 2).

Fig. 2: Pooled overall survival (OS) curves stratified by p16 status in anal cancer patients of the six studies included in the main analysis (for patients with known p16 status and outcome information) and weighted by the study's total sample size. Number of patients under observation at risk of dying at the beginning of each year are mentioned under the X-axis.

Superior survival among p16+ compared to p16- patients was observed in all six studies with unadjusted hazard ratios (HR) ranging from 0.16 (95% CI, 0.08-0.33) (36) to 0.78 (95% CI, 0.41-1.51)

(38). The pooled unadjusted HR for the six included studies was 0.39 (95% CI, 0.19-0.80) (Supplementary Fig. 6). Adjusting for age, gender, T- and N-stage the pooled HR of p16+ compared to p16- patients was 0.49 (95% CI, 0.24-0.99; Fig. 3).

Fig. 3: Forest plot of adjusted hazard ratios (HR) comparing survival in p16+ versus p16- ASCC patients, adjusted for age, gender, T- and N-stage.

Prognostic value of hrHPV DNA detection

Three-year survival rates for the hrHPV DNA+ and hrHPV DNA- groups were 84% (95% CI, 80-88%) and 52% (95% CI, 38-64%). hrHPV DNA- ASCC patients had a median survival of 38 months, while median survival was not reached during the total observation period for hrHPV DNA+ patients (Fig. 4).

Fig. 4: Pooled overall survival (OS) curves stratified by hrHPV DNA status in anal cancer patients of the five studies included in the main analysis (for patients with known hrHPV DNA-status and outcome information) and weighted by the study's total sample size. The number of patients under observation at risk of dying at the beginning of each year are mentioned under the X-axis.

The unadjusted HR for the hrHPV DNA+ compared to the hrHPV DNA- group was 0.29 (95% CI, 0.22-0.39), with HR ranging between 0.22 (95% CI, 0.07-0.69; (35)) and 0.39 (95% CI, 0.17-0.89; (33); Supplementary Fig. 7). When adjusting for age, gender, T- and N-stage, the pooled HR was 0.33 (95%CI, 0.24-0.45; Fig. 5).

Fig. 5: Forest plot of hazard ratios (HR) comparing the survival of hrHPV DNA+ to hrHPV DNA- ASCC patients, adjusted for age, gender, T- and N-stage.

Combined p16 and hrHPV DNA-status

The proportion of p16+/hrHPV DNA+ patients was 85% (36), 78% (32), 59% (33), 87% (34) and 66% (35) in the five studies. For all studies pooled, 350/459 (76%) ASCC tested p16+/hrHPV DNA+. Fifty-two patients (11%) were negative for both markers. Thirty-one tumors (7%) were p16+/hrHPV DNA- and 26 (6%) tumors were p16-/hrHPV DNA+.

Survival in patients according to the combined p16 and hrHPV DNA-status

The RE model showed that the pooled adjusted hazard was significantly lower for the p16+/hrHPV DNA+ compared to p16-/hrHPV DNA status (HR=0.26 (95% CI, 0.14-0.50); Fig. 6). The pooled adjusted hazard for p16-/hrHPV DNA+ was also significantly lower compared to the p16-/hrHPV DNA- group (HR=0.52 (95% CI, 0.33-0.83)). No significant difference was observed in pooled adjusted HR between patients with a p16+/hrHPV DNA- and a p16-/hrHPV DNA- tumor (p=0.6498) (Table 4). Patients with double positive tumors (p16+/hrHPV DNA+) showed superior survival compared to all other groups (Table 4). This relation was statistically significant when comparing double negative (p16-/hrHPV DNA-) tumors (HR=3.82 (95% CI, 2.01-7.24)) or p16+/hrHPV DNA- tumors (HR=3.19 (95% CI, 1.41-7.23)) with this group, but not significant for p16-/hrHPV DNA+ tumors (HR=2.33 (95% CI, 0.89-6.09)).

Fig. 6: Forest plot of hazard ratios (HR) comparing the survival of p16+/hrHPV DNA+ ASCC patients to p16-/hrHPV DNA- ASCC patients, adjusted for age, gender, T- and N-stage

Table 4: Pooled hazard ratios (HR) of p16 and hrHPV DNA status combinations, adjusted for age, gender, T- and N-stage for five included studies, using the double negative (top) and double positive cases (bottom), as reference.

Index group	Reference group	Adjusted HR	95% CI	p
p16+/hrHPV DNA+	p16-/hrHPV DNA-	0.26	0.14-0.50	0.0044
p16-/hrHPV DNA+		0.52	0.33-0.83	0.0205
p16+/hrHPV DNA-		0.78	0.19-3.21	0.6498
p16-/hrHPV DNA-		1.00	-	-
p16+/hrHPV DNA+	p16+/hrHPV DNA+	1.00	-	-
p16-/hrHPV DNA+		2.33	0.89-6.09	0.0672
p16+/hrHPV DNA-		3.19	1.41-7.23	0.0171
p16-/hrHPV DNA-		3.82	2.01-7.24	0.0044

The pooled three-year survival rate in patients with p16+/hrHPV DNA+ ASCC was 86% (95% CI 82-90%) compared to 39% (95% CI, 24-54%) in patients with p16-/hrHPV DNA- ASCC. In patients with discordant status, the three-year survival rate was 55% (95% CI, 35-71%) in p16-/hrHPV DNA+ and 75% (95% CI, 56-86%) in p16+/hrHPV DNA- patients.

Fig. 7: Pooled overall survival (OS) curves stratified by the combined p16 and hrHPV DNA-status in ASCC patients (for patients with known p16 and hrHPV DNA status and outcome information) and weighted by the study's total sample size. The number of patients under observation at risk of dying at the beginning of each year are mentioned under the X-axis.

Discussion

The incidence of anal cancer has been rising worldwide throughout the past decades. In Europe, incidence rates have on average increased by 23.7% and 26.6% every five years between 1988 and 2012 in men and women, respectively (5). In the United States, incidence rates have been increasing annually by 2.7% between 2001 and 2015 (46). The growing disease burden has coincided with intensive research on the etiology of anal carcinogenesis, which could support the development of refined treatment opportunities. Various studies identified oncogenic (hr) HPV types as causative agents in the majority of anal cancers, and several authors also reported better clinical outcomes for patients with HPV-associated compared to HPV-negative anal cancers (reviewed in (30, 31)). Given the emerging discussions about the value of therapy de-escalation in patients with HPV-induced anal cancer, the prognostic relevance of HPV-related markers gain particular momentum. In the light of those developments, we sought to determine the prognostic value of HPV in anal cancers by conducting an individual patient-data (IPD) meta-analysis. This allows the assessment of hrHPV DNA and p16 alone and in combination as well as potential confounding factors within multivariate analyses.

In the pooled cohort of 459 patients derived from five eligible studies, we observed a significantly reduced mortality during the observation period in patients with a combined p16+ and hrHPV DNA+ status in their anal cancers compared to patients that were negative for both markers (pooled adjusted HR=0.26 (95% CI, 0.14-0.50)). The pooled three-year survival rates in the double-positive and double-negative groups were 86% (95% CI, 82-90%) compared to 39% (95% CI, 24-54%), respectively. Combined detection of hrHPV DNA and p16 overexpression has been suggested as a reliable and practical approach to identify an etiological role of HPV in malignant tumors in different sites of the genital and head and neck region (20, 23, 45). Assuming etiological relevance of HPV in anal cancers that are both p16+ and hrHPV DNA+, the pooled prevalence of HPV-induced anal cancers in our meta-analysis was 76%. This proportion reflects an average among five studies (459 patients) with prevalence rates ranging from 59% to 87%. Importantly, our meta-analysis indicates that the average proportion of presumably HPV-induced cancers is situated in the lower range of previous estimations that were based solely on HPV DNA detection (about 70% to 95%; (8, 47-49)). The significantly better survival rates of the p16+/hrHPV DNA+ compared to the p16-/hrHPV DNA- patient group imply that HPV etiology in anal cancers is a major determinant of patient prognosis. Improved prognostication by combined p16 and hrHPV DNA detection compared to single marker analysis has also been demonstrated in a large meta-analysis on tumors in the head and neck region (25).

It has been suggested that the superior survival rates of patients with HPV-induced, i.e. p16+ and hrHPV DNA+, could be explained by distinct biological treatment responses irrespective of the anatomical origin of the tumor. This hypothesis is supported by studies reporting a higher sensitivity of HPV-transformed tumor cells compared to HPV-negative cell lines from oropharyngeal cancers towards radio(chemo)therapy (50-52), which also represents a central treatment component for anal cancer patients. The heterogeneity of reported treatment data among the included studies in our meta-analysis precluded the determination of treatment effects on patient prognosis. However, considering the favorable prognosis of patients with HPV-induced cancers observed in our study and the resulting prospect of treatment de-escalation in these patients, future studies should address the influence of treatment modalities on HPV-induced anal cancers in greater detail.

The determination of possible etiological factors in the group of p16-/hrHPV DNA- ASCC was not feasible on the basis of the obtained IPD in our meta-analysis. However, considering that patients with these cancers displayed poor survival rates and represented more than 10% of all ASCC patients in our meta-analysis, it is of high clinical relevance to investigate potential carcinogenic risk factors in this patient group in future studies, thereby exploring whether any preventive measures might be feasible.

Overall, we observed a gradient of increasing mortality among the four different groups (p16+/hrHPV DNA+ < p16-/hrHPV DNA+ < p16+/hrHPV DNA- < p16-/hrHPV DNA-). Several authors have advocated against the use of either p16 or HPV DNA alone as indicators of HPV-induced etiology in cancers, but recommend their combined use to reliably differentiate HPV-induced from HPV-unrelated tumors (45, 53, 54). This caution is on the one side based on indications that tumors with a discordant hrHPV DNA/p16 status might represent biologically and clinically distinct tumor entities that could be accompanied by different mortality rates (25). On the other side, the discordant status might also result from misclassifications of either p16 or HPV DNA: Contamination of tumor specimens with environmental hrHPV DNA during tumor collection or sample processing ((55, 56); Human papillomavirus laboratory manual, First edition, 2009) or overly sensitive detection methods may e.g. account for a false-positive hrHPV DNA test result and thus classification of the tumor as p16-/hrHPV DNA+ while it is in fact HPV-negative (p16-/hrHPV DNA-). The true HPV status and the associated clinical prognosis in cases with discordant p16/hrHPV DNA status could thus be masked, resulting in their positioning between the mortality rates for HPV-induced (p16+/hrHPV DNA+) and HPV-negative (p16-/hrHPV DNA-) cases. Considering the small patient numbers in the groups with discordant p16/hrHPV DNA status in our meta-analysis, we cannot derive firm conclusions about differential survival and relative prognosis between the two groups at this point. We therefore recommend continuing efforts to collect IPD data from more and larger studies to disentangle this question with more power. This appears particularly important considering discussions on treatment de-escalation based on the tumoral HPV status.

Prevalence rates for p16, hrHPV DNA and combined p16 and hrHPV DNA detection were significantly higher in female compared to male anal cancer patients in the pooled cohort of our meta-analysis (83% vs 65%, p<0.0001 for p16; 89% vs. 71%, p<0.0001 for hrHPV DNA; and 84% vs 63%, p=<0.0001 for combined p16+/hrHPV+). Possible explanations for the observed differences could relate to a higher risk of autoinoculation with HPV from genital sites particularly in women with genital HPV-induced precancerous lesions (57, 58) as well as to a reduced ability to clear hrHPV infections in women (59). Our meta-analysis data indicating a higher proportion of HPV-induced ASCC in female than in male patients are in line with epidemiological data demonstrating significantly higher incidence rates of anal cancer in women compared to men (4).

Strengths and limitations

To the best of our knowledge, this is the first IPD meta-analysis assessing the prognostic value of HPV-related markers in anal cancers. We could compile a large pooled cohort of 693 patients derived from seven original studies in five different countries enabling us to control for several confounding factors, such as age, gender, T- and N-stage, in our multivariate analyses. Further, the provision of data on detected HPV types in the original studies allowed us to specifically focus on DNA of high-risk HPV types, refining our studies to biologically meaningful markers that can indicate transforming relevance of HPV in the anal cancers. We could further study the relation between p16/hrHPV DNA and the clinical course over a follow-up period of up to 72 months in the total cohort, thereby providing long-term data on the prognostic significance of those markers.

Our meta-analysis also holds some limitations that may confine generalizability of the observed results. First, the prognostic relevance of p16 in ASCC was of major interest in the study design of this meta-analysis and the availability of p16 IHC data therefore represented a central inclusion criterion. Consequently, studies reporting hrHPV DNA data but no p16 IHC data were excluded, thereby limiting the number of included studies to assess the prognostic value of hrHPV DNA. Moreover, study sizes are rather small given the rarity of anal cancer. In particular, the groups with discordant p16 and hrHPV DNA status were small, downgrading the quality of evidence derivable from our meta-analysis on these groups. The methods and cut-off definitions for the detection of hrHPV DNA and p16 differed considerably among the seven included studies (Supplementary Tables 2 and 7), which could explain the large variability of prevalence rates of the markers observed in this meta-analysis. This challenge has also been recognized for HPV-related analyses in other anatomical sites (23, 60, 61) and might be resolved at least partly by the introduction of consensus cut-offs for the individual test methods in future studies. We performed an *a posteriori* modification of a HPV 16 DNA cut-off definition for one study included in this meta-analysis (38) that more closely resembled the ones used in the other studies. Sensitivity analyses (Supplementary Tables 9 and 10) showed that the prognostic value of hrHPV DNA was not considerably altered when this study was excluded from the pooled evaluation after *a posteriori* modification of the cut-off definition (Fig. 5 and Table 4). This finding corroborates the value of consensus definitions for test interpretation in HPV-related analyses. We therefore highly encourage the conduction of large cohort studies specifically comparing the prognostic value of different definitions of p16- and hrHPV DNA-positivity as a basis for a future consensus definition in ASCC. Finally, information on tumor treatment was not available for all included studies and was largely heterogeneous for the patients with available data, precluding analyses on treatment modality as a potential confounding factor. Another limitation is that certain statistical analyses are restricted to the follow-up duration of all studies, and therefore determined by the shortest study.

Conclusion

We demonstrated for the first time the superior prognostic value of combined hrHPV DNA and p16 compared to their individual detection in an IPD meta-analysis on anal cancer patients. Patients with HPV-unrelated (p16-/hrHPV DNA-) anal cancers showed a poor three-year survival rate of only 39%, whereas this rate was doubled (86%) in patients with HPV-induced (p16+/hrHPV DNA+) tumors. In contrast to previous meta-analyses that were based on extracted aggregated data, we were able to control for confounding variables. In the light of the rising incidence rates of anal cancer and the prospect of differential treatment of affected patients in relation to tumoral HPV status, our findings invite for more trials aiming to optimize future treatment of anal cancer patients according to HPV etiology.

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