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Human papillomavirus DNA prevalence and type distribution in anal carcinomas worldwide

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Abbreviations: AIN: anal intraepithelial neoplasia; DEIA: DNA enzyme immunoassay; DNA: deoxyribonucleic acid; HE: hematoxylin–eosin; HIV: human immunodeficiency virus; HPV: human papillomavirus; LiPA: line probe assay; PCR: polymerase chain reaction; SCC: squamous cell carcinoma; SPF: short PCR fragment

Additional Supporting Information may be found in the online version of this article.

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Knowledge about human papillomaviruses (HPV) types involved in anal cancers in some world regions is scanty. Here, we describe the HPV DNA prevalence and type distribution in a series of invasive anal cancers and anal intraepithelial neoplasias (AIN) grades 2/3 from 24 countries. We analyzed 43 AIN 2/3 cases and 496 anal cancers diagnosed from 1986 to 2011. After histopathological evaluation of formalin-fixed paraffin-embedded samples, HPV DNA detection and genotyping was performed using SPF-10/DEIA/LiPA$_{25}$ system (version 1). A subset of 116 cancers was further tested for p16^{INK4a} expression, a cellular surrogate marker for HPV-associated transformation. Prevalence ratios were estimated using multivariate Poisson regression with robust variance in the anal cancer data set. HPV DNA was detected in 88.3% of anal cancers (95% confidence interval [CI]: 85.1–91.0%) and in 95.3% of AIN 2/3 (95% CI: 84.2–99.4%). Among cancers, the highest prevalence was observed in warty–basaloid subtype of squamous cell carcinomas, in younger patients and in North American geographical region. There were no statistically significant differences in prevalence by gender. HPV16 was the most frequent HPV type detected in both cancers (80.7%) and AIN 2/3 lesions (75.4%). HPV18 was the second most common type in invasive cancers (3.6%). p16^{INK4a} overexpression was found in 95% of HPV DNA-positive anal cancers. In view of the results of HPV DNA and high proportion of p16^{INK4a} overexpression, infection by HPV is most likely to be a necessary cause for anal cancers in both men and women. The large contribution of HPV16 reinforces the potential impact of HPV vaccines in the prevention of these lesions.

What's new?

Human papillomavirus (HPV) is linked to anal cancer through high HPV DNA-detection rates. Here, in one of the largest international studies to date, HPV DNA was detected in more than 88% of anal cancers and more than 95% of anal intraepithelial neoplasias grades 2/3. HPV16 was the most frequently detected virus type, followed by HPV18. Overexpression of p16^{INK4a}, a surrogate marker for HPV-associated transformation, was found in 95% of HPV-positive anal cancers. The data implicate HPV as a causative factor in anal cancer.

Anal carcinomas are relatively rare, with about 27,000 new cases diagnosed worldwide in 2008 and age-adjusted incidence rates around 1 per 100,000 population.[1,2] However, recent reports indicate increasing incidences in some developed countries linked to several factors such as changes in sexual behavior.[3–5] Men having sex with men, particularly those infected by human immunodeficiency virus (HIV), represent a high risk group for the development of anal cancer.[6–8] HIV-infected women also have a high risk of developing anal intraepithelial neoplasia (AIN) and invasive anal cancer.[8,9]

Few case–control studies have evaluated the association between human papillomaviruses (HPVs) and anal cancer reporting odds ratios between 2 and 7 for HPV16 and HPV18 seropositivity for both men and women.[10] In addition, one case–control study that evaluated the presence of the virus in tumor tissue found a higher viral detection among anal carcinomas (88%, 340/388) than rectal adenocarcinomas (0%, 0/20).[11] Among HPV-related cancers, anal cancer has been linked to HPV with the highest deoxyribonucleic acid (DNA) detection rates just after cervical cancer. HPV DNA prevalence has been estimated at 94% in AIN grades 2/3 and 88% in anal cancer, with HPV16 the most frequent HPV type identified.[1,12] Information on anal cancer HPV type distribution is lacking in some world regions with most of the published reports coming from United States of America (USA) and Europe.[12] Such information is crucial to estimate the impact of HPV prophylactic vaccines in the reduction of anal cancer burden from a global perspective, particularly now that we have evidence for efficacy of HPV vaccines in the prevention of AIN and anal HPV persistent infection by types included in the vaccines.[13]

Our study aims to describe the HPV DNA prevalence and type distribution in a series of 539 AIN 2/3 and anal cancers from 24 countries. To our knowledge, and despite the underrepresentation of certain geographical regions, the data reported in this article represent the largest international effort to assess the role of HPVs in anal cancer using a standard protocol with a high sensitive HPV DNA detection test together with p16^{INK4a} expression evaluation, a cellular surrogate marker for HPV-associated transformation.

Material and Methods

Study design

A retrospective cross-sectional study was designed and coordinated by the Institut Català d'Oncologia (ICO), Barcelona,

Spain, and DDL Diagnostic Laboratory, Rijswijk, The Netherlands, to estimate the HPV DNA prevalence and type distribution in patients with AIN 2/3 and invasive anal cancers diagnosed from 1986 to 2011. Formalin-fixed paraffin-embedded (FFPE) specimens were obtained from pathology archives in 24 countries: Europe (Bosnia-Herzegovina, Czech Republic, France, Germany, Poland, Portugal, Slovenia, Spain and United Kingdom); North America (USA); Latin America (Chile, Colombia, Ecuador, Guatemala, Honduras, Mexico and Paraguay); Africa (Mali, Nigeria and Senegal); Asia (Bangladesh, India and South Korea) and Oceania (Australia). Centers were requested to provide non selected series of cases from their archives preferably consecutive in time. Information about age at and year of diagnosis, gender and original histological diagnosis was also obtained from the participating centers.

Histopathological evaluation

FFPE tissue blocks were processed under strict conditions to avoid potential contamination as described in a previous publication.[14] At least four FFPE sections were obtained from each block using the sandwich method. First and last sections were used for histopathological evaluation after hematoxylin and eosin (HE) staining. The intermediate sections were used for HPV DNA testing. The laboratory at ICO processed the FFPE tissue blocks and reviewed the resulting HE slides. The latter was performed by following the consensus criteria established by an expert panel of pathologists based on the WHO classification of the digestive system.[15] The pathology evaluation included several items such as histological diagnosis, tumor subtype and adequacy of the sample for further HPV testing. Adenocarcinomas and basal-cell carcinomas were excluded from the study as most of the adenocarcinomas likely represent a downward spread from adenocarcinomas of the rectum and basal cells arise from skin epithelium, being both HPV-unrelated types of lesions.[10,12] To confirm the non HPV relationship of the adenocarcinomas received and to support the exclusion criteria decision, a subset of more than 50% of these cases were processed and HPV was analyzed. Only two cases out of the 62 adenocarcinomas (HPV DNA analyzed) were positive for viral presence (3%), supporting the non HPV association of these tumors. A block was determined to be adequate for further HPV DNA testing if invasive cancer or an AIN 2/3 lesion was observed in the two HE-stained sections of the specimen. In case of discrepancies between the histologic diagnosis in local and in the reference pathology laboratories, the cases were re-evaluated and the results obtained at the reference lab prevailed. To control for possible sources of contamination, blocks containing non HPV-related tissues processed in the local pathology lab at the same time as the anal specimens under study were blindly processed (5% of the total anal cases).

HPV DNA detection and typing

For each specimen, a paraffin tissue section was digested with 250 μL of proteinase K solution (10 mg/mL proteinase K in 50 mM Tris-HCI, pH 8.0) to release DNA. Short PCR fragment (SPF)−10 polymerase chain reaction (PCR) was performed in a final reaction volume of 50 μL using 10 μL of extracted DNA (dilution, 1:10). The amplified PCR products were tested for the presence of HPV DNA using a DNA enzyme immunoassay (DEIA) as described previously.[16,17] DEIA can recognize at least 54 HPV types. Amplimers testing positive by DEIA for viral DNA were used to perform the reverse hybridization line probe assay ($LiPA_{25}$) (version 1: produced at Laboratory Biomedical Products, Rijswijk, The Netherlands). The $LiPA_{25}$ detection system allows for genotyping of 25 HPVs categorized by the IARC within the Group 1 (HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59), Group 2A (HPV68), Group 2B (HPV34, 53, 66, 70 and 73), Group 3 (HPV6 and 11), as well as other HPVs (HPV40, 42, 43, 44, 54 and 74).[10] All these types belong to nine species within *Alphapapillomaviruses*. The sequence variation within the SPF-10 interprimer region allows the recognition of these different HPV types, except for types 68 and 73, as their interprimer regions are identical and cannot be distinguished by $LiPA_{25}$. Specimens testing positive for HPV DNA by DEIA but that could not be typed by $LiPA_{25}$ were further analyzed by direct Sanger sequencing of PCR products as described by Geraets *et al.*[18] The cases that could not be sequenced were labeled as "*HPV undetermined*". Further, specimens with an inconclusive probe line pattern by $LiPA_{25}$ (*i.e.*, HPV68/73 or HPV39/68/73) were also sequenced to distinguish the specific HPV types. To evaluate the quality of DNA, all HPV DNA-negative samples were subjected to a PCR, targeting the human tubulin gene (forward primer: TCCTCCACTGGTACACAGGC; reverse primer: CATGTT GCTCTCAGCCTCGG), which generated a 65-bp amplicon, the same size as the SPF-10 amplicon used for assessing the presence of HPV DNA. Samples that were both negative for HPV DNA and tubulin were considered to be of inadequate quality and were therefore excluded from the final analyses.

$p16^{INK4a}$ expression

The evaluation of immunohistochemical $p16^{INK4a}$ expression was performed in all HPV DNA-negative invasive anal cancer cases with available material and in a random selection of HPV-positive cases (total, $n = 116$). $p16^{INK4a}$ was detected using the CINtec histology kit (clone E6H4, Roche mtm laboratories AG, Germany) by following the manufacturer's protocol. A pattern of diffuse staining of more than 25% stained cells (nuclear and cytoplasmic staining) was considered positive.[19,20]

Statistical analysis

Available information for the statistical analysis was as follows: country, age at and year of diagnosis, gender, histopathological diagnosis, the presence of HPV DNA, HPV type and the expression of $p16^{INK4a}$. Histological subtypes in anal cancers were grouped into the following categories: squamous cell carcinomas (SCC) 100% warty–basaloid (included

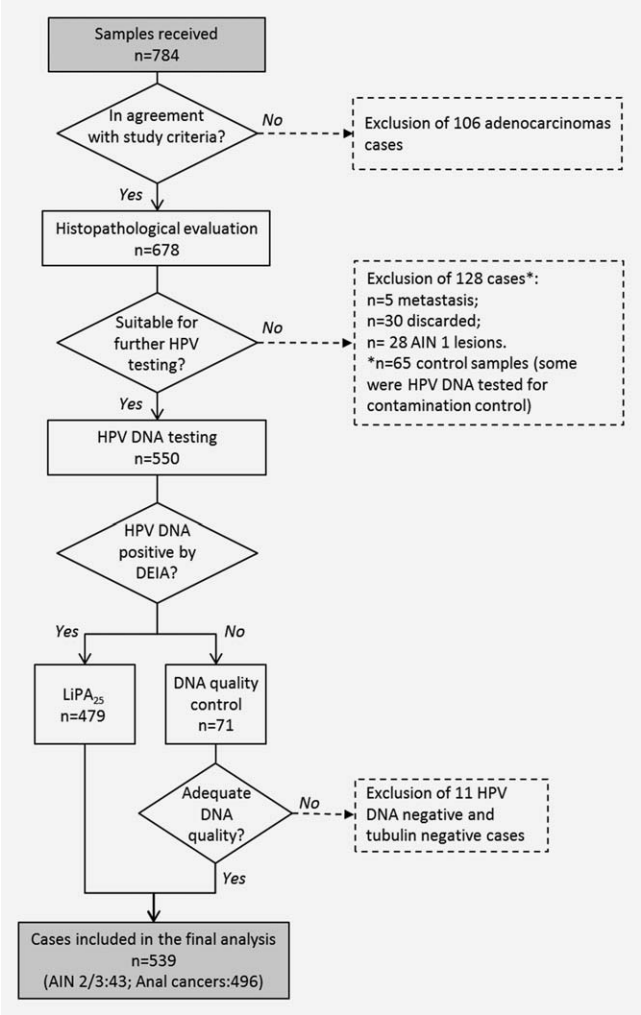

Figure 1. Study algorithm. AIN 2/3: Anal Intraepithelial Neoplasia 2/3.

exclusively or combinations of warty, basaloid or papillary basaloid histologies), SCC 100% non warty–basaloid (SCC without warty–basaloid morphological features), SCC mixed histologies (mix of the previous histological subtypes), other (undifferentiated, poorly differentiated, neuroendocrine and adenosquamous carcinomas).

The prevalence of HPV DNA and HPV type specific detection percentages was determined according to the geographical regions, histopathological categories, gender, patient's age at diagnosis and year of diagnosis. Prevalence ratios (PRs) were estimated using bivariate and multivariate Poisson regression models with robust variance.[21] In the final model, we included region, year of and age at diagnosis and gender. Histological diagnosis was not included in the regression analysis as it was considered as an intermediate variable in the carcinogenic process. The best fitting model was selected based on the log-likelihood ratio test. PRs were estimated only for anal cancers because AIN 2/3 subset of cases was small and showed a high HPV DNA detection rate (only two cases out of 43 AIN 2/3 were HPV DNA negative).

The prevalence of HPV DNA was estimated among finally included cases and HPV type specific relative contribution was calculated among the HPV DNA-positive cases. Multiple infections were added to single types under a weighting attribution proportional to the detection found in cases with single types as described previously.[14] To evaluate the increase or decrease on HPV type specific relative contributions between type of lesions, relative contribution ratios and their 95% confidence intervals (CI) were estimated (ratio of type specific relative contribution: percentage of a specific type in anal cancer/percentage of the same type in high-grade preneoplastic lesions).

Agreement between HPV DNA detection and $p16^{INK4a}$ expression was assessed by Kappa score. The McNemar test for matched pair data was used for assessing unequal distribution of discordant results.

Statistical significance for all analyses was set at the two-sided 0.05 level. Data analyses were performed with the Statistical Package for the Social Sciences (SPSS) version 13.0 (SPSS, Chicago, IL) and with STATA version 10.0 (Stata, Computing Resource Center, College Station, TX).

Ethical consideration

Specimens were received anonymously and allocated a unique identification number upon reception. All protocols were approved by international and ICO ethics committees and study progress was overseen by an international steering committee specifically formed for the supervision and advising in critical issues of the project.

Results

Initially, 784 FFPE tissue samples were collected. From these, 65 samples were non HPV-related tissues and used for contamination control; and 180 cases were excluded from the analyses: 169 were not suitable for HPV DNA testing based on the pathological criteria (*e.g.*, non preinvasive or invasive lesion observed, adenocarcinomas and basal cell carcinomas, among others) and 11 cases were finally excluded for inadequate DNA quality, being both HPV DNA and tubulin negative (Fig. 1). Therefore, 43 AIN 2/3 cases and 496 invasive anal cancers were included in the final analysis.

Patients with an AIN 2/3 diagnosis were approximately 12 years younger than patients diagnosed with an anal cancer (mean age at diagnosis, 50.8 years [standard deviation, SD = 15.8] for AIN 2/3 *vs.* 62.8 [SD = 14.7] for invasive cancer cases [$p < 0.001$]). Two-thirds of both preneoplastic and invasive cancer cases occurred in females (Table 1). There was a higher representation from European and Latin American countries and from 2000 to 2011 time period of diagnosis. Warty–basaloid SCC histological type accounted for 58.5% of the anal cancers, being basaloid the most common subtype identified in this category (76.2%) (Table 2). Less frequently, we identified non warty–basaloid SCC (33.3%), mixed warty–basaloid and non warty–basaloid histological SCC cases (6.0%) and "other" diagnoses (2.2%; 11 cases: four

Table 1. Sample description and HPV DNA prevalence in AIN 2/3 and invasive anal cancer cases

	AIN 2/3					Invasive anal cancer							
			HPV prevalence					HPV prevalence			PR		
	n	%	n	%	95 CI	n	%	n	%	95% CI	PR	95% CI	*p*-Value
Region													
Europe	23	53.5	22	95.7	[78.1–99.9]	169	34.1	148	87.6	[81.6–92.1]	**0.86**	**[0.75–0.97]**	**0.017**
North America[1]	–	–	–	–	–	96	19.4	92	95.8	[89.7–98.9]	1	–	–
Latin America	12	27.9	12	100.0	[73.5–100.0*]	157	31.7	142	90.4	[84.7–94.6]	**0.88**	**[0.77–0.99]**	**0.042**
Africa	1	2.3	1	100.0	[2.5–100.0*]	21	4.2	13	61.9	[38.4–81.9]	**0.60**	**[0.42–0.87]**	**0.006**
Asia and Oceania	7	16.3	6	85.7	[42.1–99.6]	53	10.7	43	81.1	[68.0–90.6]	**0.81**	**[0.68–0.97]**	**0.021**
Period of diagnosis													
1986–1999	4	9.3	4	100.0	[39.8–100.0*]	124	25.0	106	85.5	[78.0–91.2]	0.93	[0.85–1.01]	0.083
2000–2011[1]	39	90.7	37	94.9	[82.7–99.4]	372	75.0	332	89.2	[85.7–92.2]	1	–	–
Age at diagnosis (yo)													
<55[1]	30	69.8	29	96.7	[82.8–99.9]	135	27.2	121	89.6	[83.2–94.2]	1[2]	–	–
55–75	8	18.6	7	87.5	[47.4–99.7]	186	37.5	166	89.2	[83.9–93.3]	0.98	[0.91–1.05]	0.504
>75	4	9.3	4	100.0	[39.8–100.0*]	92	18.5	73	79.3	[69.6–87.1]	**0.86**	**[0.76–0.97]**	**0.017**
Missing information	1	2.3	1	100.0	[2.5–100.0*]	83	16.7	78	94.0	[86.5–98.0]	0.91	[0.79–1.05]	0.189
Gender													
Male	10	23.3	8	80.0	[44.4–97.5]	157	31.7	133	84.7	[78.1–90.0]	0.94	[0.87–1.01]	0.083
Female[1]	29	67.4	29	100.0	[88.1–100.0*]	329	66.3	296	90.0	[86.2–93.0]	1	–	–
Missing information	4	9.3	4	100.0	[39.8–100.0*]	10	2.0	9	90.0	[55.5–99.8]	1.01	[0.84–1.21]	0.947
TOTAL	43	100.0	41	95.3	[84.2–99.4]	496	100.0	438	88.3	[85.1–91.0]	–	–	–

[1]Reference category for multivariate analysis. Model adjusted for region, period of diagnosis, age at diagnosis and gender.
[2]*p*-Trend test, 0.016 (excluding missing category). The numbers that are highlighted in bold font are PRs with a *p*-value of <0.05. *One-sided, 97.5% CI.
Abbreviations: AIN 2/3: Anal Intraepithelial Neoplasia 2/3; HPV prevalence: HPV DNA positivity; yo: years old; 95% CI: 95% Confidence Interval.

Table 2. Histological diagnosis of invasive anal cancer cases

			HPV prevalence and PR					
	n	%	n	%	95% CI	PR	95% CI	*p*-Value
Histological diagnosis								
SCC 100% warty–basaloid[1]	290	58.5	278	95.9	[92.9–97.8]	1	–	–
SCC 100% nonwarty–basaloid	165	33.3	129	78.2	[71.1–84.2]	**0.82**	**[0.75–0.89]**	**<0.001**
SCC mixed histologies	30	6.0	28	93.3	[77.9–99.2]	0.97	[0.88–1.07]	0.595
Other[2]	11	2.2	3	27.3	[6.0–61.0]	**0.28**	**[0.11–0.75]**	**0.011**
TOTAL	496	100.0	438	88.3	[85.1–91.0]	–	–	–

[1]Reference category for univariate analysis.
[2]Other histological diagnosis category includes: four undifferentiated carcinomas, three neuroendocrine, three adenosquamous and one poorly differentiated. The numbers highlighted in bold font are PRs with a *p*-value of <0.05.
Abbreviations: HPV prevalence: HPV DNA positivity; SCC: Squamous Cell Carcinoma; 95% CI: 95% Confidence Interval.

undifferentiated, one poorly differentiated, three neuroendocrine and three adenosquamous tumors).

HPV DNA positivity was 95.3% (95% CI: 84.2–99.4%) for AIN 2/3 and 88.3% (95% CI: 85.1–91.0%) in invasive anal cancer (Table 1). Within invasive cancer cases, HPV prevalence varied by geographical region with the highest prevalence in North America (95.8%; 95% CI: 89.7–98.9%) and the lowest in Africa (61.9%; 95% CI: 38.4–81.9%). No statistically significant differences were observed for gender or for period of diagnosis, neither in a 10-year nor in a 5-year period. Patients with anal cancer positive for HPV DNA were diagnosed at a younger age than patients with HPV-negative tumors (age, 62.2 years; SD = 14.3 *vs.* 66.9, SD = 17.0; $p = 0.027$); there was a decreasing HPV DNA detection with

Table 3. HPV type specific relative contribution among HPV DNA-positive AIN 2/3 and invasive anal cancer cases

	AIN 2/3 (HPV+, n=41)				Invasive anal cancer (HPV+, n=438)					
	Single		Single + multiple[1]		Single		Single + multiple[1]		Relative contribution ratio (cancer:AIN)[2]	
HPV type	n	%	n	%	n	%	n	%	Ratio	95% CI
HPV6	–	–	–	–	8	(1.8)	8	(1.8)	–	–
HPV11	2	(4.9)	2	(5.0)	4	(0.9)	5	(1.1)	0.21	(0.04–1.06)
HPV16	27	(65.9)	31	(75.4)	332	(75.8)	354	(80.7)	1.07	(0.89–1.28)
HPV18	–	–	–	–	15	(3.4)	16	(3.6)	–	–
HPV30	–	–	–	–	1	(0.2)	1	(0.2)	–	–
HPV31	1	(2.4)	2	(3.7)	5	(1.1)	8	(1.9)	0.51	(0.09–2.83)
HPV33	–	–	–	–	10	(2.3)	12	(2.7)	–	–
HPV35	–	–	–	–	7	(1.6)	7	(1.6)	–	–
HPV39	–	–	–	–	1	(0.2)	2	(0.5)	–	–
HPV42	–	–	–	–	1	(0.2)	1	(0.2)	–	–
HPV45	1	(2.4)	1	(2.4)	4	(0.9)	4	(0.9)	0.37	(0.04–3.27)
HPV51	1	(2.4)	2	(3.7)	–	–	–	–	–	–
HPV52	–	–	–	–	2	(0.5)	3	(0.7)	–	–
HPV56	–	–	–	–	2	(0.5)	2	(0.5)	–	–
HPV58	–	–	–	–	8	(1.8)	8	(1.8)	–	–
HPV59	–	–	–	–	1	(0.2)	2	(0.5)	–	–
HPV67	–	–	–	–	1	(0.2)	1	(0.2)	–	–
HPV68	–	–	–	–	1	(0.2)	1	(0.3)	–	–
HPV97	–	–	–	–	1	(0.2)	1	(0.2)		
HPV undetermined	–	–	–	–	2	(0.5)	2	(0.5)	–	–
Multiple infections	9	(22.0)	–	–	32	(7.3)	–	–	**0.33**	**(0.17–0.65)**

[1]Three multiple infections were not counted in the proportional attribution estimation as the HPV types were not found as single infections: HPV18 and 58, HPV6, 44, and 74 and HPV58 and 68/73.
[2]Considering single + multiple columns estimation.
Abbreviations: AIN 2/3: Anal Intraepithelial Neoplasia 2/3; HPV +: HPV DNA positive; Single: single and multiple infections counted separately; Single + multiple: multiple infections were added to single types under a weighting attribution proportional to the detection found in cases with single types as described in the methodology; 95% CI: 95% Confidence Interval.

increasing age at diagnosis (p-trend test = 0.016). HPV prevalence varied according to the histological diagnosis (Table 2). Warty–basaloid SCC cases showed the highest HPV prevalence (95.9%; 95% CI: 92.9–97.8%) with no variation within the different histological subtypes included in this category, whereas the "other" histology category showed the lowest prevalence (27.3%; 95% CI: 6.0–61.0%). The three HPV-positive cases among the "other" category were one undifferentiated carcinoma, one neuroendocrine and one adenosquamous cell carcinoma.

Among HPV DNA-positive samples (Table 3), the percentage of multiple infections was higher for AIN 2/3 (22.0%) than for invasive anal cancers (7.3%) ($p = 0.005$). The most frequent HPV type was HPV16 for both AIN 2/3 (75.4% including multiple infections) and invasive anal cancer (80.7%). Among cancers, the second most common type was HPV18 (3.6%), accounting together with HPV16 for 84.3% of HPV DNA-positive cases. Other HPV types detected were HPV33 (2.7%), HPV31 (1.9%), HPV6 and HPV58 (both 1.8%), HPV35 (1.6%), and other types were identified in <1.5% of the specimens. Figure 2 shows the relative contribution of HPV16, HPV18 and other types, displayed by region, time at and age of diagnosis, gender and histology (Supporting Information Tables 1–5; there is the complete type distribution by the available information). We observed a higher proportion of types other than HPV16/HPV18 in Africa, and in males; but none of these comparisons were statistically significant.

Concordance between the expression of $p16^{INK4a}$ and the presence of HPV DNA was observed in 87.1% of anal cancer cases analyzed (95% CI: 79.6–92.6%); with a Kappa score of 0.741 (95% CI: 0.620–0.862, $p < 0.001$), indicating substantial agreement. The McNemar test indicated that the discordant results were not equally distributed ($p = 0.039$). The discordant pairs of HPV DNA-negative and $p16^{INK4a}$-positive cases were more frequent than the HPV DNA-positive cases with a $p16^{INK4a}$-negative result

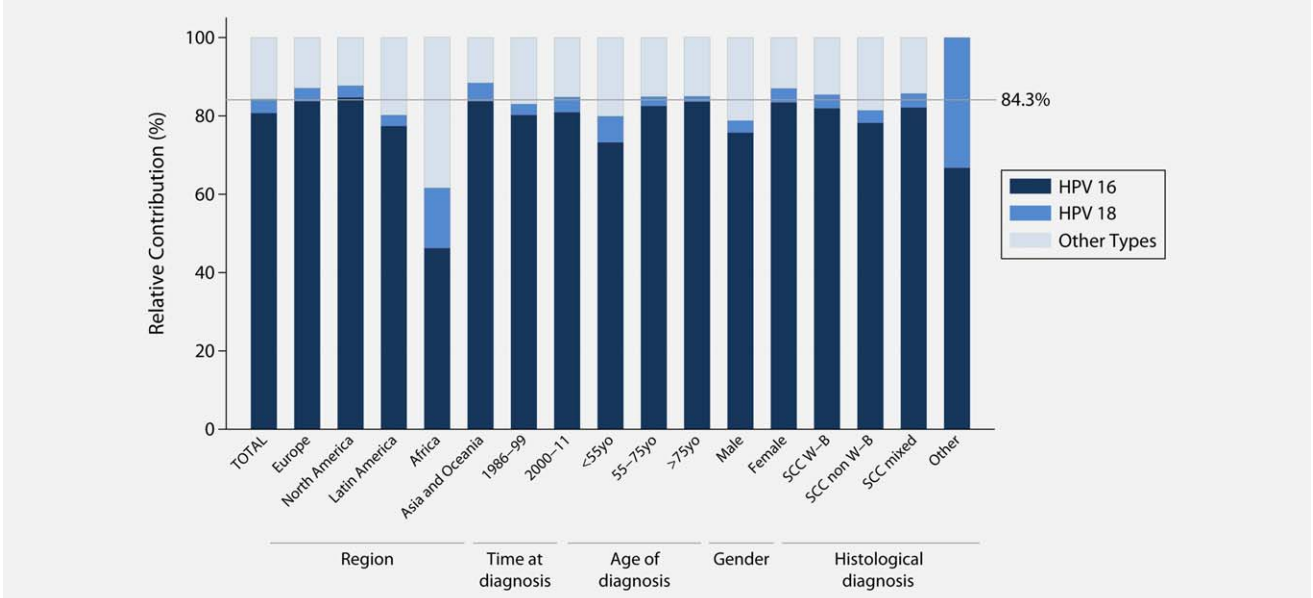

Figure 2. HPV16, HPV18 and other HPV types relative contribution among HPV DNA-positive invasive anal cancers, by case characteristics. SCC: Squamous Cell Carcinoma; W–B: Warty–Basaloid; yo: years old. None of the comparisons, HPV16/18 versus other HPV types by the different variables, were statistically significant.

(Table 4). Almost all HPV DNA-positive cases showed a $p16^{INK4a}$ overexpression pattern (56/59 = 94.9%), and only three of the HPV-positive cases were $p16^{INK4a}$ negative (HPVs identified in these cases: 11, 16 and 18). Among HPV DNA-negative cases 12/57 (21.1%) showed $p16^{INK4a}$ overexpression.

Discussion

In our study, we have analyzed the presence of HPV DNA in more than 500 precancerous and cancerous anal lesions collected across the five continents. A very high prevalence of HPV DNA was observed in both precancerous anal lesions and in invasive anal cancer at a similar magnitude to that observed in a meta-analysis on HPV detection in cervical cancer[22] and in our own previously published series on cervical cancer lesions.[14] In addition, $p16^{INK4a}$, a cellular surrogate marker for HPV-associated transformation, was overexpressed in almost all the HPV DNA-positive cancer cases evaluated (95%), confirming the etiological involvement of the detected HPVs in the carcinogenic process. This $p16^{INK4a}$ overexpression figure resembles also that found in our own series on cervical cancer lesions (98%).[23] The most important individual contributor was HPV16 in both types of lesions.

Overall, the HPV DNA positivity in AIN 2/3 cases was 95.3%, which is similar to that reported in a previous meta-analysis from de Vuyst *et al.*,[12] (93.9%). In previous studies, HIV-positive individuals showed higher HPV DNA detection and different type distributions, with more multiple infections and lower proportion of HPV16, than HIV negative.[12] Unfortunately, the small sample size for the precancerous lesions in our study (43 cases) precluded any further investigation using the available information and information on HIV status was not available in the analyzed series.

Table 4. Concordance of HPV DNA and $p16^{INK4a}$ results in invasive anal cancer cases

	$p16^{INK4a}$		
HPV DNA	Negative	Positive	Total
Negative	45 (78.9%)	12 (21.1%)	57[1]
Positive	3[2] (5.1%)	56 (94.9%)	59
Total	48	68	116

[1]In only one HPV DNA-negative anal cancer case from the 58, the p16 was not performed owing to lack of material.
[2]These cases had a single HPV infection: HPV11, HPV16 or HPV18; %: Row % (p16 results among each of the HPV results category).

Regarding invasive anal cancer, HPV DNA prevalence was 88.3% which was in accordance with a recent report on 146 anal cancers from USA (91.1%)[24]; but higher than results from the meta-analysis from de Vuyst *et al.* (84.3%) and lower than the results obtained in large studies such as EDiTH V study ($n = 366$, France) in which 97% of cases were HPV DNA positive.[12,25] Our higher viral DNA detection rate compared to the meta-analysis could be attributed to the variability in material and methods in the included reports. Some studies in the systematic review used type-specific assays targeting a smaller set of HPVs than broad-spectrum SPF-10 PCR, which could explain the lower detection rate. In addition, we performed a systematic pathological review of all cases and excluded potential HPV-unrelated diagnoses, such as the adenocarcinomas to avoid potential misclassification with rectal adenocarcinomas. Our lower

detection compared to the EDiTH V study could be explained by our assessment of DNA quality targeting through PCR a small portion of the human tubulin gene, of precisely the same size as the HPV DNA target of the SPF-10 (65 bp). In contrast, in the EDiTH V study, a larger amplicon was used as a reference gene to assess the quality of DNA (HLA-DPB1, 270 bp).[25] The use of longer amplicons increases the probability of labeling a FFPE sample as not suitable for DNA assays, and thus excluding it from the denominator in rate estimations.

As observed in other HPV-related cancers, we found a higher prevalence of viral DNA in warty–basaloid anal cancers.[12] Significant differences were also found between SCC and "other diagnosis" for which the HPV prevalence was lower. However, the low number of samples labeled as "other diagnosis" precluded establishing any conclusion. The prevalence of HPV was higher among the patients diagnosed with anal cancer at a younger age, which has also been observed in other HPV-related cancers.[14,26] Some geographical differences were observed, with a noteworthy lower HPV prevalence in anal cancer specimens from Africa. There is not a clear explanation for this finding and technical reasons cannot be discarded. A report on HPV detection in anal cancers from six countries using HPV16 DNA hybridization also found a lower viral presence in specimens from South Africa and India than from Swiss, Polish or Brazilian cases.[27] Finally, although a higher HPV detection in women than in men was observed, the difference was not statistically significant. Such lack of difference on the prevalence of HPV in anal cancer by gender has also been observed in another recent report[24]; however, other previous reports have observed a statistically significant higher HPV prevalence in women than men.[12]

Regarding samples positive for HPV DNA, the most frequent type in AIN 2/3 was HPV16 with a prevalence of 75.4%. In invasive anal cancer, frequently found types were in decreasing order: HPV16 (80.7%), HPV18 (3.6%), HPV33 (2.7%), HPV31 (1.9%), HPV6 and HPV58 (both 1.8%) and HPV35 (1.6%). Our findings are in agreement with other reports in which HPV16 has been identified as the predominant HPV type in anal cancer, with a relative contribution among HPV DNA-positive cases that is much higher than in other anogenital HPV-related sites.[12,14,24–26] This increased contribution of HPV16 in anal cancer compared to other anatomic sites may have a biological basis, reflecting a differential tropism of HPV16 toward anal mucosa, a differential mucosal immune response or a differential increased probability of that type to lead to malignant transformation in the anal mucosa. HPV18 and HPV45, which are commonly overrepresented in glandular lesions, showed lower relative contributions than observed in cervical cancer tissue.[14,22] This observation in anal compared to cervical lesions might be explained by the exclusion of glandular lesions from the study to avoid misclassification with rectal adenocarcinomas. In the stratified analysis by geographical region, we observed a higher proportion of types other than HPV16/HPV18 in Africa, but these observed differences were based on small number of cases (13 HPV DNA-positive cases in Africa) and were not statistically significant.

It is important to highlight that the detection rates of low-risk HPV types (*e.g.*, HPV6 and HPV11) were higher in anal cancer (1.8 and 1.1%, respectively) compared to those observed in cervical cancers (below 1% for both types). The increased prevalence of these types in the anal region is consistent with the high prevalence of warts at this anatomic site. From our sample series, a subset of four anal cancer cases harboring single low-risk infections (three HPV6 and one HPV42) was further analyzed by laser capture microdissection. In all four cases, DNA for these specific HPVs was found in tumor cells, and thus confirming the initial detection in the whole tissue section and excluding potential contamination from adjacent tissue.[28] Other investigators have also identified by laser capture microdissection HPV6 specifically in anal tumor cells.[29] Thus, our results confirm that low-risk types can be occasionally associated with invasive anal neoplastic lesions.

Twenty-two percent of AIN 2/3 cases harbored HPV DNA from multiple HPV types compared to 7.3% in anal cancers. This decrease in multiplicity of infection with neoplastic disease progression has been also described for CIN 2/3 (cervical intraepithelial neoplasia) and cervical cancer,[30] and may reflect the concept of clonal development of invasive neoplasia resulting from persistent infection with a single HPV type.[31,32]

The accumulated knowledge on the natural history of anal cancer closely resembles that of cervical cancer. Both types of tumors share similar transmission mechanisms for HPVs as etiological agents, are modulated by similar risk factors and seem to arise from the same type of cells occurring at a transition zone between columnar–glandular and stratified epithelia. This squamous–columnar junction may thus represent a susceptible spot in which the HPVs target cells that could be more prone to be transformed upon infection, leading to malignant transformation. Recently, a report identified that carcinogenic HPV-related cervical intraepithelial lesions and cervical cancers are linked to a small cell population localized at the squamous–columnar junction of the cervix that expresses a unique gene expression profile, and that is not regenerated after excision.[33] The determination of whether the anal cancers arise from a similar kind of cell population remains unknown and deserves further investigation. In addition, in the anal canal there could be an increased access of the virus to the basal cells linked to microlesions resulting from receptive intercourses. Our finding that $p16^{INK4a}$ was overexpressed in almost all the HPV DNA-positive anal cancer cases analyzed (95%), resembles the figure found in our own cervical cancer series (98%)[23] and strongly points towards an etiological implication of the virus in the oncogenic process as it has been stated by others in the previous reports.[34,35] Overexpression of $p16^{INK4a}$ is detected in HPV-associated tumors but nearly absent in HPV-unrelated carcinomas. The biological link between HPV and $p16^{INK4a}$ overexpression originates through the interaction of the viral oncoprotein E7 with the pRb protein, and is therefore a

cellular surrogate marker of the causal link between viral infection and cancer.[36]

The added value of our study compared to the previous reports is the use of highly sensitive HPV DNA detection and genotyping system (SPF-10/DEIA/LiPA$_{25}$) under a thorough contamination control process; the processing and testing for HPV DNA in a single central laboratory with long standing experience with archival clinical specimens; the use of a DNA quality control PCR that generates an amplicon with the same length as the one used for viral DNA; the addition of a surrogate marker of HPV-associated cellular transformation (p16^{INK4a}), suggesting the etiological involvement of the detected HPVs; and the detailed pathological evaluation performed to exclude potential HPV-unrelated misclassified diagnosis (like rectal adenocarcinomas). In addition, despite the under-representation of certain geographical regions, our data represent, to our knowledge, the largest international effort evaluating the involvement of HPVs in anal cancer under a common protocol.

Conclusions

In our study, we observed a major contribution of HPV to anal precancerous and cancerous lesions strongly suggesting the necessary role of HPV in the etiology of anal cancers as it has been reported previously.[34,35] The most common type in AIN 2/3 and in invasive anal cancers was HPV16, followed to a lesser extent by HPV18, which altogether accounted for 84.3% of HPV DNA-positive anal cancer cases. Our results suggest that both women and men would benefit from HPV vaccination and anal HPV screening for high risk groups to prevent anal cancerous lesions. HPV vaccination with currently licensed HPV prophylactic vaccines could potentially prevent 84.3% of the anal cancers and 75.4% of AIN 2/3 lesions.

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