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Variants of Squamous Cell Carcinoma of the Anal Canal and Perianal Skin and Their Relation to Human Papillomaviruses[1]

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ABSTRACT

High-risk types of human papillomaviruses (hrHPVs) may be a necessary cause in cervical cancer and in some subtype of anal, vulvar, and penile cancers. Large studies aimed at characterizing hrHPV-associated and non-hrHPV-associated subtypes of anal carcinomas are, however, lacking. We searched for human papillomavirus type 16 and 13 other hrHPVs in tumor tissue by PCR and performed a systematic histological evaluation of specimens from 386 patients with anal cancer (86% invasive; 302 women and 84 men). Cancers in women and homosexual men were more often hrHPV positive ($P < 0.01$) and located in the anal canal ($P \leq 0.01$) than were cancers in heterosexual men. In both women and men, anal canal cancers contained hrHPV clearly more often than did perianal skin cancers, and increasing hrHPV positivity was seen with higher localization in the anal canal. Indeed, 95 and 83% of cancers involving the anal canal in women and men, respectively, were hrHPV positive *versus* 80 and 28% of perianal skin cancers (P-trend < 0.001). Basaloid feature, adjacent anal intraepithelial neoplasia, poor or absent keratinization, and a predominance of small or medium neoplastic cells were all strongly positively associated with hrHPV status. Like cancer of the uterine cervix, the development of cancer of the anal canal may require infection with hrHPV, whereas a dual etiology of perianal skin cancers bears parallels to vulvar and penile cancers.

INTRODUCTION

Certain types of HPVs,[3] referred to as hrHPVs, are centrally involved in the etiology of anogenital squamous cell carcinomas (1). For cervical carcinoma, hrHPV may, indeed, be a necessary cause (2). A large proportion of vulvar and penile cancers categorized as basaloid or warty carcinoma also harbor HPV, whereas keratinizing squamous cell carcinoma, the other major histological subtype of cancer at these sites, is not linked to HPV to any similar extent (3–5). Recently, we showed that hrHPVs are involved in the etiology of most but not all anal squamous cell carcinomas. Indeed, hrHPV, particularly HPV-16, was present in the majority of anal cancers, but a higher proportion of male than female patients had tumors in which hrHPV was not detected with the PCR technique (6).

Previous investigations have used molecular biological techniques to detect HPV in anal carcinomas, but only few have examined more than 25 patients by PCR (7–11). To characterize HPV-associated and non-HPV-associated variants of anal squamous cell carcinoma, we combined the results of our PCR-based examination for HPV with a detailed histological scrutiny of neighbor sections from the same anal cancer specimens.

MATERIALS AND METHODS

On the basis of routine pathology reports, we included a total of 417 patients with histologically verified invasive or *in situ* anal cancer as cases in a population-based case-control study in Denmark and Sweden. Telephone interviews were conducted with focus on sexual behavior, smoking habits, and other potential factors influencing the risk (6). Through searches in computerized pathology registers, we identified and collected formalin-fixed, paraffin-embedded tumor material from 394 patients (94%) in over 60 different pathology departments in the two countries. The study was approved by the scientific ethical committees in both participating countries.

HPV Detection in Tumor Tissue. The tumor material was analyzed for HPV by PCR using a sandwich method. In this approach, the outer sections in a series of consecutive 4-μm sections were stained with H&E. The area of tissue examined and the percentage of neoplastic cells were estimated to qualify HPV status assessment in cases with questionable or negative HPV results. For analytical purposes, we used these estimates to calculate an estimated neoplastic tissue volume [thickness of section (4 μm) $\times$ estimated area $\times$ estimated percentage neoplastic cells].

We used tissue sections between the two outer H&E-stained sections for PCR analyses. Samples of liver tissue (negative controls) were cut before each tumor specimen and subjected to the same PCR procedures as the anal cancer specimens. Each 4-μm section was digested in 250 μl of proteinase K mix consisting of 10 mM Tris-HCl (pH 7.4), 0.45% Tween 20, and 100 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at 37°C for 20–24 h (12). Samples were then treated at 100°C for 10 min and centrifuged. Ten μl of the supernatant were used for subsequent PCR. After a human β-globin-specific PCR (to exclude tumors without analyzable target DNA), we did GP5+/6+ general primer-mediated PCR, known to detect most mucosotropic HPV genotypes (13). In an enzyme immunoassay analysis, the PCR products were captured on streptavidin-coated microwells, denatured by alkaline treatment, and hybridized to two probe cocktails of digoxigenin-labeled specific oligonucleotides, one comprising 14 hrHPVs (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, -66, and -68) and another comprising six low-risk HPVs (HPV-6, -11, -40, -42, -43, and -44). Positive samples were subjected to individual typing for the hrHPVs HPV-16, -18,- 31, and -33 (14).

Histological Evaluation of Tumor Specimens. One of us (C. F.) examined systematically the H&E-stained sections from tumors successfully analyzed for HPV. Blinded to questionnaire data and HPV status, the histological category of the tumor was determined as: (*a*) invasive squamous cell carcinoma; (*b*) AIN (any grade), including squamous cell carcinoma *in situ*; or (*c*) tissue that could not be classified as either invasive squamous cell carcinoma or AIN. Whenever possible, the *anatomical localization* in the anal canal (colorectal zone, anal transitional zone, or squamous zone) and/or in the perianal skin was assessed (15).

Specimens categorized as invasive squamous cell carcinoma were examined for the presence of: (*a*) AIN in the adjacent epithelium (hereafter referred to as associated AIN); (*b*) basaloid features (peripheral palisading and/or eosinophilic necrosis); (*c*) keratinization with a semiquantitative assessment in poorly keratinized (+), moderately keratinized (++), or well keratinized (+++); and (*d*) the size of the predominant neoplastic cell type (small, medium, and large).

Specimens from five patients contained no tumor cells, and another was

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[3] The abbreviations used are: HPV, human papillomavirus; hrHPV, high-risk type of HPV; AIN, anal intraepithelial neoplasia; OR, odds ratio.

negative on β-globin PCR. On histological examination, one tumor was considered a basal cell carcinoma, and another was considered a malignant melanoma. These tumors were excluded, thus leaving 386 tumors in the study. In most cases, histological examination confirmed the invasive nature of lesions initially included as invasive anal cancers. Likewise, most patients included on the basis of routine pathology reports of *in situ* anal cancer exhibited varying grades of AIN. Ten tumors initially included as *in situ* anal cancers showed evidence of at least minimal invasion, and eight originally included as invasive cancers exhibited only AIN. All 18 lesions were considered invasive anal cancers in subsequent analyses. Seven tumors retained the initial assessment as either invasive or *in situ* anal cancer, based on routine pathology reports, because tumor material was too sparse for proper evaluation. Consequently, the material consisted of tumors from 331 patients (253 women and 78 men) with tumors categorized as invasive squamous cell carcinoma and 55 patients (49 women and 6 men) with tumors categorized as *in situ* squamous cell carcinoma.

Statistical Methods. Invasive and *in situ* lesions were analyzed separately. We compared hrHPV-positive and hrHPV-negative cancers with respect to selected demographic and behavior characteristics by means of χ^2 test, Fisher's exact two-tailed test (when a cell contained less than five subjects), and Wilcoxon's two-sample test. Associations of hrHPV status with histological characteristics and anatomical localization were evaluated by χ^2 test, Fisher's exact two-tailed test, Mantel-Haenszel χ^2 test for trend, logistic regression, and generalized estimating equations in a logistic regression with unstructured correlation (16). Correlations between anatomical and histopathological characteristics were evaluated by Spearman's rank correlation test.

RESULTS

Invasive Anal Cancers

Anal carcinomas in women contained hrHPV much more often (90%) than in men (63%; $P < 0.001$). All men reporting homosexual experience had hrHPV-positive anal cancer ($n = 11$) *versus* 58% of 66 men without such experience ($P = 0.006$; Table 1). The predominant hrHPV was HPV-16, detected in 87% of all hrHPV-positive invasive cancers, followed by HPV-18 (7%), HPV-33 (6%), HPV-31 (1%), and untyped hrHPV (2%). Seven patients had double hrHPV infection: three had HPV-16 and HPV-18 and four had HPV-18 and HPV-33.

Anal Localization. Among assessable women, 166 of 222 (75%) had cancers involving the anal canal while 56 (25%) were restricted to the perianal skin (Table 2). All men reporting homosexual experience who had tissue examined histologically ($n = 10$) had cancers involving the anal canal; among men without homosexual experience, only 56% ($n = 30$) had cancers involving the anal canal, whereas 44% ($n = 24$) had perianal skin cancers. These proportions of anal canal cancers were significantly larger in women ($P = 0.005$) and homosexual men ($P = 0.01$) than in men without homosexual experience.

hrHPV and Age. Among women, hrHPV-status did not vary significantly according to age (median = 63 years in hrHPV-positive and 59 years in hrHPV-negative women), sexual behavior, or smoker status, whereas in men, the age distribution was considerably younger among hrHPV-positive (median = 58 years) than hrHPV-negative patients (median = 68 years, $P = 0.03$; Table 1). This age pattern was due partly to a younger age distribution among men with homosexual experience (median = 56 years). After restriction of the analysis to men without reported homosexual experience, hrHPV-positive men remained younger (median = 59 years) than hrHPV-negative patients ($P = 0.04$).

hrHPV and Anal Localization. Considerably more cancers involving the anal canal were hrHPV positive (95% among women and 83% among men) compared with cancers confined to the perianal skin (80 and 28%, respectively). Indeed, the higher the examined tissue originated in the anal canal, the larger the proportion hrHPV-positive cancers (*P*-trend < 0.001 in either sex; Table 2). In an additional analysis, we took into account the fact that 18% of assessable lesions extended over two or more zones in the anal region. This analysis revealed similar statistical trends (Table 2). The lower hrHPV positivity in perianal skin cancers was not due to insufficient amounts of tissue. The estimated neoplastic tissue volumes were actually larger for perianal skin cancers (median = 147×10^{-3} mm^3) than for anal canal cancers (median = 94×10^{-3} mm^3; $P = 0.04$).

Table 1 *Selected characteristics for 331 patients with invasive anal cancer examined for HPV in Denmark and Sweden, 1991–1994*

	No. of patients (% hrHPV-positive patients)	
	Women	Men
No. of patients[a]	253 (90)	78 (63)
Nationality		
Danish	108 (90)	44 (61)
Swedish	145 (90)	34 (65)
	$P = 0.89$	$P = 0.76$
Age at diagnosis		
<50	50 (94)	15 (80)
50–59	57 (82)	20 (70)
60–69	62 (94)	18 (61)
≥70	84 (90)	25 (48)
	$P = 0.88$	$P = 0.03$
Lifetime no. of partners[b]		
0 or 1	52 (87)	13 (62)
2 or 3	77 (91)	7 (71)
4 to 9	69 (91)	17 (65)
≥10	40 (88)	32 (63)
	$P = 0.95$	$P = 0.96$
Heterosexual anal intercourse		
Yes	34 (91)	NA[c]
No	216 (90)	
	$P = 1.00$	
Visit to a prostitute		
Yes	NA	17 (76)
No		58 (60)
		$P = 0.26$
Homosexual experience		
Yes	NA	11 (100)
No		66 (58)
		$P = 0.006$
Current smoker		
Yes	91 (87)	40 (58)
No	162 (92)	38 (68)
	$P = 0.18$	$P = 0.32$

[a] Numbers do not add up to 253 for women and 78 for men for all variables because of missing values.
[b] Partners refer to partners of the opposite sex.
[c] NA, not applicable.

hrHPV and Histopathological Characteristics. Associations between hrHPV-status and specific histopathological characteristics were similar in men and women (Table 2). Presence of basaloid features, lack of keratinization, associated AIN, and predominantly small or medium neoplastic cells were all positively associated with hrHPV status. To examine whether hrHPV-status and histopathological characteristics could be misclassified due to insufficient amounts of tumor tissue, we excluded those small sections with an estimated neoplastic tissue volume of less than 10×10^{-3} mm^3. This restricted analysis in 231 women and 74 men (92% of the patients) revealed unchanged overall hrHPV positivity (90% in women and 61% in men) and virtually identical associations with all characteristics presented in Tables 1 and 2 (data not shown). However, among women, associations with basaloid features ($P = 0.04$) and keratinization (*P*-trend = 0.05) achieved formal statistical significance in this restricted analysis, reflecting that detection of these characteristics, unlike detection of hrHPV, depended significantly on the size of the tumor specimen examined (Table 3).

Anal Localization and Age. Patients with anal canal cancers were older (median = 64 years for women and 62 years for men) than those with perianal skin cancers (median = 56 and 58 years, respectively), a difference that was statistically significant for women ($P = 0.002$).

Table 2 *Anatomic localization, histological characteristics, and predominant neoplastic cell size in 331 invasive anal squamous cell carcinomas*

	No. of cancers (% hrHPV-positive)	
	Women (n = 253)	Men (n = 78)
Anatomic localization[a]		
Most proximal lesion extent (87%)		
Anal canal, colorectal zone	96 (99)	21 (90)
Anal canal, anal transitional zone	15 (80)	2 (100)
Anal canal, squamous zone	55 (91)	17 (71)
Perianal skin	56 (80)	25 (28)
	P-trend[b] < 0.001	P-trend[b] < 0.001
Anal epithelial zones involved[c] (87%)		
Anal canal, colorectal zone	96 (99)	21 (90)
Anal canal, anal transitional zone	36 (92)	5 (100)
Anal canal, squamous zone	77 (92)	20 (70)
Perianal skin	64 (81)	30 (33)
	P-trend[d] < 0.001	P-trend[d] = 0.01
Histological characteristics[a]		
Basaloid features[e] (95%)		
No	117 (87)	51 (49)
Yes	122 (94)	23 (87)
	P = 0.06	P = 0.002
Keratinization (95%)		
No	127 (92)	31 (84)
Yes, + (poorly keratinized)	54 (94)	13 (69)
Yes, ++ (moderately keratinized)	30 (87)	12 (42)
Yes, +++ (well keratinized)	28 (82)	18 (28)
	P-trend[b] = 0.10	P-trend[b] < 0.001
Associated AIN (95%)		
No	155 (88)	54 (50)
Yes	84 (96)	20 (90)
	P = 0.03	P = 0.001
Neoplastic cell type[a]		
Predominant cell-size in lesion (98%)		
Large	48 (81)	32 (25)
Medium	97 (93)	24 (88)
Small	105 (93)	20 (90)
	P = 0.04	P < 0.001

[a] Numbers do not add up to 253 for women and 78 for men because not all characteristics could be assessed for all specimens. Percentages indicate the proportion of cancer specimens assessable for the individual characteristic.
[b] Mantel-Haenszel χ^2 test for trend.
[c] Each cancer specimen examined may extend over more than one of the four epithelial zones.
[d] Wald test for trend based on generalized estimating equations method for correlated data.
[e] Basaloid features comprise eosinophilic necrosis and/or peripheral palisading.

Women and men with hrHPV-positive perianal skin cancers were considerably younger (median = 53 and 47 years, respectively) than women (median = 73 years; P = 0.01) and men (median = 66 years; P = 0.002) with hrHPV-negative perianal skin cancers.

Correlation between Anatomical and Histopathological Characteristics. We evaluated correlations between anatomical and histopathological characteristics for invasive anal cancers regardless of HPV status. Anatomical localization was dichotomized (anal canal *versus* perianal skin), as was keratinization (any *versus* none) and predominant neoplastic cell type (small or medium *versus* large). Associated AIN was not correlated in either sex with any of the other histopathological characteristics or with anatomical localization ($P > 0.05$). In both men and women, however, statistically significant correlations ($P \leq 0.01$) existed between each pair of all other variables, thus strongly linking involvement of the anal canal with small or medium neoplastic cells, presence of basaloid features, and lack of keratinization (data not shown).

hrHPV and Histopathological Characteristics by Anal Localization. Because correlations between anatomical and histopathological characteristics were similar in the two sexes, we combined men and women in a logistic regression analysis to evaluate associations between these characteristics and hrHPV status in intra-anal and perianal cancers (Table 4). Anal canal cancers were significantly more likely (OR = 7.5; 95% confidence interval, 3.5–16.0) than perianal skin cancers to contain hrHPV. Stratified analyses showed that, in both anatomical localizations, the odds of hrHPV positivity were 6–7 times higher for cancers with predominantly small or medium neoplastic cells compared with cancers dominated by large neoplastic cells. Well-keratinized cancers were much less likely to be hrHPV positive than were cancers without keratinization, and associated AIN (OR = 9.3) was strongly positively associated with hrHPV status in anal canal cancers, as was the presence of basaloid features (OR = 6.8) in perianal skin cancers (Table 4).

Histopathological Prediction of hrHPV. Each characteristic examined was a strong predictor of the cancer being hrHPV positive, particularly for cancers involving the anal canal. Positive predictive values for basaloid features, poor or absent keratinization, associated AIN, and small/medium neoplastic cells were each 95% or more for cancers in this localization. On the other hand, no characteristic had negative predictive value above 28% in anal canal cancers or 50% in perianal skin cancers (Table 4).

In Situ Anal Cancers

All six *in situ* anal cancers in men were hrHPV positive. The anatomical localization was not evaluable in one patient; the remaining five were restricted to the perianal skin.

Women (n = 49) with *in situ* anal cancer were significantly younger than women with invasive anal cancer (median = 48 years *versus* 63 years, P < 0.001). Tumor specimens from 44 patients (90%) were hrHPV positive. Anatomical localization was inestimable in four lesions. All 11 lesions involving the anal canal were hrHPV positive, as were 30 (88%) of the 34 lesions confined to the perianal skin.

Low-Risk HPV in Invasive and *in Situ* Anal Cancers

Tumors in only 10 women (4%), 1 *in situ* and 9 invasive tumors, contained low-risk HPV types. One low-risk HPV-positive invasive cancer was also hrHPV positive. The nine women with low-risk HPV-positive invasive anal cancer had a higher lifetime number of

Table 3 *PCR-determined hrHPV positivity and histopathological characteristics in 331 invasive anal squamous cell carcinomas according to estimated neoplastic tissue volume*

	No. of cancers (% hrHPV positive)		No. (%) of cancers (women and men) with			
	Women	Men	Basaloid features	++/+++ keratinization	Associated AIN	Small or medium neoplastic cells
Estimated neoplastic tissue volume ($\times 10^{-3}$ mm^3)						
<10	22 (86)	4 (100)	6 (23)	2 (8)	6 (23)	20 (77)
10–24	18 (94)	7 (71)	8 (32)	8 (32)	7 (28)	19 (76)
25–49	39 (87)	13 (69)	18 (35)	12 (23)	16 (31)	41 (79)
50–99	46 (93)	13 (54)	28 (47)	14 (24)	19 (32)	47 (80)
100–249	72 (89)	18 (56)	45 (50)	24 (27)	32 (36)	62 (69)
≥250	56 (91)	23 (61)	40 (51)	28 (35)	24 (30)	57 (72)
P-trend[a]	0.81	0.54	0.02	0.02	0.82	0.33

[a] Mantel-Haenszel χ^2-test for trend.

Table 4 *Odds ratios and predictive values of histological characteristics and predominant neoplastic cell size in the identification of hrHPV-positive invasive cancers of the anal canal and perianal skin*

Characteristic	Anal canal cancers				Perianal skin cancers			
	No. of hrHPV-positive cancers	No. of hrHPV-negative cancers	OR[a] (95% CI)	Positive *vs.* negative predictive value of characteristic	No. of hrHPV-positive cancers	No. of hrHPV-negative cancers	OR[a] (95% CI)	Positive *vs.* negative predictive value of characteristic
Basaloid features								
No	81	11	1 (reference)		30	25	1 (reference)	
Yes	104	5	2.6 (0.9–7.9)	95 *vs.* 12%	18	3	6.8 (1.2–38.8)	86 *vs.* 45%
Keratinization[b]								
Absent	113	8	1 (reference)	] 95 *vs.* 23%	14	4	1 (reference)	] 68 *vs.* 40%
+ (poorly keratinized)	45	0			7	6	0.5 (0.1–3.6)	
++ (moderately keratinized)	15	3	0.3 (0.1–1.4)		13	7	0.4 (0.1–2.3)	
+++ (well keratinized)	12	5	0.2 (0.1–0.9)		14	11	0.2 (0.03–0.95)	
Associated AIN								
No	115	15	1 (reference)		29	24	1 (reference)	
Yes	70	1	9.3 (1.2–73.4)	99 *vs.* 12%	19	4	2.8 (0.7–12.1)	83 *vs.* 45%
Size of predominant neoplastic cell type								
Large	21	8	1 (reference)		22	22	1 (reference)	
Small or medium	169	8	6.8 (2.3–20.6)	95 *vs.* 28%	30	7	6.6 (1.6–27.0)	81 *vs.* 50%

[a] Odds ratios are adjusted for sex and age (<60 years *vs.* ≥60 years); CI, confidence interval.
[b] Positive and negative predictive values for keratinization are based on a dichotomization in unkeratinized or poorly keratinized (+) *vs.* moderately keratinized (++) or well-keratinized (+++).

male partners (median = 6) than other women (median = 3; $P = 0.02$), and they were more often current smokers (78 *versus* 34%; $P = 0.01$). Histopathological characteristics and anatomical localization did not differ significantly according to low-risk HPV status, except basaloid features that were present in only one (13%) low-risk HPV-positive cancer *versus* 52% of other invasive cancers ($P = 0.03$). Five anal tumors in men (6%), all invasive, were low-risk HPV positive. Of these, two were also hrHPV positive.

DISCUSSION

We studied 386 anal cancers to examine the association between HPV status and histopathological characteristics in detail. Overall, we detected hrHPV in 90% of invasive anal cancers in women and 63% of those in men. Using *in situ* hybridization and HPV-16-specific PCR in 99 patients, Holm *et al.* (9) found HPV in similar proportions of anal canal cancers in women (89%) and men (56%). Another study of 93 women and 36 men with *in situ* or invasive anal cancer showed 70% of the tumors in women and 67% of those in men to be PCR positive to one or more of HPV-6, -11, -16, and -18, but a distinction between anal canal cancers and perianal skin cancers was not presented (8).

Whether cancers involving the anal canal contain HPV more often than perianal skin cancers has been the subject of only few investigations. One study reported more HPV-16 in cancers originating above than below the dentate line (17, 18), and another small study detected HPV-16 by DNA *in situ* hybridization in 81% of anal canal cancers *versus* 25% of anal margin cancers (19). This study documents a clear association between the localization of the cancer in the anal region and the presence of hrHPV. Indeed, 95% of female cancers and 83% of male cancers involving the anal canal contained hrHPV. Perianal skin cancers were hrHPV positive to a lesser extent, suggesting that some perianal skin cancers, particularly in elderly patients, may have a causal pathway in which HPV is not involved.

Using RNA *in situ* hybridization, Higgins *et al.* (20) found 73% of 41 anal cancers to be HPV positive. Like the men in our study, HPV-negative patients were older than HPV-positive patients, but the exact cancer localization in the anal region was not presented (20). Two other studies suggested an older age distribution among patients with HPV-negative anal cancer. In a combined analysis of 37 men and 30 women, Scholefield *et al.* (18) found HPV-negative patients to be older than HPV-positive patients, and although Heino *et al.* (21) detected HPV by DNA *in situ* hybridization in only 35% of anal cancers from 46 men and women, they reported HPV-negative patients to be, on average, 11 years older than HPV-positive patients (21).

Previous studies of HPV in subtypes of anal cancer have been limited by low statistical power. Also, considerable differences in sensitivity of the HPV tests used have produced inconsistent results, and the plethora of terms describing the anatomy of the anal region and the histological patterns in variants of anal carcinomas have contributed to the current lack of clarity (15). Moreover, male:female ratios and proportions of patients with perianal skin cancers and of men with homosexual experience have varied considerably (6, 9, 22), and these variations may have each contributed to the variation in reported HPV positivity.

Only one previous study showed significantly more hrHPV positivity in anal cancers with basaloid features (19), but other studies are consistent with this association (11, 18, 20, 21, 23, 24). However, unlike any previous study, we found that the major discriminator between hrHPV-positive and hrHPV-negative anal cancer is the anatomical localization. Anal canal cancers were much more likely to harbor hrHPV than perianal skin cancers whether specific histological characteristics were present or not. Perianal skin cancers with basaloid features were clearly more likely to be hrHPV-positive than those without. Some of these lesions could represent advanced anal canal cancers with extension to the perianal skin for which the exact site of origin could not be assessed in the sections examined.

Keratinizing squamous cell carcinomas of the vulva and penis have looser links to HPV than basaloid or warty carcinomas (3–5). According to this study, a parallel may be drawn to perianal skin cancers, among which we found almost 60% to be moderately or well keratinized. We observed a clear inverse association between the degree of keratinization and hrHPV positivity, thus lending statistical support to previous observations (9, 10, 17, 18, 20). Anal cancers with associated AIN were more often hrHPV positive than those without. This association, supported by one previous study (20), represents another parallel to vulvar cancer. Vulvar cancers with intraepithelial neoplasia in the adjacent epithelium have close etiological links to HPV (3, 4).

Our finding of a strong association between hrHPV-status and the size of the predominant neoplastic cell type has not been reported before. Scholefield *et al.* (18) failed to identify an association between cell size and HPV status. Logistic regression revealed that both

intra-anal and perianal cancers with small or medium neoplastic cells as the predominant cell type were much more likely to be hrHPV positive than cancers dominated by large neoplastic cells. Like perianal skin cancers with basaloid features, those with predominantly small or medium neoplastic cells may comprise some anal canal cancers that were inappropriately categorized as perianal because of insufficient amounts of tumor material examined.

We applied a sensitive technique to detect HPV and assessed histopathological characteristics in immediately neighboring sections, thereby minimizing the risk that tissue examined for HPV was not representative of the tumor tissue examined histologically. We detected hrHPV in even the smallest tissue sections, but some small lesions may have been categorized wrongly with respect to basaloid features or degree of keratinization. Likewise, we may have missed the involvement of the anal canal in some lesions categorized as perianal skin cancer. However, such misclassification would tend to dilute the trend toward increasing hrHPV positivity with higher localization in the anal canal and obscure the association with histological characteristics such as basaloid features and keratinization. Consequently, our observations are likely to be conservative estimates of the underlying true associations.

In conclusion, we found the great majority of cancers involving the anal canal to be hrHPV positive, suggesting that, as for cervical cancer, hrHPV may be a necessary cause for squamous cell carcinomas of the anal canal. Presence of basaloid features or adjacent AIN lesion, poor or absent keratinization, and a predominance of small or medium neoplastic cells are all strong positive predictors of hrHPV positivity. However, like vulvar and penile cancers, a proportion of anal cancers, particularly those located perianally in elderly patients, may not involve hrHPVs in their etiology.

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