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Biological relevance of human papillomaviruses in vulvar cancer

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The carcinogenic role of high-risk human papillomavirus (HR-HPV) types in the increasing subset of vulvar intraepithelial neoplasia and vulvar cancer in young women has been established. However, the actual number of vulvar cancer cases attributed to HPV is still imprecisely defined. In an attempt to provide a more precise definition of HPV-driven vulvar cancer, we performed HPV-type-specific E6*I mRNA analyses available for 20 HR-/possible HR (pHR)-HPV types, on tissue samples from 447 cases of vulvar cancer. HPV DNA genotyping was performed using SPF10-LiPA$_{25}$ assay due to its high sensitivity in formalin-fixed paraffin-embedded tissues. Data on p16^{INK4a} expression was available for comparative analysis via kappa statistics. The use of highly sensitive assays covering the detection of HPV mRNA in a broad spectrum of mucosal HPV types resulted in the detection of viral transcripts in 87% of HPV DNA+ vulvar cancers. Overall concordance between HPV mRNA+ and p16^{INK4a} upregulation (strong, diffuse immunostaining in >25% of tumor cells) was 92% (K = 0.625, 95% confidence interval (CI) = 0.531–0.719). Among these cases, 83% were concordant pairs of HPV mRNA+ and p16^{INK4a}+ and 9% were concordant pairs of HPV mRNA − and p16^{INK4a} −. Our data confirm the biological role of HR-/pHR-HPV types in the great majority of HPV DNA+ vulvar cancers, resulting in an HPV-attributable fraction of at least 21% worldwide. Most HPV DNA+ vulvar cancers were associated with HPV16 (85%), but a causative role for other, less frequently occurring mucosal HPV types (HPV26, 66, 67, 68, 70 and 73) was also confirmed at the mRNA level for the first time. These findings should be taken into consideration for future screening options as HPV-associated vulvar preneoplastic lesions have increased in incidence in younger women and require different treatment than vulvar lesions that develop from rare autoimmune-related mechanisms in older women.
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With an annual incidence of 2 cases per 100 000 women, vulvar cancer is a rare malignancy.[1,2] The estimated global burden of vulvar cancer is 27 000 cases annually and it accounts for 3–5% of all gynecological cancers in developed countries.[3] Most cases of vulvar cancer present as squamous cell carcinoma (>90%).[1,2] The International Agency for Research on Cancer and World Health Organization distinguish two pathways for the development of vulvar cancer. The first pathway is activated by underlying autoimmune-related processes while the second pathway is triggered by mucosal human papillomavirus (HPV) infection. Differentiated-type vulvar intraepithelial neoplasia is non-HPV associated and appears in older women (median age 70 years) who develop chronic inflammation of the anogenital area often diagnosed as lichen sclerosus or lichen planus.[4] In 2–5% of cases, these lesions further develop into differentiated keratinizing squamous cell carcinoma of the vulva.[5] Usual-type vulvar

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intraepithelial neoplasia is HPV associated and appears in younger women. Morphologically, it can be either basaloid, warty or mixed histology, and if untreated, 9–16% of lesions can progress to basaloid or warty squamous cell carcinoma of the vulva.[5] In some countries, HPV-associated vulvar intraepithelial neoplasia increased four-fold between 1973 and 2000, particularly among younger women (median age 40 years).[6,7] Changes in sexual behavior, early onset of sexual activities and transmission of high-risk/possible high-risk HPV types (HR-/pHR-HPV), especially HPV16, are considered contributing factors to this observed increase in vulvar lesions.

Differentiating between HPV-associated and non-HPV-associated vulvar lesions has been recognized as an important distinction among clinicians as it has both therapeutic and postoperative management implications.[8–10] Non-HPV-associated vulvar intraepithelial neoplasia is a rapidly progressive lesion requiring immediate excision and treatment.[8] By contrast, HPV-associated vulvar intraepithelial neoplasia develops slowly and can spontaneously regress or regress postlocalized treatment (eg, with topical immune modulators).[8,10] However, patients with HPV-associated vulvar lesions are at increased risk of developing additional HPV-associated lesions within the anogenital tract. Consequently, careful examination and monitoring of the cervix and the perianal area is needed in these women.[11]

True estimation of the HPV-attributable fraction in vulvar cancer is still imprecise. World estimation of HPV DNA-positive (HPV DNA+) vulvar cancers stands currently at 43%.[3] This estimation is based on a recent meta-analysis that assessed 63 epidemiological studies including the evaluation of 1873 vulvar cancer cases collected from across the globe.[12] However, the mere presence of HPV DNA has shown to be insufficient to define HPV-driven mucosal cancers outside of the cervix.[13–16] Previously, we compared HPV DNA positivity alone and in combination with $p16^{INK4a}$ upregulation by assessing these markers directly in 1709 vulvar cancer tissue specimens collected from 39 countries worldwide.[17] Our results showed a lower fraction of HPV DNA+ vulvar cancers compared with previous HPV DNA reports; 29%[17] *versus* 43% based on a meta-analysis.[3] In addition, adding $p16^{INK4a}$ upregulation data to the sole HPV DNA presence in tumor tissues lowered estimate of the HPV-attributable fraction of vulvar cancer from 29% to 25%.[17]

In the continuous effort to better define HPV-driven vulvar cancer, we focused on collecting biological evidence of HPV-transformed phenotype in the vulva by investigating the expression and concordance of HPV mRNA and $p16^{INK4a}$ in 447 HPV DNA+ cases of vulvar cancer. Making this distinction is particularly important toward the adequate assessment of potential clinical differences between HPV-driven and non-HPV-driven malignancies of the vulva.

Materials and methods

Ethics Statement

The samples obtained and analyzed here were selected from HPV VVAP (International Survey on HPV prevalence and type distribution in Vulvar, Vaginal, Anal, Penile neoplasias)[17] for which the Institutional Review Board approval was received. All samples were anonymized. All protocols applied were approved by local and Catalan Institute of Oncology ethics committees.

Study Approach

All specimens analyzed were formalin fixed and paraffin embedded. Vulvar cancer cases with sufficient tissue quality and quantity that had been previously analyzed for the presence of HPV DNA and $p16^{INK4a}$ expression were eligible for further analyses. Based on these criteria, 447 HPV DNA+ histologically diagnosed vulvar cancers were analyzed for the expression of viral transcripts. HPV E6*I mRNA RT-PCR assays were available for 20 HR-/pHR-HPV types: HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, and 82.

HPV mRNA Analysis

The mRNA extraction and mRNA detection from tissue ribbons were performed as previously described.[18,19] For all cases analyzed, hematoxylin and eosin stain before and after sectioning for RNA showed $\leq 10\%$ tumor reduction. For each case, HPV-type-specific E6*I mRNA RT-PCR assays were performed for an HPV type(s) previously determined by genotyping and for a cellular ubiquitin C gene as a control for tissue quality. A second assay was performed to assess the presence of HPV16 E6*I mRNA in all cases, irrespective of HPV DNA result. Cases with HPV mRNA positive (HPV mRNA+) and/or ubiquitin C mRNA+ signal were considered 'RNA valid'. All 'RNA invalid' samples, ie, cases that were HPV mRNA-negative (HPV mRNA –) and ubiquitin C mRNA – , were analyzed a second time, and according to signals obtained, classified as 'RNA valid' or 'RNA invalid'. Of the 11 vulvar cancer cases initially classified as 'RNA invalid', six cases were reclassified as 'RNA valid' and five remained classified as 'RNA invalid' upon re-analyses.

Testing for HPV type mRNA in HPV DNA-negative (HPV DNA –) cases was not included as part of the primary scope of this work owing to the complexity of such analyses (Figure 1). However, a subset of 20 HPV DNA – cases were tested for HPV16 E6*I mRNA as a negative control (10 HPV DNA – / $p16^{INK4a}$ – and 10 HPV DNA – / $p16^{INK4a}$ + cases). The 10 HPV DNA – / $p16^{INK4a}$ – cases were 'RNA

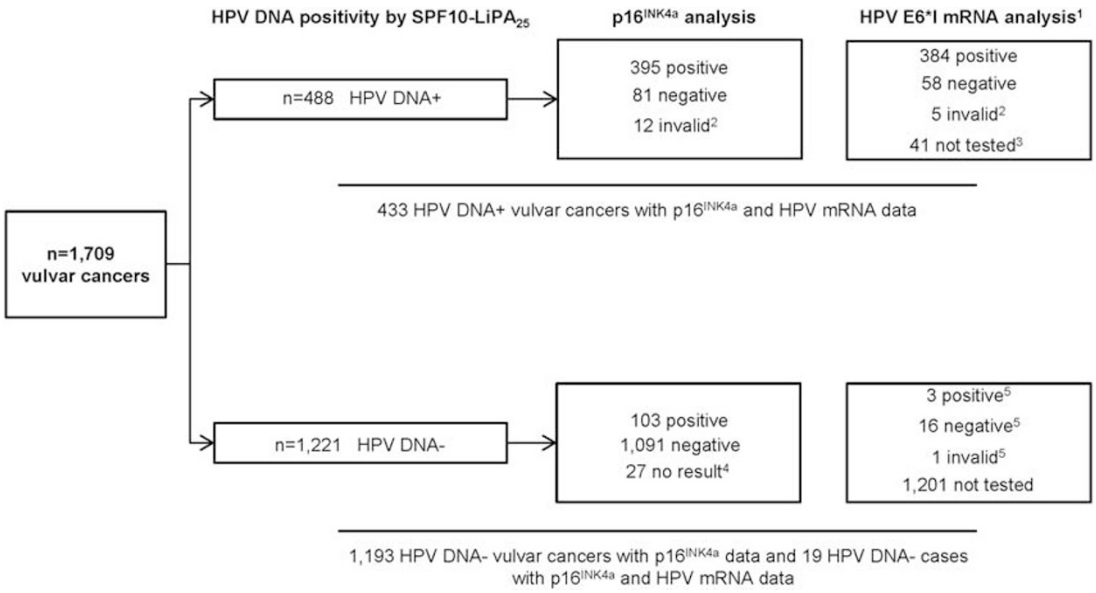

Figure 1 Study algorithm. (1) HPV E6*I mRNA assays were developed for 12 HR-HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59; and for 8 pHR-HPV: 26, 53, 66, 67, 68, 70, 73, and 82. (HPV-type classification was according to IARC classification.[1]) (2) In $p16^{INK4a}$ analysis: Tissue quality insufficient to analyze $p16^{INK4a}$ expression ($n=12$). In mRNA analysis: both HPV and ubiquitin C mRNA are negative and therefore invalid ($n=5$). (3) No splice site HPV mRNA assays available for the HPV types identified by SPF10-$LiPA_{25}$ or sequencing; LR-HPV: HPV6 ($n=5$), HPV11 ($n=2$), HPV44 ($n=3$), HPV61 ($n=2$), HPV74 ($n=3$), HPV102 ($n=1$); pHR-HPV: HPV30 ($n=1$), HPV69 ($n=2$); HPV undetermined, ie, not identified by SPF10-$LiPA_{25}$ or sequencing but positive with DEIA ($n=20$); not enough material ($n=2$; one HPV16 and one HPV6/16/39 or 68 or 73 case). (4) No result: In $p16^{INK4a}$ analysis: Insufficient tumor tissue in tissue blocks for $p16^{INK4a}$ analysis. (5) A random selection of 20 HPV DNA – vulvar cancer cases (10 HPV DNA – /$p16^{INK4a}$ – and 10 HPV DNA – /$p16^{INK4a}$+ cases) were analyzed for HPV mRNA as control tissues. One case was invalid in mRNA analysis.

Table 1 Expression of HPV DNA and HPV mRNA in a worldwide collection of vulvar cancer cases ($n=1709$)

	Vulvar cancers								*Vulvar cancers with available p16INK4a results*							
	Total		*HPV mRNA*						*Total*		*HPV mRNA*					
			Positive		*Negative*		*Invalid*				*Positive*		*Negative*		*Invalid*	
	n/N	*(%)*	n	*(%)*[a]	n	*(%)*[a]	n	*(%)*[a]	n/N	*(%)*	n	*(%)*[a]	n	*(%)*[a]	n	*(%)*[a]
HPV DNA negative	1221/1709	(71)							1194/1670	(71)						
Tested for HPV mRNA[b]	20/1221	(2)	3	(15)	16	(80)	1	(5)	20/1194	(2)	3	(10)	16	(80)	1	(5)
HPV DNA positive	488/1709	(29)							476/1670	(29)						
Tested for HPV mRNA	447/488	(92)	384	(86)	58	(13)	5	(1)	438/476	(92)	377	(86)	56	(13)	5	(1)
Single HPV type	418/447	(94)	360	(86)	53	(13)	5	(1)	409/438	(93)	353	(86)	51	(12)	5	(1)
Multiple HPV types	29/447	(6)	24	(83)	5	(17)	0	(0)	29/438	(7)	24	(83)	5	(17)	0	(0)

HPV mRNA results were available for 20 HR-/pHR-HPV types: HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, and 82.
[a]Among cases tested for HPV mRNA.
[b]For HPV DNA-negative cases, HPV E6*I mRNA was performed in a random control sample of 20 vulvar cancers: 10 HPV DNA – /$p16^{INK4a}$+ and 10 HPV DNA – /$p16^{INK4a}$ – cases, respectively.

valid' and also HPV16 mRNA – . From 10 HPV DNA – /$p16^{INK4a}$+, 9 were 'RNA valid' and 3 of the 9 were HPV16 mRNA+ (33%). This observation had minimal impact on overall estimates owing to the small number of $p16^{INKa}$+ cases among the HPV DNA – cases (9%; 103 of 1,194). Two out of the 10 HPV DNA – /HPV mRNA – /$p16^{INK4a}$ – cases showed partial basaloid features, whereas 2 out of the 3 HPV DNA – /HPV mRNA+/$p16^{INK4a}$+ cases were basaloid.

HPV Genotyping, $p16^{INK4a}$ Immunohistochemistry and Histological Assessment

HPV genotyping, $p16^{INK4a}$ immunohistochemistry and histological analyses were performed as

Table 2 Concordance of HPV mRNA and $p16^{INK4a}$ expression in HPV DNA-positive vulvar cancers ($n = 433$) across variables

		HPV mRNA and $p16^{INK4a}$ concordance in vulvar cancer cases										
	Total HPV DNA positive	*$p16^{INK4a}$+ and HPV mRNA+*		*$p16^{INK4a}$+ and HPV mRNA −*		*$p16^{INK4a}$ − and HPV mRNA+*		*$p16^{INK4a}$ − and HPV mRNA −*		*Overall concordance*	*Kappa*	
	n	n	*(%)*	n	*(%)*	n	*(%)*	n	*(%)*	*(%)*	*Index*	P-*value*[a]
Total	433	360	(83)	19	(4)	17	(4)	37	(9)	(92)	**0.625**	
By number of HPV types detected												0.572
Single HPV type	404	339	(84)	19	(5)	14	(3)	32	(8)	(92)	**0.614**	
Multiple HPV types	29	21	(72)	0	(0)	3	(10)	5	(17)	(90)	**0.707**	
By year of diagnosis												0.665
1980–1989	3	3	(100)	0	(0)	0	(0)	0	(0)	(100)	—	
1990–1999	105	86	(82)	4	(4)	6	(6)	9	(9)	(90)	**0.588**	
2000–2009	313	260	(83)	14	(4)	11	(3)	28	(9)	(92)	**0.636**	
2010–2011	12	11	(92)	1	(8)	0	(0)	0	(0)	(92)		
By age (years)												**0.000**
< 56	151	130	(86)	9	(6)	7	(5)	5	(3)	(89)	**0.327**	
56–66	86	74	(86)	4	(5)	5	(6)	3	(3)	(90)	**0.343**	
67–74	50	40	(80)	2	(4)	2	(4)	6	(12)	(92)	**0.702**	
7580	58	46	(79)	1	(2)	0	(0)	11	(19)	(98)	**0.946**	
≥ 81	60	47	(78)	3	(5)	2	(3)	8	(13)	(92)	**0.712**	
By continent												0.054
Europe	151	122	(81)	8	(5)	2	(1)	19	(13)	(93)	**0.753**	
North America	20	14	(70)	0	(0)	3	(15)	3	(15)	(85)	**0.583**	
Center South America	118	103	(87)	6	(5)	2	(2)	7	(6)	(93)	**0.600**	
Africa	17	10	(59)	1	(6)	5	(29)	1	(6)	(65)	**0.089**	
Asia	45	36	(80)	2	(4)	2	(4)	5	(11)	(91)	**0.662**	
Oceania	82	75	(91)	2	(2)	3	(4)	2	(2)	(94)	**0.413**	
By histological diagnosis												0.321
SCC 100% warty/basaloid/ papilar basaloid	221	200	(90)	10	(5)	4	(2)	7	(3)	(94)	**0.468**	
SCC 100% non-warty/basaloid	173	130	(75)	9	(5)	9	(5)	25	(14)	(90)	**0.671**	
SCC mixed (any, %)	30	23	(77)	0	(0)	4	(13)	3	(10)	(87)	**0.535**	
Other diagnosis	9	7	(78)	0	(0)	0	(0)	2	(22)	(100)	**1.000**	
By presence of keratinizing component												0.053
Keratinizing	163	123	(75)	6	(4)	8	(5)	26	(16)	(91)	**0.734**	
Non-keratinizing	70	59	(84)	5	(7)	3	(4)	3	(4)	(89)	**0.367**	
Missing in keratinizing	200	178	(89)	8	(4)	6	(3)	8	(4)	(93)	**0.496**	

$p16^{INK4a}$ positivity ($p16^{INK4a}$+) is defined as >25% of tumor cells with strong, diffuse immunostaining for $p16^{INK4a}$.
HPV mRNA and $p16^{INK4a}$ results were available for 433 out of the 488 HPV DNA-positive vulvar cancers (89%).
Bold numbers indicate statistically significant kappa index test results (P-value < 0.05).
[a]P-value obtained from the comparison of the different kappa indices (different categories from each variable).

previously described.[17] For simplicity of reporting, we denote $p16^{INK4a}$-positive ($p16^{INK4a}$+) cases as cases that showed strong, diffuse immunostaining in >25% of tumor cells ($p16^{INK4a}$ upregulation) and $p16^{INK4a}$ negative ($p16^{INK4a}$ −) cases as cases with focal, weak immunostaining or immunostaining in ≤25% of tumor cells.[16]

Statistical Analysis

Information was available for 'country', 'age at diagnosis', 'year of diagnosis', 'histopathological diagnosis', 'HPV DNA positivity', 'HPV type' and 'HPV mRNA and/or $p16^{INK4a}$ expression'. Chi-squared test and Student's *t*-test were used to evaluate associations between variables and HPV DNA, HPV mRNA and $p16^{INK4a}$ positivity. Agreement between HPV DNA and $p16^{INK4a}$ and between HPV mRNA and $p16^{INK4a}$ was assessed by kappa score. The McNemar test was used for matched pair data to assess the unequal distribution of discordant results. The agreement was evaluated globally and by the different assays explored. All statistical inferences were based on two-sided tests. Results were statistically significant at $P < 0.05$. Data analyses were performed using Statistical Package for the Social Sciences (SPSS) (Version 13.0, SPSS, Chicago, IL, USA) and

Table 3 Transcriptional activity of individual HPV types in vulvar cancers harboring multiple HPV types ($n = 29$)

HPV DNA type detected[a]	*Number of cases*	*HPV mRNA*			*p16^{INK4a}*
		Positive	*Negative*	*NT*	
16, 18	1	16, 18			+
31, 33	1	31, 33			+
16, 33	1	16	33		+
16, 51	1	16	51		+
33, 56	1	33	56		+
35, 66	1	35	66		+
31, 33, 58	1	33, 58	31		+
51, 68 or 73	1	73	51, 68		+
16, 31	1	16, 31			−
16, 18	1		16, 18		−
6, 16	4	16		6	+
11, 18	1	18		11	+
18, 44	1	18		44	+
18, 44	1	18		44	+
18, 74	1	18		74	+
31, 42	1	31		42	+
42, 70	1	70		42	+
44, 45	1	45		44	+
59, 74	1	59		74	+
6, 33	1	33		6	+
31, 33, 45, 44	1	31	33, 45	44	+
11, 39, 51	1	39, 51		11	−
18, 44	1		18	44	−
44, 58	1		58	44	−
44, 66	1		66	44	−
51, 53, 58, 54	1		51, 53, 58	54	−

HPV mRNA+: positive results in HPV E6*I mRNA RT-PCR assays available for 20 HPV types: HPV16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 67, 68, 70, 73, and 82. NT: not tested.
[a]HPV DNA was assayed by SPF10-LiPA$_{25}$ genotyping.

with STATA (Version 10.0, Stata Corporation, Computing Resource Center, College Station, TX, USA).

Results

A total of 1709 vulvar cancer cases collected from 39 countries were HPV genotyped; 29% (488/1709) of these cases were HPV DNA+ (Figure 1).[17] From 488 HPV DNA+ cases, 447 were analyzed in the current study for the expression of viral mRNA and 99% (442/447) were 'RNA valid' (HPV mRNA+ and/or ubiquitin C mRNA+) (Table 1). A subset of 433 HPV DNA+ vulvar cancers had available HPV mRNA and p16^{INK4a} data (Table 1) included in the final statistical analysis of HPV mRNA and p16^{INK4a} concordance (Table 2).

HPV E6*I mRNA and p16^{INK4a} Expression in Vulvar Cancers Harboring Single or Multiple HPV Infections

Overall, 87% (384/442) of vulvar cancer cases were HPV DNA+/HPV mRNA+ (Table 1).

Table 4 Concordance of HPV mRNA and p16^{INK4a} expression in HPV DNA-positive vulvar cancers ($n = 433$) according to the p16^{INK4a} positivity cutoff

	HPV mRNA and p16^{INK4a} concordance in vulvar cancers										
	p16^{INK4a}+ and HPV mRNA+		*p16^{INK4a}+ and HPV mRNA−*		*p16^{INK4a}− and HPV mRNA+*		*p16^{INK4a}− and HPV mRNA−*		*McNemar test*	*Overall concordance*	*Kappa*	
p16^{INK4a} cutoff[a]	n	(%)	n	(%)	n	(%)	n	(%)	P-*value*	(%)	*Index*	*Strength of agreement*
> 25%	360	(83)	19	(4)	17	(4)	37	(9)	0.868	(92)	**0.625**	Good
> 50%	310	(72)	16	(4)	67	(15)	40	(9)	**0.000**	(81)	**0.387**	Fair
> 75%	235	(54)	13	(3)	142	(33)	43	(10)	**0.000**	(64)	**0.198**	Poor

HPV mRNA and p16^{INK4a} data were available for 433 out of the 488 HPV DNA+ vulvar cancers (89%). Strength of agreement in kappa index value: < 0.20 Poor; 0.21–0.40 Fair; 0.41–0.60 Moderate; 0.61–0.80 Good; > 0.80 Very good. Bold numbers indicate statistically significant kappa index and McNemar *P*-values. Kappa index *P*-value shows significantly different values depending on p16^{INK4a} cutoffs. When cutoff for p16^{INK4a} positivity was set to > 25%, 83% (360/433) of tumors were classified as p16^{INK4a}+/HPV mRNA+ yielding good agreement between p16^{INK4a} and HPV mRNA positivity. When cutoff for p16^{INK4a} positivity was set to > 50%, 69% (297/433) of tumors were classified as p16^{INK4a}+/HPV mRNA+ yielding fair agreement. Finally, > 75% cutoff, classified 54% (234/433) of tumors as p16^{INK4a}+/HPV mRNA+ yielding poor agreement between p16^{INK4a} and HPV mRNA positivity.
[a]p16^{INK4a} cutoff: a percentage of tumor cells with strong, diffuse immunostaining for p16^{INK4a}.

Table 5 Distribution of keratinizing and non-keratinizing HPV DNA+/HPV mRNA+ vulvar cancers according to the $p16^{INK4a}$ positivity cutoff

	HPV DNA+/HPV mRNA+ vulvar cancers						
	Total (n = 193)		*Keratinizing (n = 131)*[b]		*Non-keratinizing (n = 62)*[c]		*Chi-square*
$p16^{INK4a}$ cutoff[a]	n	%	n	%	n	%	P-*value*
>25%	182	(94)	123	(94)	59	(95)	1.000
>50%	155	(80)	98	(75)	57	(92)	**0.006**
>75%	103	(53)	58	(44)	44	(71)	**0.000**

With >25%, 94% (123/131) of keratinizing and 95% (59/62) of non-keratinizing tumors were classified as HPV DNA+/HPV mRNA +/$p16^{INK4a}$+.
This is in contrast to 75% (98/131) of keratinizing and 92% (57/62) of non-keratinizing HPV DNA+/HPV mRNA+/$p16^{INK4a}$+ tumors with >50% cutoff or 44% (58/131) of keratinizing and 71% (44/62) of non-keratinizing HPV DNA+/HPV mRNA+/$p16^{INK4a}$+tumors with >75% cutoff, respectively.
[a]$p16^{INK4a}$ cutoff: a percentage of tumor cells with strong, diffuse immunostaining for $p16^{INK4a}$. Bold numbers indicate statistically significant *P*-values.
[b]One hundred and thirty-one of the 163 HPV DNA+ keratinizing tumors had available HPV mRNA and $p16^{INK4a}$ data.
[c]Sixty-two of the 70 HPV DNA+ non-keratinizing tumors had available HPV mRNA and $p16^{INK4a}$ data.

Of the 413 'RNA valid' vulvar cancer cases harboring single HPV type DNA, 87% (360/413) were HPV mRNA+ (Table 1). Among 317 HPV16 single DNA+ cases, 88% (280/317) were HPV16 mRNA+. Among the 96 non-HPV16 DNA+ cases, 83% (80/96) were mRNA+ for the HPV type defined by SPF10-$LiPA_{25}$ genotyping, including one HPV33 DNA+ case that expressed both HPV33 and HPV16 mRNA transcripts. We have identified nine vulvar cancer cases that were HPV DNA+ for a single pHR-HPV type (ie, HPV26, 53, 66, 67, 68, 70 and 73, respectively) (Supplementary Table S1). The biological activity of HPV26, 66, 67, 68, 70 and 73 in vulvar cancer was confirmed by the presence of HPV-type mRNA and upregulation of $p16^{INK4a}$. Two HPV53 single DNA+ cases were HPV53 mRNA – and $p16^{INK4a}$ – .

Twenty-nine 'RNA valid' vulvar cancers harboring DNA of multiple HPV types were identified of which 83% (24/29) were HPV mRNA+ for at least one HPV type identified by genotyping (Table 1 and Table 3). From these 29 cases, 10 could be fully analyzed (ie, mRNA assays were available for all the types detected by genotyping). Overall, 40% (4/10) of these cases expressed transcripts of multiple HR-/pHR-HPV types (Table 3). Three of these four cases had a preneoplastic lesion adjacent to the invasive tumor.

Among vulvar cancer cases with HPV mRNA and $p16^{INK4a}$ data, 83% (360/433) were concordantly HPV mRNA+ and $p16^{INK4a}$+ and 9% (37/433) were concordantly HPV mRNA – and $p16^{INK4a}$ – , yielding an overall concordance of 92% ($K = 0.625$; 95% CI: (0.531–0.719); Table 4). A total of 36 cases (8%) showed discordant HPV mRNA and $p16^{INK4a}$ data: 19 HPV mRNA – /$p16^{INK4a}$+ and 17 HPV mRNA +/$p16^{INK4a}$ cases (Table 4). McNemar tests ($P = 0.868$) indicated that the discordant cases are equally distributed. Furthermore, there was no difference in kappa index concordance between histological diagnosis, region of origin or year of diagnosis (Table 2). However, differences in kappa index concordance were observed according to age group; agreement being higher among women aged >66 years compared with women aged <66 years (Table 2).

Cutoff for $p16^{INK4a}$ in HPV-Driven Vulvar Cancer

To define $p16^{INK4a}$ upregulation in vulvar cancer, we used the cutoff of >25% $p16^{INK4a}$+ tumor cells, with strong staining intensity and in a diffuse pattern. As there is no current standardization for $p16^{INK4a}$ cutoff to define HPV-associated vulvar cancer, we challenged our >25% cutoff by different scenarios as shown in Tables 4 and 5. The >25% cutoff showed 83% (360/433) of HPV DNA+/HPV mRNA+ tumors to be $p16^{INK4a}$+, yielding good agreement between $p16^{INK4a}$ and HPV mRNA positivity (Table 4). Increasing this cutoff to >50% or >75% resulted in a fair and poor agreement between $p16^{INK4a}$ and HPV mRNA positivity, respectively (Table 4). With >25% cutoff, 94% of HPV DNA +/HPV mRNA+ keratinizing tumors were defined as $p16^{INK4a}$+ compared with only 44% when >75% cutoff was applied (Table 5). This difference was lower for non-keratinizing vulvar cancers where 95% of non-keratinizing HPV DNA+/HPV mRNA+ tumors were defined as $p16^{INK4a}$+ with >25% cutoff against 71% with >75% cutoff (Table 5). Keratinizing vulvar cancers tend to present with a lower percentage of $p16^{INK4a}$+ tumor cells, compared with non-keratinizing vulvar cancers (Table 5). Examples of vulvar cancer regarding histology, $p16^{INK4a}$ staining and HPV status are shown in Figure 2.

Patients' Age and Tumor Histology, Origin and Year of Diagnosis

All discordant cases were equally distributed within each variable-dependent category (McNemar test yielded non-significant results). All vulvar cancers (100%) identified between 1980 and 1989 ($n = 3$) and after 2010 ($n = 12$) were HPV DNA+/HPV mRNA +/$p16^{INK4a}$+ compared with 82% of such cases identified between 1990 and 2009 ($n = 418$) (Table 2). Prevalence of HPV DNA+/HPV mRNA +/$p16^{INK4a}$+ cases varied by geographic region with the highest prevalence observed in Oceania (91%) and the lowest in Africa (59%) (Table 2). In addition, the total number of vulvar cancer cases from Africa

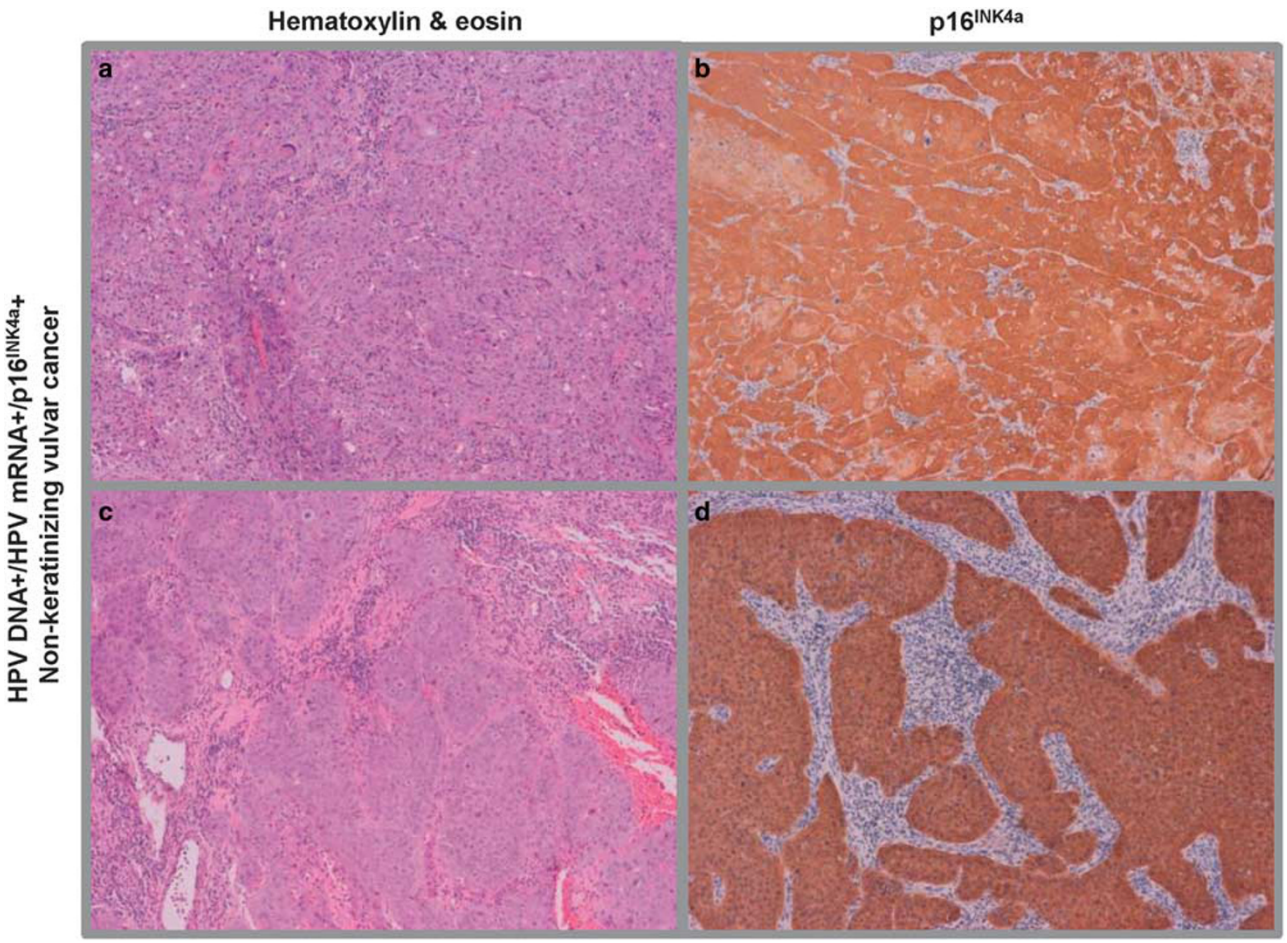

Figure 2 Examples of vulvar cancer cases regarding HPV status, $p16^{INK4a}$ immunostaining and the presence of keratinizing component. (**a** and **b**) HPV45 DNA+/HPV45 mRNA+ non-keratinizing vulvar cancer with >75% $p16^{INK4a}$+ tumor cells (ID 20271). (**c** and **d**) HPV52 DNA+/HPV52 mRNA+ non-keratinizing vulvar cancer with >75% $p16^{INK4a}$+ tumor cells (ID 20365). (**e** and **f**) HPV16 DNA+/HPV16 mRNA+ keratinizing vulvar cancer with 26–50% diffuse $p16^{INK4a}$+ tumor cells (ID 22143). (**g** and **h**) HPV16 DNA+/HPV16 mRNA+ keratinizing vulvar cancer with 26–50% diffuse $p16^{INK4a}$+ tumor cells (ID 22245). (**i** and **j**) HPV DNA –/HPV16 mRNA –/$p16^{INK4a}$ – keratinizing vulvar cancer (ID 20072). (**k** and **l**) HPV DNA –/HPV16 mRNA –/ $p16^{INK4a}$ – keratinizing vulvar cancer (ID 20462). ×10 magnification.

($n=17$) was also low in comparison with Oceania ($n=75$) (Table 2). HPV DNA+/HPV mRNA+/$p16^{INK4a}$+ vulvar cancers varied across five age groups and ranged between 78% (≥81 years) and 86% (<66 years) (Table 2).

Discussion

The etiological role of HPV in the development of vulvar cancer has been well recognized.[1] However, the true attributable fraction of HPV in vulvar cancer remains unclear. It has become increasingly evident that a functional evidence of HPV activity or HPV transformation is necessary in addition to the HPV DNA presence, in order to define true HPV-driven tumors outside of the cervix uteri.[20] In our study, we focused on obtaining evidence of HPV transcriptional activity, ie, the presence of HPV mRNA in HPV DNA+ vulvar cancer tissues. In an examination of 447 HPV DNA+ vulvar cancers, we combined HPV mRNA data with data on a well-established marker of HPV-transformed phenotype in mucosal cancers, $p16^{INK4a}$. Viral mRNA was identified in 87% of the HPV DNA+ vulvar cancer cases. Among the 433 cases with both HPV mRNA and $p16^{INK4a}$ data available, 83% were concordant pairs of HPV mRNA+ and $p16^{INK4a}$+. These data indicate that a proportion of HPV DNA+ cases (9%) does not express an additional marker of HPV activity, therefore questioning HPV attribution in that subset. Thus, in the absence of mechanistic data to define an HPV-driven cancer, markers of HPV activity and HPV-transformed phenotype demonstrated in addition to HPV DNA, should allow for more robust etiologic attribution.

To identify transcriptionally active HPV types in vulvar lesions, we applied HPV-type-specific and highly sensitive E6*I mRNA assays developed for 20 HR-/pHR-HPV types and validated for use in

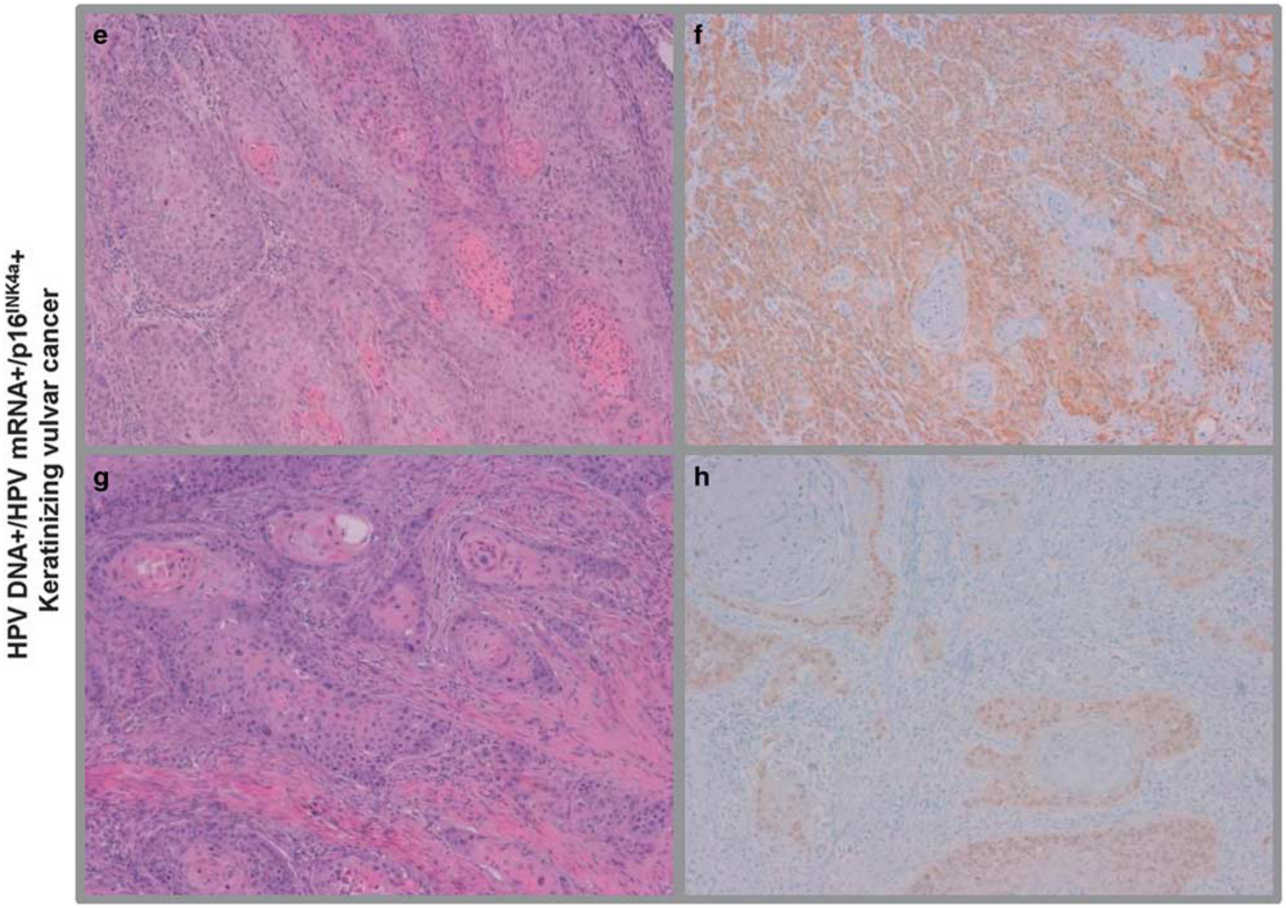

Figure 2 Continued

formalin-fixed paraffin-embedded tissues.[18,21] HPV transcripts are indicative of active virus but are not transformation specific.[22,23] But the expression of viral mRNA in mucosal tumors is a requirement for expression of E7 oncoprotein, which drives malignant transformation and induces $p16^{INK4a}$ upregulation. Our study is not the first to examine the expression of viral transcripts in vulvar lesions. However, in terms of the number of cases and range of HPV types analyzed, it is the broadest in scope. We identified eight earlier studies that provided data on viral E6/E7 transcripts in vulvar lesions, which focused on HPV16 and/or HPV18 transcripts only.[24–31] Our study also demonstrates that HR-HPV types other than HPV16 and 18, as well as a subset of pHR-HPV types, have an etiological role in the development of vulvar cancer.

Expression of $p16^{INK4a}$ protein in vulvar preneoplastic lesions and vulvar cancer has also been assiduously investigated.[2,27,32–35] In the context of HPV infection, $p16^{INK4a}$ upregulation is a result of a cellular defense mechanism referred to as 'oncogene-induced senescence'.[36] In cell lines of cervical cancer, an HPV-transformed tumor model, CDKN2A gene that encodes for $p16^{INK4a}$ protein is present as a wild-type, and $p16^{INK4a}$ upregulation is induced by HR-HPV E7 oncogene expression.[36–38] In the vulva, up to 20% of cancers carry CDKN2A mutations but these are usually 'silencing' mutations primarily identified in HPV DNA – cancers and resulting in a loss of $p16^{INK4a}$ expression.[39,40] This confirms the value of $p16^{INK4a}$ upregulation as a marker of HPV-transforming activity in vulvar cancer in addition to HPV DNA and/or HPV mRNA or as a single marker once the precise cutoff has been defined.

Numerous immunohistochemical studies have confirmed $p16^{INK4a}$ upregulation as an excellent biomarker to define HR-HPV-associated lesions at different mucosal sites.[13,15,21,41–44] In the vulva, $p16^{INK4a}$ also seems to be a superior marker to assess the effectiveness of imiquimod treatment suggested for HPV DNA+ vulvar intraepithelial neoplasia.[10,45] Our findings demonstrate $p16^{INK4a}$ upregulation in all HPV DNA+/HPV mRNA+ vulvar cancers harboring single pHR-HPV types 26, 66, 67, 70 or 73 (1 case each) and HPV DNA+ (no RNA assay available) pHR-HPV types 30 (1 case) or 69 (2 cases). These HPV types are not included in commercial HPV genotyping assays and might be missed when analyzing for HPV DNA only. However, the likelihood of identifying such cases is very low ($< 2\%$). The specificity of $p16^{INK4a}$ upregulation in support

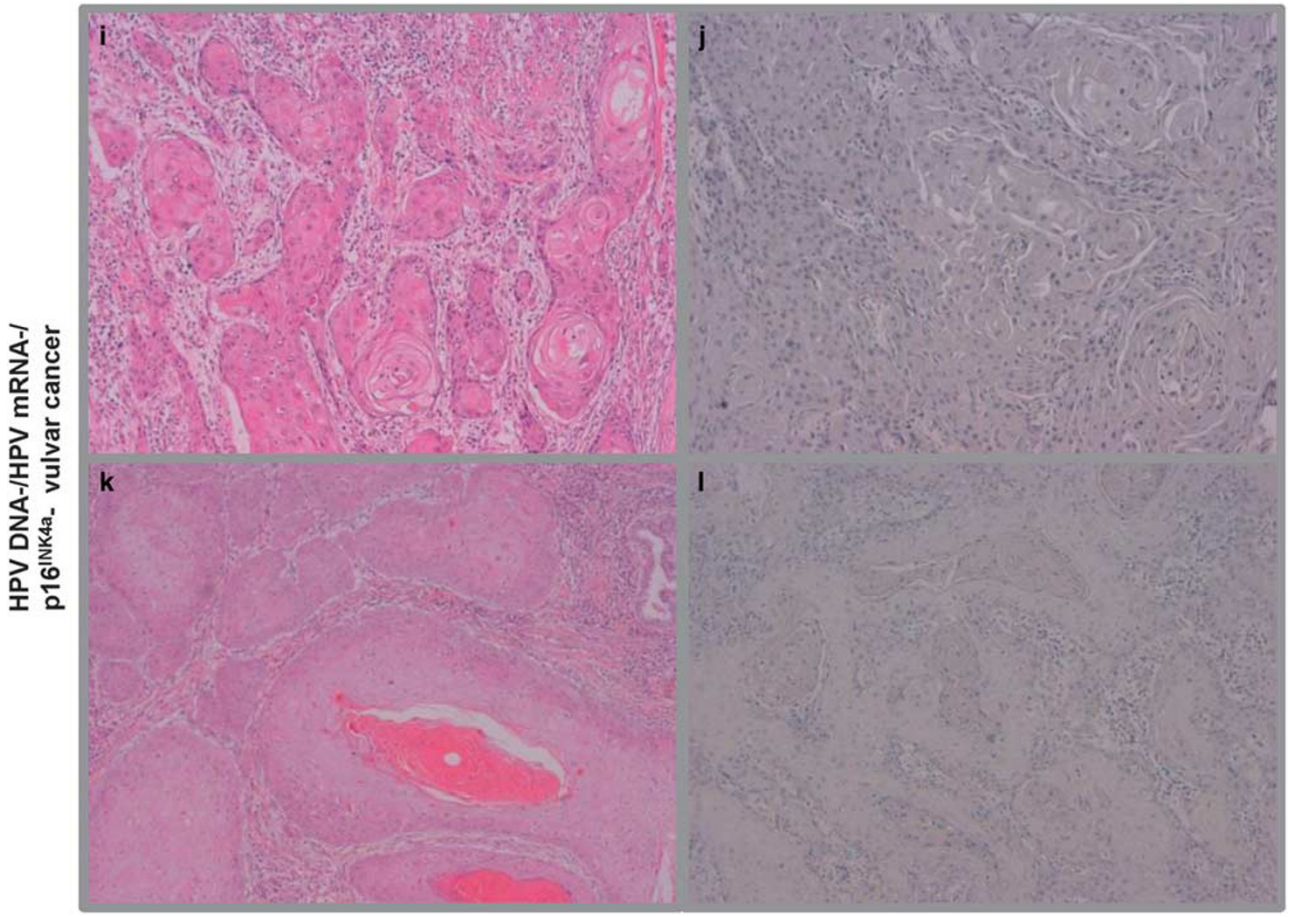

Figure 2 Continued

of HR-/pHR-HPV-type carcinogenicity is further substantiated by our findings that 89% (353/396) and 83% (10 of 12) of vulvar cancers harboring a single, active infection with HR-HPV or pHR-HPV type, respectively, showed $p16^{INK4a}$ upregulation. On the contrary, only 7% (1/15) of vulvar cancers harboring single LR-HPV type DNA showed $p16^{INK4a}$ upregulation (Supplementary Table S2). In this single LR-HPV6 DNA+/$p16^{INK4a}$+ vulvar cancer case in our study (with low-intensity $p16^{INK4a}$ staining in >75% of tumor cells), HPV6 DNA was found in tumor cells, and not in the surrounding stroma, speculating that LR-HPV6 might be just less efficient in inducing $p16^{INK4a}$ upregulation.[46] In such cases, as well as cases discordant for HPV mRNA and $p16^{INK4a}$ expression, investigation via CDKN2A gene sequencing should be explored in future studies. It is also possible that, in such cases, HPV DNA or HPV mRNA are present below detection limits of the applied assays. Or perhaps $p16^{INK4a}$ upregulation was a consequence of a CDKN2A gene mutation, although such CDKN2A mutations seem to be rare in vulvar malignancies.[39,40]

The cutoff for upregulation of $p16^{INK4a}$, as a biomarker to identify HPV-associated vulvar lesions, has not been specifically defined. The Lower Anogenital Squamous Terminology Standardization Project for HPV-Associated Lesions recommends when $p16^{INK4a}$ should be used in combination with histology to describe HPV-associated precancerous lesions.[47,48] The experts suggest that strong and diffuse, block-positive $p16^{INK4a}$ result should be used to support a categorization of precancerous disease.[47,48] However, no percentage of $p16^{INK4a}$+ tumors cells has been specified as a guideline to help define HPV-associated vulvar cancer. In this study, we have applied a cutoff including >25% of $p16^{INK4a}$+-stained tumor cells in a diffuse pattern and with high staining intensity. This cutoff was meticulously defined by a team of expert pathologists in a study on 321 cervical cancers.[21] To test how the distribution of HPV-associated vulvar cancers would change if that cutoff would be increased, we have tested different scenarios (Tables 4 and 5). We found that keratinizing vulvar cancers, the most common histological subtype in this cancer series,[17] were affected the most when cutoff for $p16^{INK4a}$ positivity was >25%. Application of >75% cutoff to define $p16^{INK4a}$ positivity resulted in 66% of HPV DNA+/HPV mRNA+ keratinizing tumors to be classified as $p16^{INK4a}$−, compared with only 6% when >25% $p16^{INK4a}$ cutoff

was applied. If $p16^{INK4a}$ positivity would be the only biomarker to assess HPV association, these cancers would be classified as non-HPV associated. Similar observation was made for non-keratinizing tumors but to a lesser extent—27% of HPV DNA+/HPV mRNA+ non-keratinizing tumors were to be classified as $p16^{INK4a}-$ with >75% cutoff, in contrast to 5% with >25% cutoff. As HPV DNA and HPV mRNA expression are considered to precede the upregulation of $p16^{INK4a}$ in HPV-associated mucosal tumors, the lack of correlation between high percentage of $p16^{INK4a}+$ tumor cells and HPV mRNA positivity in this vulvar cancer series remains to be investigated. At present, we conclude that lower percentage of $p16^{INK4a}+$-stained tumor cells is associated with a degree of keratinizing component, an observation already made in our series of 321 cervical cancers.[21]

A single study by Riethdorf *et al*[27] also correlated the use of HPV mRNA and $p16^{INK4a}$ expression in vulvar lesions and found 90% (52 of the 58) of vulvar intraepithelial neoplasia and vulvar cancer cases to be HPV16 mRNA+/$p16^{INK4a}+$. More specifically, the authors showed that 31% of vulvar cancers were HPV16 mRNA+ while 34% were $p16^{INK4a}+$.[27] In our study, the HPV mRNA+ and $p16^{INK4a}+$ concordance among vulvar cancers yielded good agreement ($K=0.625$, 95% CI: (0.531–0.719)), with 83% concordant pairs of HPV mRNA+ and $p16^{INK4a}+$ and 9% concordant pairs of HPV mRNA− and $p16^{INK4a}-$. HPV DNA+/HPV mRNA +/$p16^{INK4a}+$ vulvar cancers did not significantly vary among different age groups or different time periods. The highest fraction of HPV DNA+/HPV mRNA +/$p16^{INK4a}+$ vulvar cancers was identified in Oceania (92%) and the lowest in Africa (59%), which was significantly different. However, we cannot exclude that this difference might be due to the number of cases available from these two continents (Africa: $n=17$ *versus* Oceania: $n=82$).

A clear definition of HPV-driven vulvar cancer is important for assessing potential clinical differences in HPV-associated vulvar cancers compared with those vulvar cancers that develop through autoimmune processes. Clinical studies have demonstrated better overall and disease-free survival for patients with HPV-associated head-and-neck[15,23,41,49] and anal cancers[42] *versus* mutation-induced cancers at these anatomical sites. Several recent studies have also investigated whether survival of patients with vulvar cancer is associated with tumor HPV status or $p16^{INK4a}$ expression.[32,50–52] At present, there has not been a definitive indication of a need for different treatment or clinical management of HPV-associated and non-HPV-associated vulvar cancers. However, few recent studies demonstrated the association of $p16^{INK4a}$ positivity in vulvar cancer with prolonged overall survival rate,[51,52] lower in-field relapse[51] and lower recurrence rate[50] compared with vulvar cancers that were $p16^{INK4a}-$. It remains to be seen whether classifying HPV-driven vulvar cancers through HPV mRNA positivity together with $p16^{INK4a}$ upregulation should further improve the classification and evaluation of HPV-driven *versus* non-HPV-driven vulvar cancers.

Although the set of vulvar cancer cases analyzed here represents the collection of tissue samples worldwide, our study is limited by the small number of cases originating from North America and Africa (<5% of all cases) as opposed to the other four continents. Other limitations include the absence of fully functional data for all of the samples tested as we did not analyze the transcriptional activity of pHR-HPV30 and 69 or LR-HPV types, for which no E6*I mRNA RT-PCR assays have been developed. In addition, as our study is not population based, there remains a question of potential selection bias. However, we believe that this is unlikely as the cases selected were obtained from large pathology laboratories some of which served as the unique national laboratory for the country. In addition, we requested that selection of consecutive cases should be based on an overall diagnosis of vulvar cancer or on the availability of tissues in a given period without any additional selection criteria, such as 'histology' or 'age'.[17]

The biggest asset of the present study is the contribution of HPV mRNA data, in addition to the provision of data on $p16^{INK4a}$ expression in vulvar cancer; in this regard, the study undertakes the task of providing a more reliable description of vulvar cancers with HPV-transformed phenotype. The estimate of HPV attribution in vulvar cancer in our study is lower when compared with previous HPV DNA reports and meta-analyses.[3] This might be due to the use of: (i) strict HPV protocols and different laboratories to avoid potential cross-contamination during regulated procedures (tissue sectioning, DNA and RNA extraction, PCR, RT-PCR and Luminex hybridization); (ii) control samples for assessing potential cross-contamination; and (iii) additional markers indicative of active virus in addition to the sole HPV DNA presence. Finally, a clear and accurate characterization of HPV-driven tumors is the essential starting point to define possibly required altered approaches in patient management or evaluation of therapeutic response.

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Disclosure/conflict of interest

MP has received research support through cooperation contracts of DKFZ with Roche and Qiagen in the field of development of HPV diagnostics. He is an inventor on patents owned by DKFZ in the field of HPV diagnostics. Authors LA and SdS have received occasional travel fund to attend scientific meetings from Merck and Sanofi Pasteur MSD. The other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)