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# Human papillomavirus, p16<sup>INK4A</sup>, and Ki-67 in relation to clinicopathological variables and survival in primary carcinoma of the vagina

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**Background:** This study aimed to determine human papillomavirus (HPV) status and to investigate p16<sup>INK4A</sup> and Ki-67 expression and their correlation with clinical parameters and survival in women with primary carcinoma of the vagina (PCV).

**Methods:** The presence of HPV DNA was evaluated by PCR. Genotyping was performed by Luminex in 68 short-term ( $\leq 2$  years) and long-term ( $\geq 8$  years) PCV survivors. p16<sup>INK4A</sup> and Ki-67 expression was evaluated by immunohistochemistry.

**Results:** Human papillomavirus DNA was detected in 43% of patients, the majority (63%) of whom were HPV16 positive. High p16<sup>INK4A</sup> expression was significantly correlated with low histopathological grade ( $P=0.004$ ), HPV positivity ( $P=0.032$ ), and long-term survival ( $P=0.045$ ). High Ki-67 expression was negatively correlated with histopathological grade ( $P<0.001$ ) and tumour size ( $P=0.047$ ). There was an association between HPV positivity and low histopathological grade, but not between HPV positivity and survival.

**Conclusion:** High p16<sup>INK4A</sup> expression was associated with long-term survival, but the only independent predictors for survival were tumour size and histopathological grade. Our results indicate that p16<sup>INK4A</sup> and Ki-67 expression might be useful in tumour grading, and that it might be possible to use p16<sup>INK4A</sup> expression as a marker for HPV positivity, but this has to be further elucidated.

Primary carcinoma of the vagina (PCV) is a rare malignancy, comprising only 1–2% of the malignancies of the female genital tract (Beller *et al*, 2006). The most prevalent histological type of PCV is squamous cell carcinoma (SCC). Primary carcinoma of the vagina mostly affects women over 60 years of age and has a poor prognosis (Beller *et al*, 2006; Hellman *et al*, 2006). Due to its rarity, biological and prognostic factors of PCV have not been frequently studied, in contrast to other malignancies of the female genital tract.

The importance of human papillomavirus (HPV) and its oncogenic potential in the female genital tract, as well as in the oropharynx, has received considerable attention in the last decades. In accordance with other SCCs of the genital tract, such as cervical, vulvar, and anogenital carcinoma, the majority of PCV is associated with high-risk HPV types, with a reported range of 51.4–81% (Insinga *et al*, 2008; De Vuyst *et al*, 2009; Smith *et al*, 2009; Fuste *et al*, 2010; Brunner *et al*, 2011; Alonso *et al*, 2012). Two etiopathogenic pathways have been postulated for PCV: one

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related to high-risk HPV positive, and the other to high-risk HPV-negative PCV (Koyamatsu *et al*, 2003; Hellman *et al*, 2004; Fuste *et al*, 2010; Alonso *et al*, 2012). Knowledge of HPV status is important in PCV because of its suggested clinical and prognostic significance (Brunner *et al*, 2011; Alonso *et al*, 2012), but HPV status is also important in understanding the aetiology of PCV.

When HPV integrates in the host cell genome in squamous cells, the result is overexpression of the viral oncogenic proteins E6 and E7, which interact with a number of specific cellular proteins to initiate neoplastic transformation. The E7 protein binds to the tumour suppressor protein, retinoblastoma gene product (pRB), leading to upregulation of CDKs, degradation of pRB, and enhanced expression of p16<sup>INK4A</sup>. Thus, an oncogenic HPV infection results in an accumulation of p16<sup>INK4A</sup> in the cell's nucleus and cytoplasm (Klaes *et al*, 2001). p16<sup>INK4A</sup> expression measured by immunohistochemistry may be useful in clinical application as an indicator of degraded pRB caused by overexpression of the E7 protein. Studies in other SCCs have shown that p16<sup>INK4A</sup> expression measured by immunohistochemistry correlates well with HPV positivity (Mellin Dahlstrand *et al*, 2005; Santos *et al*, 2006; Reimers *et al*, 2007; Fuste *et al*, 2010), making it a useful biomarker for HPV-related oncogenic activity and malignant transformation in cervix, vulva, and vagina (Klaes *et al*, 2001; O'Neill and McCluggage, 2006; Fuste *et al*, 2010). In the cervix p16<sup>INK4A</sup> has proven as useful in identifying dysplasia, and correlates with the severity of dysplasia (Norman *et al*, 2007). In the cervix and other sites, it has also been demonstrated to be of prognostic significance (Masoudi *et al*, 2006; Reimers *et al*, 2007; Schwarz *et al*, 2012).

Ki-67 is a proliferation antigen, which is expressed in the nuclei of growing cells. It is used to assess the proliferation index of a cell population and can be used for grading dysplasia in cervical biopsies (Baak and Kruse, 2005). MIB-1 staining is reduced in atrophy and increased in dysplasia (Mittal *et al*, 1999), making it useful in histopathologically uncertain cases as it can help distinguish between postmenopausal changes and dysplasia. It has been suggested that higher MIB-1 immunostaining intensity is an indicator of increased proliferation activity, which is associated with unfavourable clinical outcome, increased tumour size, and more advanced stage of cancer (Heatley 1998; Kruse *et al*, 2003). In cervical adenocarcinoma, increased Ki-67 expression was seen in tumours of lower grade and tumours with a higher stage at diagnosis, and correlated with a worse prognosis (Muller *et al*, 2008). It was observed that Ki-67 expression had a similar prognostic value in vulvar carcinoma (Hantschmann *et al*, 2000). Increased Ki-67 expression in PCV has previously been demonstrated, but has not been associated with survival (Koyamatsu *et al*, 2003; Habermann *et al*, 2004; Hellman *et al*, 2013).

Many prognostic factors for PCV have been studied, including tumour stage, tumour size, and women's age (Habermann *et al*, 2004; Hellman *et al*, 2006; Brunner *et al*, 2011; Jang *et al*, 2012). Human papillomavirus-positive PCV has a better prognosis than HPV-negative PCV in early stages (Alonso *et al*, 2012). p16<sup>INK4A</sup> expression measured by immunohistochemistry may be used to determine the HPV status of PCV (Fuste *et al*, 2010; Alonso *et al*, 2012). Prognostic factors and biomarkers of PCV need to be further elucidated to improve diagnostic tools and treatment. The aim of this study is to determine HPV status and to investigate p16<sup>INK4A</sup> and Ki-67 expression and their correlation with clinical parameters and survival in patients with PCV.

## MATERIALS AND METHODS

**Patients and tumour biopsies.** This study is based on a population of 130 consecutive patients who were diagnosed with

PCV, and subsequently treated at Karolinska University Hospital between 1978 and 1995. To get two groups with a significant difference in survival, patients with PCV were divided into short-term survivors (dying within  $\leq 2$  years of diagnosis) and long-term survivors (surviving  $\geq 8$  years after diagnosis). Of the 130 consecutive patients, 77 fell into one of the two defined groups. Archived tumour biopsies from these 77 patients were examined at the Department of Pathology, Karolinska University Hospital. All tumour biopsies were fixed in buffered formaldehyde, paraffin-embedded and diagnosed on haematoxylin and eosin-stained tissue sections.

For the present study, four sections from each archived tumour biopsy were prepared and used for histological diagnosis and immunohistochemistry (thickness: 4  $\mu$ m). Sections for haematoxylin and eosin staining were prepared before and after each section to confirm tumour representativity. Primary carcinoma of the vagina diagnoses, as well as the representativity of the sections used for immunohistochemical studies, were reviewed and confirmed by two pathologists (C Silfversward and E Wilander) at the Department of Pathology, Karolinska University Hospital, and the Departments of Genetics and Pathology, and Clinical Pathology and Cytology, Rudbeck Laboratory, Uppsala University Hospital and Uppsala University, Uppsala. The histopathologist reviewed and confirmed the histopathological grade and the International Federation of Gynecology and Obstetrics (FIGO) stage before immunohistochemistry was performed. Histopathological evaluation was done according to the World Health Organization classification 1975, no 13, and staging was performed according to the FIGO staging system (accepted for PCV in 1963), based on the original clinical records and new histopathological results.

Nine tumour biopsies were excluded due to poor material with necrosis. Thus, 68 patients with PCV, 39 short-term and 29 long-term survivors, were included in the final analyses. Sixty-three tumour biopsies were classified as SCC, two as adenocarcinoma, and three as small cell carcinoma (Table 1). The study protocol was accepted by the ethical committee of the Stockholm County Council (Dnr 01-194). Patient permission for use of archived tumour biopsies was not required.

Fifty-one patients received a combination of external beam irradiation and brachytherapy, eleven patients received only brachytherapy, and six patients were treated with surgery and/or chemotherapy alone. The medium external dose was 40 Gy (range 19–60 Gy) and the medium dose to the vaginal tumour was 57 Gy (range 14–100 Gy). Patients were followed up at 4-month intervals for 2 years and at 6-month intervals thereafter for an additional 3 years. After 5 years, patients were referred to a general gynaecologist for annual follow-up. All patients included in the study had a reliable documented follow-up, with a minimum of 8 years for the long-term survivors (Table 2).

Recurrence was defined as disease reappearance 3 months or more after completion of primary treatment in a patient considered to be in complete remission. Evidence of disease  $< 3$  months after completion of treatment was defined as persistent disease (Table 2).

**HPV status.** Briefly, analyses were performed on extracted DNA obtained from a 10- $\mu$ m thick section of paraffin blocks, the preceding section of which had been used for morphological diagnosis. These sections of archived tumour biopsies were dewaxed with xylene-ethanol. DNA was extracted by a MagNA Pure LC Robot (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

**HPV detection and typing.** The quality of DNA samples was analysed using a  $\beta$ -globin real-time PCR using 1  $\mu$ l of the sample. All samples that we included for future analysis were  $\beta$ -globin positive. Human papillomavirus testing was performed by PCR

Table 1. Patient and tumour characteristics	
Patients included in the study	68
<b>Age at diagnosis (years)</b>	
Median	69
Mean	68
<b>History of CIN</b>	
Yes	21
No	47
<b>Previous gynaecological malignancy</b>	
Yes	11
No	57
<b>Hysterectomy before diagnosis</b>	
Yes	24
No	44
<b>Histology</b>	
Squamous cell carcinoma	63
Adenocarcinoma	2
Small cell carcinoma	3
<b>Histopathological grade (n = 67)</b>	
Well differentiated	10
Moderately differentiated	34
Poorly differentiated	23
<b>FIGO stage</b>	
I	34
II	13
III	11
IV	10
<b>Tumour size</b>	
<4 cm	29
4–8 cm	27
>8 cm	12
<b>Tumour localisation</b>	
Upper third	36
Lower third	15
All other locations	17
<b>Growth pattern (n = 59)</b>	
Exophytic	20
Ulcerating	31
Endophytic	8
<b>Regional metastasis (inguinal node metastasis)</b>	
Yes	4
No	64
<b>Distant metastasis</b>	
Yes	5
No	63
Abbreviations: CIN = cervical intraepithelial neoplasia; FIGO = International Federation of Gynecology and Obstetrics.	

amplification of a fragment in the L1 gene. Samples were tested for the presence of HPV DNA by amplifying 1 µl of DNA with the MGP primer system as previously described (Soderlund-Strand

Table 2. Follow-up data for 68 patients with PCV	
<b>Relapse/persistent disease</b>	
Yes	40
No	28
<b>Follow-up time (days)</b>	
Median	89
Mean	2808
<b>Short- vs long-term survival</b>	
≤2 years	39
≥8 years	29
<b>Vital status at last follow-up</b>	
Alive	8
Dead of PCV	38
Dead of other disease	21
Alive with relapse	1
<b>Disease specific survival</b>	
Dead of PCV	38
All other	30
<b>Overall survival</b>	
Dead	59
Alive	9
Abbreviation: PCV = primary carcinoma of the vagina.	

et al, 2009). The Bioplex 200 Luminex system (Bio-Rad, Hercules, CA, USA) was used for HPV detection and genotyping using multiplex bead-based hybridisation with Luminex technology as described by Schmitt et al (2006). Briefly, 10 ml of the biotinylated MGP-PCR product was mixed with beads coupled with different HPV probes. After 10 min of denaturation at 95 °C, the samples were hybridised at 41 °C for 30 min. After washing, streptavidin-R-phycoerythrin was incubated with the samples for 30 min at room temperature. One hundred beads of each HPV type from each sample were analysed using the Luminex system. Probes for 14 oncogenic, high-risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68a, and 68b, including probes for variant sequences of HPV18, 35, 51, and 58) and 22 non-oncogenic types including low-risk and possible high-risk types (6, 11, 26, 30, 40, 42, 43, 53, 54, 61, 67, 70, 73, 74, 81, 82, 83, 86, 87, 89, 90, and 91) were used. Eleven negative controls (H<sub>2</sub>O) and eight positive controls (HPV plasmid pools) were included in each test.

**Immunohistochemistry for p16<sup>INK4A</sup> and MIB-1.** Immunohistochemical staining was performed with the CINtec Histology Kit (Code No. K5336; DAKO Cytomation, Glostrup, Denmark) according to the manufacturer’s recommendations using the Dako Autostainer. This kit contains Tris EDTA buffer (×10) pH 9, intended for epitope retrieval. A Coplin jar filled with epitope retrieval buffer, diluted 1:10 with distilled water, was placed in a water bath and heated to 95–99 °C. Deparaffinised sections were then incubated for 10 min while maintaining a temperature of 95–99 °C. Jars with slides were removed from the water bath and left to cool at room temperature for 20 min, followed by washing for 5 min in Wash Buffer (Code No. S3006; DAKO Cytomation) diluted 1:10 with distilled water. The automated procedure began with 1 × rinse (1 × rinse equals 4 min) in wash buffer diluted 1:10 with distilled water (Wash Buffer (×10), Code No. S3006; DAKO Cytomation). Endogenous peroxidase activity was abolished by incubating slides for 5 min in a peroxidase-blocking

solution (Dako REAL, Code No. S2023; DAKO Cytomation). Slides were rinsed  $1 \times$ , after which 200  $\mu$ l of the primary antibody against the p16<sup>INK4a</sup> protein clone E6H4 was dropped onto each slide, followed by incubation for 30 min. Slides were rinsed  $1 \times$ . Reaction products were visualised by incubating slides for 30 min with a visualisation reagent (a horseradish peroxidase/goat-anti-mouse immunoglobulin-labelled dextran polymer) and, after  $2 \times$  rinses, incubated for 10 min in a 1:40 solution of DAB chromogen (3,3'-diaminobenzidine) in DAB buffered substrate, also from the CINTec Histology Kit. Slides were then washed in distilled water for 1 min and counterstained for 2 min in Harris Hematoxylin solution diluted 1:2 with distilled water. After 2 min of washing in water, slides were dehydrated in ethanol to xylene and mounted in a water-free permanent mounting medium with mounting glass. Tissue sections containing cervical cancer were used as positive controls for p16<sup>INK4a</sup>, while negative controls consisted of incubated doublet slides in the negative control reagent contained in the kit, instead of primary antibodies.

Immunostaining was independently evaluated by two observers (C Flores-Staino and E Wilander) and was considered as positive for p16<sup>INK4a</sup> when both observers agreed that the nuclei were clearly stained. In addition, cells with a distinct cytoplasmic immunoreaction were scored as positive. Image analysis was carried out as previously described. Immunohistochemistry results were scored based on both staining intensity and percentage of immunoreactive epithelial cells (Sano *et al*, 1998; Klusmann *et al*, 2003). Scoring criteria for p16<sup>INK4a</sup> were no expression (negative); weak expression (<30% positive cells); moderate expression (31–50% positive cells); and strong expression (>50% positive cells). Samples scored as moderate or higher were considered as positive for p16<sup>INK4a</sup> (Sano *et al*, 1998; Klusmann *et al*, 2003).

To detect the Ki-67 antigen, we used the monoclonal mouse antibody (clone MIB-1) (Code No. M7240; DAKO Cytomation). The Ki-67 antigen is a marker for mitotically active cells and is expressed in the nuclei of growing cells. Tumour biopsy sections were deparaffinised, rehydrated, and microwave treated in target retrieval solution diluted 1:10 with distilled water (Dako REAL Target Retrieval Solution ( $\times 10$ ) Code No. S2023; DAKO Cytomation) for  $2 \times 5$  min at 500 W. Thereafter, slides were subjected to the autostainer procedure and treated together with the p16<sup>INK4a</sup> slides as described above. Ki-67 positivity was scored with respect to nuclear staining and with attention to heterogeneity in distribution as follows: <10% positive cells; 10–50% positive cells; and >50% positive cells.

**Statistical analysis.** Associations between ordinal variables were tested using the Chi-square test or the Fisher's exact test. When investigating possible correlations between immunohistochemical staining and clinical parameters, the semi-quantitative groups described above were analysed with Spearman's rank correlation coefficients.

In the multivariate analyses, the median value was used to generate two groups of equal size for the immunohistochemical staining of Ki-67, with a cutoff at 50% positive cells. All parameters that showed a significant difference when comparing short- and long-term survivors were then included in a multivariate logistic regression model to evaluate the independence of each factor. The median age in different groups was compared using the Mann-Whitney test.

The covariates used in the statistical analysis are described in Table 1.

## RESULTS

**Immunohistochemical expression of p16<sup>INK4A</sup> in relation to clinical parameters and HPV status.** Seven patients with PCV

(10.3%) had no p16<sup>INK4A</sup> expression, eight (11.7%) had weak expression, sixteen (23.5%) had moderate expression, and thirty-seven (54.4%) had strong expression. A significant correlation was detected between p16<sup>INK4A</sup> expression and histopathological grade, HPV status, and survival (Table 3). Increasing p16<sup>INK4A</sup> expression correlated significantly with moderately and poorly differentiated tumours ( $P=0.004$ ). Furthermore, higher p16<sup>INK4A</sup> expression correlated significantly with long-term survival ( $P=0.045$ , Spearman correlation  $-0.24$ ). Patients with moderate/high expression of p16<sup>INK4A</sup> had better survival if the tumours were HPV negative compared with HPV-positive tumours ( $P=0.028$ ), data not shown.

All patients with regional or distant metastasis had moderate or strong p16<sup>INK4A</sup> expression; however, this was not significant due

**Table 3.** p16<sup>INK4A</sup> expression in correlation with clinical parameters and HPV status in 68 patients with PCV

Clinical parameters	Expression of p16		P-value
	None/weak N (%)	Moderate/strong N (%)	
Age at diagnosis, year (range)	75 (60–84)	66 (32–89)	0.084
<b>Histopathological grade (n = 67)</b>			
Well	6 (60)	4 (40)	0.004
Moderate	5 (15)	29 (85)	
Poor	3 (13)	20 (87)	
<b>Tumour size</b>			
< 4 cm	5 (17)	24 (83)	0.20
4–8 cm	5 (19)	22 (81)	
> 8 cm	5 (42)	7 (58)	
<b>FIGO stage</b>			
I–II	13 (28)	34 (72)	0.12
III–IV	2 (10)	19 (90)	
<b>Local metastasis</b>			
Yes	3 (50)	3 (50)	0.12
No	12 (19)	50 (81)	
<b>Regional metastasis</b>			
Yes	0 (0)	4 (100)	0.27
No	15 (23)	49 (77)	
<b>Distant metastasis</b>			
Yes	0 (0)	5 (100)	0.28
No	15 (24)	48 (76)	
<b>HPV status (n = 44)</b>			
Positive	1 (5)	18 (95)	0.032
Negative	8 (32)	17 (68)	
<b>Short- vs long-term survival</b>			
$\leq 2$ years	12 (31)	27 (69)	0.045
$\geq 8$ years	3 (10)	26 (90)	
<b>Relapse</b>			
Yes	5 (28)	13 (72)	0.50
No	10 (20)	40 (80)	

Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus; PCV = primary carcinoma of the vagina.

to the small number of patients that had metastasis. Patients with strong p16<sup>INK4A</sup> expression were younger than those with weak p16<sup>INK4A</sup> expression, although this difference was not significant. There was no correlation between relapse and the expression of p16.

**Immunohistochemical expression of Ki-67 in relation to clinical parameters.** Seven patients with PCV (10.3%) had <10% Ki-67-positive cells, 38 (55.9%) had 10–50% positive cells, and 23 (33.8%) had >50% positive cells. A significant correlation between Ki-67 expression and histopathological grade ( $P < 0.001$ ) and tumour size ( $P = 0.047$ ) was found (Table 4). However, there was no correlation detected between Ki-67 expression and long- and short-term survival or relapse.

**HPV types and status in correlation with clinical parameters and expression of p16<sup>INK4A</sup> and Ki-67.** Forty-four out of sixty-eight patients with PCV could be evaluated for HPV status. Nineteen (43%) were positive for high-risk HPV and twenty-five (57%) were HPV negative (Table 5). The majority (12 out of 19, 63%) of HPV-positive patients were positive for HPV16. The others were positive for HPV45 (3 patients, 16%), HPV18

(1 patient, 5.3%), HPV35 (1 patient), HPV56 (1 patient), and HPV68 (1 patient). Human papillomavirus positivity was significantly correlated with strong p16<sup>INK4A</sup> expression ( $P = 0.032$ ). One HPV16-positive patient was negative for p16<sup>INK4A</sup> expression. All the other HPV-positive patients with PCV (18 out of 19, 95%) showed either moderate or strong p16<sup>INK4A</sup> expression. The majority of HPV16-positive patients with PCV showed strong p16<sup>INK4A</sup> expression, while those infected with other HPV types showed either moderate or strong p16<sup>INK4A</sup> expression. In all, 3 out of the 25 HPV-negative patients (12%) were negative for p16<sup>INK4A</sup> immunostaining, while the remaining 88% showed varying expression: 17 out of 25 (68%) showed moderate or strong p16<sup>INK4A</sup> expression.

All HPV-positive patients with PCV showed moderate to strong proliferation activity; however, there was no significant correlation between Ki-67 expression and HPV status (Table 4).

The mean age of HPV-positive and HPV-negative patients was 72 years (range 51–89) and 68 years (range 32–84), respectively (Table 6). Hysterectomy was reported by 8 out of 19 HPV-positive patients (42%), and 9 out of 25 (36%) HPV-negative patients. There was no difference in HPV positivity among women with a history of CIN (seven HPV positive and nine HPV negative) or hysterectomy (eight HPV positive and nine HPV negative). Seven HPV-positive patients (7 out of 19, 37%) underwent hysterectomy due to CIN 0–6 years before PCV diagnosis. Five HPV-negative patients with PCV (5 out of 25, 20%) underwent hysterectomy due to CIN 1–9 years before PCV diagnosis.

All of the HPV-positive patients with PCV had moderate to poor histopathological grade ( $P = 0.011$ ). The two adenocarcinomas were negative for HPV and had low expression of p16. Only one of the three small cell carcinomas was evaluable for HPV and that case was negative with moderate expression of p16. The other two small cell carcinomas showed high expression of p16. Human papillomavirus-positive patients were diagnosed at more advanced stages and the tumour was more commonly localised in the upper third of the vagina. The growth pattern was more often ulcerative in HPV-positive patients and exophytic in HPV-negative patients, but this difference was not statistically significant. No significant difference was seen in survival or relapse by HPV status.

**Survival analyses.** Reliable follow-up data were available for all 68 patients with PCV. In the univariate analysis, there was a significant correlation between higher p16<sup>INK4A</sup> expression and long-term survival (Table 4). Larger tumour size and higher FIGO stage correlated negatively with survival (Table 7). Locally advanced tumours with growth in the septum rectovaginale correlated significantly with short-term survival ( $P = 0.030$ ). In the multivariate analysis, the presence of local metastasis could not be included since there were no long-term survivors with local

Table 4. Ki-67 expression in correlation with clinical parameters and HPV status in 68 patients with PCV

Clinical parameters	Ki-67 expression			P-value
	<10% N (%)	10–50% N (%)	>50% N (%)	
Histopathological grade (n = 67)				<0.001
Well	5 (50)	4 (40)	1 (10)	
Moderate	1 (3)	23 (68)	10 (29)	
Poor	1 (4)	10 (43)	12 (52)	
Tumour size				0.047
<4 cm	3 (10)	16 (55)	10 (35)	
4–8 cm	3 (11)	11 (41)	13 (48)	
>8 cm	1 (8)	11 (92)	0 (0)	
FIGO stage				0.096
I–II	7 (15)	23 (49)	17 (36)	
III–IV	0 (0)	15 (71)	6 (29)	
Local metastasis				0.056
Yes	2 (33)	4 (67)	0 (0)	
No	5 (8)	34 (55)	23 (37)	
Regional metastasis				0.67
Yes	0 (0)	2 (50)	2 (50)	
No	7 (11)	36 (56)	21 (33)	
Distant metastasis				0.73
Yes	0 (0)	3 (60)	2 (40)	
No	7 (10)	35 (56)	21 (33)	
HPV status (n = 44)				0.18
Positive	0 (0)	12 (63)	7 (37)	
Negative	4 (16)	14 (56)	7 (28)	
Short- vs long-term survival				0.46
≤2 years	5 (13)	23 (59)	11 (28)	
≥8 years	2 (7)	15 (52)	12 (41)	
Relapse				0.50
Yes	2 (11)	8 (44)	8 (44)	
No	5 (10)	30 (60)	15 (30)	

Abbreviations: FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus; PCV = primary carcinoma of the vagina.

Table 5. p16<sup>INK4A</sup> expression in relation to HPV status and different HPV types

p16 <sup>INK4A</sup> expression	HPV negative N (%)	HPV positive N (%)	
		HPV16	Other HPV types (18, 35, 45, 56, 68)
None	3 (12)	1 (5.3)	
Weak (>30%)	5 (20)		
Moderate (30–50%)	7 (28)	3 (16)	3 (16)
Strong (>50%)	10 (40)	8 (42)	4 (21)
Total	25 (100)	19 (100)	

Abbreviation: HPV = human papillomavirus.

**Table 6.** HPV status in relation to patient and tumour characteristics (n = 44)

Patient and tumour characteristics	HPV positive N (%)	HPV negative N (%)	P-value
Mean age at diagnosis (years)	73 (range 51–89)	68 range (32–84)	
<b>History of CIN</b>			
Yes	7 (44)	9 (56)	1.00
No	12 (43)	16 (57)	
<b>Previous gynaecological malignancy</b>			
Yes	3 (50)	3 (50)	1.00
No	16 (42)	22 (58)	
<b>Hysterectomy before diagnosis</b>			
Yes	8 (47)	9 (53)	0.76
No	11 (41)	16 (59)	
<b>Histopathological grade (n = 43)</b>			
Well	0 (0)	8 (100)	0.011
Moderate	13 (62)	8 (38)	
Poor	6 (43)	8 (57)	
<b>FIGO stage</b>			
I + II	11 (34)	21 (66)	0.054
III + IV	8 (67)	4 (33)	
<b>Tumour size</b>			
< 4 cm	6 (32)	13 (68)	0.40
4–8 cm	9 (53)	8 (47)	
≥ 8 cm	4 (50)	4 (50)	
<b>Tumour localisation</b>			
Upper third	13 (54)	11 (46)	0.14
Lower third	2 (18)	9 (82)	
All other locations	4 (44)	5 (56)	
<b>Growth pattern (n = 39)</b>			
Exophytic	5 (31)	11 (69)	0.11
Ulcerating	12 (63)	7 (37)	
Endophytic	1 (25)	3 (75)	
<b>Local metastasis</b>			
Yes	2 (50)	2 (50)	1.00
No	17 (43)	23 (57)	
<b>Regional metastasis</b>			
Yes	1 (50)	1 (50)	1.00
No	18 (43)	24 (57)	
<b>Distant metastasis</b>			
Yes	1 (50)	1 (50)	1.00
No	18 (43)	24 (57)	
<b>Short- vs long-term survival</b>			
≤ 2 years	13 (52)	12 (48)	0.18
≥ 8 years	6 (32)	13 (68)	
<b>Relapse</b>			
Yes	3 (25)	9 (75)	0.18
No	16 (50)	16 (50)	

Abbreviations: CIN = cervical intraepithelial neoplasia; FIGO = International Federation of Gynecology and Obstetrics; HPV = human papillomavirus.

**Table 7.** Correlation between clinical characteristics, human papillomavirus (HPV), and survival

	Survival		P-value
	Short term, ≤ 2 years N (%)	Long term, ≥ 8 years N (%)	
<b>Histopathological grade</b>			
Well	7 (70)	3 (30)	0.072
Moderate	23 (68)	11 (32)	
Poor	9 (39)	14 (61)	
<b>Tumour size</b>			
< 4 cm	5 (17)	24 (83)	< 0.001
4–8 cm	22 (81)	5 (19)	
> 8 cm	12 (100)	0 (0)	
<b>FIGO stage</b>			
I–II	23 (49)	24 (51)	0.036
III–IV	16 (76)	5 (24)	
<b>Local metastasis (tumour growth in septum rectovaginale)</b>			
Yes	6 (100)	0 (0)	0.030
No	33 (53)	29 (47)	
<b>Regional metastasis (inguinal node metastasis)</b>			
Yes	2 (50)	2 (50)	1.00
No	37 (58)	27 (42)	
<b>Distant metastasis</b>			
Yes	3 (60)	2 (40)	1.00
No	36 (57)	27 (43)	
<b>HPV status</b>			
Positive	13 (68)	6 (32)	0.18
Negative	12 (48)	13 (52)	

Abbreviation: FIGO = International Federation of Gynecology and Obstetrics.

metastasis. The FIGO stage was also excluded from the analysis since it did not add any additional influence after tumour size was included. Thus, the parameters included in the analysis were histopathological grade, tumour size, Ki-67 expression, and p16<sup>INK4A</sup> expression. Small tumour size and low histopathological grade remained statistically significantly associated with long-term survival, whereas Ki-67 expression and p16<sup>INK4A</sup> expression did not.

### DISCUSSION

In this study, HPV status and the expression of molecular markers p16<sup>INK4A</sup> and Ki-67 were evaluated as prognostic markers in 68 patients diagnosed with PCV. There are some limitations to our study that should be addressed. This study was retrospective and relied on archived tumour biopsies that were >10 years old. On the other hand, the study was based on one of the largest collections of biological material in the literature to-date on HPV and biological markers for this rare disease. Other studies addressing the prognostic value of HPV in PCV have included 35–69 patients (Brunner *et al*, 2011; Alonso *et al*, 2012; Larsson *et al* 2013), thus our study are among the larger ones.

Ki-67 expression correlated with histopathological grade and tumour size, and p16<sup>INK4A</sup> expression correlated with

histopathological grade, HPV status, and survival. Interestingly, both moderate and strong p16<sup>INK4A</sup> expression correlated with better survival, which has not been demonstrated previously. As in other HPV-related cancer sites, there was a correlation between strong p16<sup>INK4A</sup> expression and the presence of HPV DNA. It has previously been reported that HPV is a positive prognostic factor in PCV (Brunner *et al*, 2011; Alonso *et al*, 2012; Larsson *et al*, 2013); p16<sup>INK4A</sup> expression was investigated in one of these studies (Alonso *et al*, 2012) and was not a marker for survival. Thus, the role of p16<sup>INK4A</sup> expression as a marker for survival in PCV is still unclear. The inconsistent results might be explained by the limited number of patients included in both studies, and also by the different methods used to evaluate p16<sup>INK4A</sup> expression. In both studies, there was a clear correlation between p16<sup>INK4A</sup> expression and HPV status. In our study, HPV status could only be evaluated in 65% of the patients, which could be one reason why we found no prognostic value. Likewise, Brunner *et al* (2011) found that prognosis did not significantly differ between HPV-positive and HPV-negative tumours in the entire cohort; however, patients with unfavourable tumour stage and HPV positivity had improved disease-free and overall survival. In multivariate analysis, Alonso *et al* (2012) confirmed better disease-free and overall survival of HPV-positive patients independent of age and stage. This reduced risk of progression and mortality in HPV-positive patients was limited to patients with stage I and II tumours.

Human papillomavirus positivity was detected in 43% of the patients with PCV in our study, which is slightly lower compared with previously reported data from meta-analyses (Smith *et al*, 2009) and other studies (Ferreira *et al*, 2008; Insinga *et al*, 2008; De Vuyst *et al*, 2009; Fuste *et al*, 2010; Brunner *et al*, 2011; Alonso *et al*, 2012; Larsson *et al*, 2013), with a prevalence ranging between 51.4% and 81%. This variation is most likely due to differences in the detection methods used and in the selection of patients, but the geographical variation in HPV prevalence is another possibility. In the present study, HPV16 was the most prevalent type, as it was found in 63% of patients, which is in accordance with previous case series of patients with PCV (Fuste *et al*, 2010; Alonso *et al*, 2012; Larsson *et al*, 2013), as well as patients with cervical and vulvar cancer. Other HPV types found were HPV18, 35, 45, 56, and 68, which are all considered as high-risk types, and are also occasionally found in other HPV-related cancers.

Studies on the spectrum of HPV types present in PCV are of particular importance with regard to the introduction of HPV vaccines, to evaluate their future effect on different diseases, including PCV. Larsson *et al* (2013) showed that HPV16-positive patients with PCV had better survival than those infected with other HPV types. In a study of patients with cervical carcinoma treated with radiotherapy, patients infected with HPV16, or other types from the alpha-9 species, had a more favourable prognosis (Wang *et al*, 2010; Lai *et al*, 2013).

HPV16 is also the predominant type in oropharyngeal cancer. Human papillomavirus positivity has been shown to be a strong and independent prognostic factor of survival among patients with oropharyngeal cancer, and has been related to better response to chemoradiation (Lindquist *et al*, 2007; Ang *et al*, 2010; Marur *et al*, 2010; Gillison *et al*, 2012). This could be attributed to different pathways of p53 dysfunction in HPV-positive and HPV-negative tumours (Crook *et al*, 1991), but also to other cell cycle-related changes that could affect radiosensitivity (Pyeon *et al*, 2007). Therefore, determination of HPV status is now part of routine diagnostic evaluation when assigning prognoses for these malignancies (Gillison *et al*, 2012).

HPV18 is associated with less apoptosis than HPV16, which might result in increased radioresistance in HPV18-positive cervical tumours. A possible mechanism could be a difference in E6 oncoprotein activity (Arends *et al*, 1995; Hampson *et al*, 2001).

It has been demonstrated that oropharyngeal tumours with strong p16<sup>INK4A</sup> expression have a more favourable prognosis regardless of HPV status, which is why the authors suggested that p16<sup>INK4A</sup> immunohistochemistry alone is the best test to use for risk stratification and for predicting response to radiotherapy in this type of cancer (Lewis *et al*, 2010). p16<sup>INK4A</sup> expression has also been shown to predict improved survival after chemoradiation therapy for advanced-stage invasive cervical carcinoma (Schwarz *et al*, 2012). p16<sup>INK4A</sup> is strongly expressed in HPV-related vulvar intraepithelial neoplasia but p16<sup>INK4A</sup>-negative vulvar intraepithelial neoplasia is not associated with HPV infection. Similarly, HPV-positive invasive vulvar SCCs are p16<sup>INK4A</sup> positive, whereas the more common non-HPV-related neoplasms are largely negative, or focally positive (O'Neill and McCluggage, 2006). However, the prognostic and predictive value of p16<sup>INK4A</sup> expression has never been investigated in vulvar carcinoma. In the present study, we found that p16<sup>INK4A</sup> expression correlated with survival and also with HPV positivity. However, we observed that 17 out of 25 of the HPV-negative PCVs were p16<sup>INK4A</sup> positive and these also had better prognosis than the p16-positive/HPV-positive tumours. This fact that many of the tumours showed moderate/high expression of p16<sup>INK4A</sup> also were HPV negative might indicate that HPV-independent mechanisms also lead to overexpression of p16 in PCV, unless they are due to an undetected past or present infection. The p16 expression in HPV-negative tumours needs to be further investigated to get increased knowledge in the aetiology of PCV.

The prognostic value of Ki-67 expression has been evaluated previously in PCV, but none was revealed (Hellman *et al*, 2013). In this study, other cutoff values were used to investigate Ki-67 expression more in detail. As in the earlier studies (Habermann *et al*, 2004; Hellman *et al*, 2013), we observed high proliferative activity in nearly all patients with PCV, and a correlation with tumour size was observed. However, no prognostic value was found in the present study, leading us to conclude that Ki-67 expression might not contribute any prognostic information to cases of PCV. On the other hand, our results indicate that Ki-67 expression and p16<sup>INK4A</sup> expression might be useful in the histopathological grading of PCV, that is, high Ki-67 and p16<sup>INK4A</sup> expression would indicate a low-grade tumour. Similar results have been reported for cervical adenocarcinoma, where Ki-67 and p16<sup>INK4A</sup> expression may be a helpful marker in histopathological grading (Muller *et al*, 2008).

Furthermore, Ki-67 and/or p16<sup>INK4A</sup> expression have been found to be markers of progression for intraepithelial neoplastic lesions in cervix, vulva, vagina, and anus (Cameron *et al*, 2002; Norman *et al*, 2007). We observed a correlation between HPV positivity, lower histopathological grade, and high p16<sup>INK4A</sup> expression. In addition, p16<sup>INK4A</sup> expression correlated significantly with low histopathological grade. Likewise, Ki-67 expression was associated with low histopathological grade. Taken together, these findings might indicate that HPV-positive tumours have high proliferative activity, and therefore respond better to radiation treatment, leading to a better prognosis. We also observed that smaller tumours had higher proliferative activity (higher Ki-67 expression) than larger tumours, which might also lead to a better response to radiation therapy. On the other hand, different proliferative activity may exist in different parts of the tumour, and a small biopsy only reflects the proliferative activity in that specific part of the tumour. However, small tumours are more homogenous than large tumours, which have more genetic alterations. These alterations lead to more complex cellular dysfunctions (Heselmeyer *et al*, 1997), resulting in increased resistance to therapy and a worse prognosis.

Human papillomavirus-positive tumours are known to be associated with less genetically complex tumours that respond better to therapy and have improved outcomes (Schwarz *et al*,

2012). In the present study, almost all patients with PCV received radiation treatment, which is why it was difficult to compare the response to radiation treatment between HPV-positive and -negative patients in this study. But with regard to the findings in oropharyngeal, cervical, and recent vaginal cancer studies, the possibility to select patients for less aggressive therapies based on HPV status and p16<sup>INK4A</sup> expression should be evaluated in larger studies.

In previous studies, we have found different clinical features and expressions of molecular markers according to tumour location in the vagina (Hellman *et al*, 2004, 2006, 2013). Tumours located in the upper third of the vagina have a better prognosis, and are often associated with a history of CIN and younger age at diagnosis (Hellman *et al*, 2004, 2006). These findings have been correlated with HPV positivity (Fuste *et al*, 2010; Alonso *et al*, 2012; Larsson *et al*, 2013). Fuste *et al* (2010) demonstrated that all tumours in the upper third of the vagina were HPV positive (none of the HPV-negative tumours were located in the upper third of the vagina), and that HPV-positive tumours tend to affect younger women and women with a history of CIN. Alonso *et al* (2012) and Larsson *et al* (2013) found superior survival among patients with HPV-positive tumours. In the present study, HPV-positive tumours were more often located in the upper third of the vagina than HPV-negative tumours. However, we found no difference in age at diagnosis or history of CIN between HPV-positive and HPV-negative patients.

In a case series of advanced cervical carcinoma, Schwarz *et al* (2012) found an association between p16<sup>INK4A</sup>-negative patients with SCC increased age at presentation, and suggested that p16<sup>INK4A</sup> expression might be an age-related factor. This finding is consistent with the results from the head-and-neck literature, which document an increased incidence of HPV-related SCC with p16<sup>INK4A</sup> upregulation in younger patients with no other known carcinogen exposure, whereas HPV-negative tumours are more common in older patients with documented carcinogen exposure (Marur *et al*, 2010; Gillison *et al*, 2012). In our study, we also found that older patients had lower p16<sup>INK4A</sup> expression than younger patients. In addition, HPV-negative vaginal tumours are more often seen in older patients (Fuste *et al*, 2010; Alonso *et al*, 2012), but this could not be verified in our material.

Furthermore, HPV-positive PCV is more frequently of non-keratinising, basaloid, and warty type than HPV-negative PCV (Fuste *et al*, 2010). In addition, keratinising PCV occurs more frequently in older patients, whereas non-keratinising PCV more frequently affects younger women (Ferreira *et al*, 2008). It has been reported that keratinising PCV is less radiosensitive, and is associated with shorter overall survival (Kumar *et al*, 2009). Clearly, several factors are associated with age, and other pathogenetic mechanisms that may also influence response to therapy and outcome seem to operate at older age.

Genital HPV infections are sexually transmitted and in HPV-positive women the vaginal mucosa is exposed to higher concentrations of virus particles than the cervical canal (Gustavsson *et al*, 2009). Despite this, the oncogenic effects of HPV infection are more profound in the cervix compared with the vagina, since almost all cervical carcinomas are caused by HPV, whereas HPV is only found in around 50% of patients with PCV. The reason for this discrepancy is probably the existence of the transitional zone between the stratified squamous epithelium and cervical glandular epithelium. In this area, special target cells have been identified that are highly susceptible to malignant transformation due to HPV infection (Herfs *et al*, 2012). The vaginal mucosa, like vulvar mucosa and penis, lacks such vulnerable sites and for this reason they are probably more resistant to HPV-related SCC. In HPV-positive PCV, HPV16 seems to predominate to a higher degree than in cervical SCC. It is possible that the lack of glandular epithelium in the vagina contributes to this picture,

since HPV18 is more prevalent in the cervix, where a glandular transitional zone is present.

Obviously, other pathogenetic mechanisms are operating in the development of PCV. As PCV predominantly occurs in post-menopausal women, which is in direct contrast to cervical carcinoma where the majority of cases occur before 60 years of age, the response of these organs to the same carcinogenic stimulus (e.g., HPV) seems to vary with age, and the response rate of the cervix may be more rapid (probably due to the transformation zone) than the response rate of the vagina. There could also be an additional factor that occurs predominantly in the older age group, for example oestrogen deficiency, which makes the vaginal mucosa more responsive to carcinogenic stimuli, be it HPV or other carcinogenic agents. However, what these other carcinogenic agents might be is still relatively unknown. Irrespective of this, the vaginal squamous mucosa appears to be relatively resistant to malignant transformation in comparison with the squamous mucosa of other topographic sites.

In summary, there is now accumulating evidence that there are two types of PCV, like vulvar carcinoma, which seem to develop along different pathogenetic pathways and have different risk factor profiles: one subset that is HPV related, and one that is not. Studies have shown that HPV-positive tumours have a better prognosis, are diagnosed at a younger age, are associated with a history of CIN, and are often located in the upper third of the vagina. Histopathologically, HPV-positive tumours are mostly non-keratinising, and have strong p16<sup>INK4A</sup> expression. In contrast, HPV-negative PCV is often of the keratinising type, and occurs in older patients.

In conclusion, we have for the first time found that p16<sup>INK4A</sup> expression might be a prognostic marker in PCV. However, the only factors we found that could independently predict survival were tumour size and histopathological grade. There was no difference in survival by HPV status in this study population, but we found an association between HPV positivity and p16<sup>INK4A</sup> expression, and between p16<sup>INK4A</sup> expression, Ki-67 expression, and histopathological grade that might be useful in tumour grading. p16<sup>INK4A</sup> immunohistochemistry could be a marker for diagnosis of HPV-positive PCV and also a prognostic marker for therapeutic guidance. Further studies are needed to confirm these findings, and to evaluate p16<sup>INK4A</sup> and HPV status as markers for diagnosis, prognosis, and therapy. Furthermore, studies on the aetiology of HPV-negative PCV are warranted.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.



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