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Original Research Article

# Human Papillomavirus and p16 Expression in the Female Genital Tract and Its Value in Diagnosis

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## Abstract

***Introduction*:** The p16 gene is a tumor suppressor gene located at chromosome 9p21, that is a cyclin-dependent kinase inhibitor and is essential in regulating the cell cycle. In human papilloma virus (HPV) infection, the HPV oncogenes E6 and E7 can inactivate pRB and thus lead to p16 overexpression. ***Materials and Methods:*** This is prospective and descriptive study conducted in the Department of Pathology, IMS, BHU and HIMS over a period of 1 year from 1st January 2016 to 31st December 2016. Archival, formalin fixed tumour specimens from patients were retrieved from the department of pathology for immunohistochemical staining by means of an anti-p16 monoclonal antibody. In total, there were 90 patients. We evaluated p16 expression for its clinicopathological significance. ***Result:*** HPV types and status in correlation with clinical parameters and expression of p16. Eighty five out of 90 patients with primary carcinoma of the vagina (PCV) could be evaluated for HPV status. 26 were positive for high-risk HPV and 59 were HPV negative. The majority (17 out of 26, 65%) of HPV-positive patients were positive for HPV16. The others were positive for HPV45 (4 patients, 16.6%), HPV18 (2 patient, 8.3%), HPV35 (1 patient), HPV56 (1 patient), and HPV68 (1 patient). Human papillomavirus positivity was significantly correlated with strong p16 expression (p= 0.045). In all, 7 out of the 59 HPV-negative patients were negative for p16 immunostaining, while the remaining 83% showed varying expression: 39 out of 59 (60.9%) showed moderate or strong p16 expression. ***Conclusion:*** The vast majority of HPV positive vaginal cancers showed p16 overexpression, suggesting active involvement of HPV in the malignant transformation process. More in-depth studies are needed to understand the molecular carcinogenesis pathway in these p16-negative tumors and to improve outcomes for this population.

**Keywords:** Human papillomavirus, p16 expression, female genital tract.



## INTRODUCTION

The p16 gene is a tumor suppressor gene located at chromosome 9p21, that is a cyclin-dependent kinase inhibitor and is essential in regulating the cell cycle [1]. p16 inactivates cyclin-dependent kinases that phosphorylate retinoblastoma (Rb); therefore, p16 can decelerate the cell cycle. Rb phosphorylation status in turn influences expression of p16. In human papilloma virus (HPV) infection, the HPV oncogenes E6 and E7 can inactivate pRB and thus lead to p16 overexpression [2]. Therefore, p16 overexpression is a surrogate biomarker of HPV infection (in particular high-risk HPV types), which makes it useful in evaluating HPV-associated squamous and glandular neoplasia of the lower gynecologic tract [3]. The intensity and distribution of p16 is important in interpretation as well as in nuclear versus cytoplasmic localization. HPV-independent mechanisms of p16 overexpression also exist, so one may observe p16 expression in tumors that do not necessarily harbor HPV infection, such as ovarian serous carcinoma [4].

Functional loss of p16 has been reported for many human cancers [5]. Hypermethylation, rather than mutation or deletion, is the main cause of p16 dysfunction [6]. The p16-positive immunostain is an indirect marker for the presence of altered high-risk human papillomavirus (HPV)–induced growth cycle transformation. Human papillomavirus (HPV) is the most common sexually transmitted infection, posing an 85% lifetime risk of infection for sexually active

individuals [7]. The vast majority of HPV infections are transient and it is estimated that up to 90% of infections are cleared spontaneously within 2 years [8]. However, in some cases, persistent infection with high- risk HPV types can lead to development of precancerous and cancerous lesions in the genital tract. Several studies have suggested an etiologic role of high- risk HPV infection in a proportion of VaIN and VaSCC lesions. Better understanding of the association between HPV infection and female genital cancerous lesions could have important implications for diagnosis and prevention of these rare and elusive diseases.

There are only a few small series that have studied the expression of p16 in female genital tract [9]. The clinicopathological significance of p16 expression has not been reported in female genital tract. Our study aimed to evaluate the clinicopathological significance of p16 expression in the treatment of female genital tract. For a better analysis of prognosis in relation to treatment, we only recruited patients who m*et al*l certain criteria.

## MATERIALS AND METHODS

This is prospective and descriptive study conducted in the Department of Pathology, Heritage Institute of Medical Sciences and Institute of Medical Sciences, Banaras Hindu University, Varanasi over a period of 1 year from 1st January 2016 to 31st December 2016.

Archival, formalin fixed tumour specimens from patients were retrieved from the department of pathology for immunohistochemical staining done at IMS, BHU, Varanasi by means of an anti-p16 monoclonal antibody. In total, there were 90 patients. We evaluated p16 expression for its clinicopathological significance.

Immunohistochemical (IHC) staining was carried out by means of an avidin–biotin complex (ABC) immunoperoxidase method. We used similar protocols to those used previously for the study of p16 expression. The specimens dewaxed in xylene for 15 minutes, and rehydrated with ethanol. The slides were treated with 3% hydrogen peroxide for 30 minutes at room temperature. After three washes in phosphate buffered saline, antigen retrieval was performed by microwaving in citrate buffer (pH 6) for five minutes. We used a 1/50 dilution of the monoclonal anti-p16 antibody and incubation was carried out overnight at 4°C. The sections were then incubated with secondary antibody for 30 minutes. Staining was performed using ABC reagents and 3,3`-diaminobenzidine/hydrogen peroxide as substrate. The sections were counterstained with Mayer's haematoxylin for 30 seconds and blued in Scot's tap water for three minutes. The normal epithelium adjacent to the tumour nests served as an internal positive control. Squamous cell carcinomas known to be positive for p16 were used in each run of the experiment as external positive controls.

**HPV Status**

Briefly, analyses were performed in the Department of Microbiology, BHU, Varanasi. In extracted DNA obtained from a 10-mm 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 according to the manufacturer's instructions.

**HPV Detection and Typing**

The quality of DNA samples was analysed using a b-globin real-time PCR using 1 ml of the sample. All samples that we included for future analysis were b-globin positive. Human papillomavirus testing was performed by PCR amplification of a fragment in the L1 gene. Samples were tested for the presence of HPV DNA by amplifying 1 ml of DNA with the MGP primer system as previously described. The Bioplex 200 Luminex system 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 1C, the samples were hybridised at 41 1C for 30 min. After washing, streptavidin-Rphycoerythrin 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 (H2O) and eight positive controls (HPV plasmid pools) were included in each test.

Scoring criteria for p16INK4a were no expression (negative); weak expression (o30% positive cells); moderate expression (31–50% positive cells); and strong expression (450% positive cells).

## RESULTS

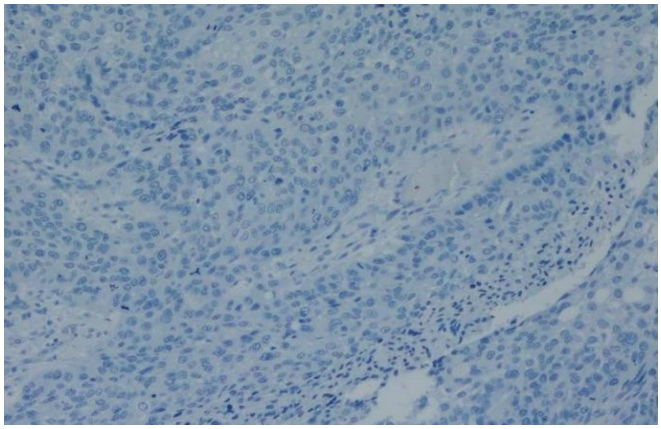

**Figure 1: Negative p16 staining in tumor cells (×10)**

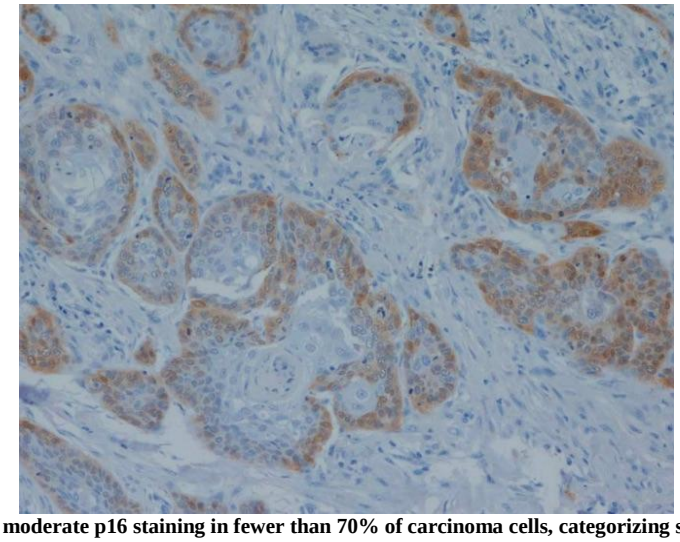

**Figure 2: Focally weak to moderate p16 staining in fewer than 70% of carcinoma cells, categorizing such cases as negative (×10)**

**Table-1: Patient and tumour characteristics**

| Parameters | Frequency | Percentage |
|---|---|---|
| **Histology** | | |
| Squamous cell carcinoma | 81 | 90 |
| Adenocarcinoma | 4 | 4.4 |
| Small cell carcinoma | 5 | 5.5 |
| **Histopathological grade** | | |
| Well differentiated | 11 | 12.2 |
| Moderately differentiated | 46 | 51.1 |
| Poorly differentiated | 33 | 36.6 |
| **FIGO stage** | | |
| I | 41 | 45.5 |
| II | 19 | 21.1 |
| III | 17 | 18.8 |
| IV | 13 | 14.4 |
| **Tumour size** | | |
| <4 cm | 39 | 43.3 |
| 4–8 cm | 34 | 37.7 |
| >48 cm | 17 | 18.8 |
| **Tumour localisation** | | |
| Upper third | 48 | 53.3 |
| Lower third | 19 | 21.1 |
| All other locations | 23 | 25.5 |

**Table-2: Characteristics of tumour**

| Growth pattern | Frequency | Percentage |
|---|---|---|
| Exophytic | 31 | 34.4 |
| Ulcerating | 48 | 53.3 |
| Endophytic | 11 | 12.2 |
| **Regional metastasis (inguinal node metastasis)** | | |
| Yes | 8 | 8.8 |
| No | 82 | 91.1 |
| **Distant metastasis** | | |
| Yes | 9 | 10 |
| No | 81 | 90 |

**Table-3: p16 expression in relation to HPV status and different HPV types**

| | | HPV positive N (%) | |
|---|---|---|---|
| **p16 expression** | **HPV negative N (%)** | **HPV16** | **Other HPV types (18, 35, 45, 56, 68** |
| None | 9 (15.2) | 3 (11.5) | |
| Weak (430%) | 11 (18.6) | | |
| Moderate (30–50%) | 17 (28.8) | 4 (15.3) | 4 (15.3) |
| Strong (450%) | 22 (37.2) | 10 (38.4) | 5 (19.2) |
| Total | 59 (100) | 26 (100) | |

In In table 3, HPV types and status in correlation with clinical parameters and expression of p16. Eighty five out of 90 patients with PCV could be evaluated for HPV status. 26 were positive for high-risk HPV and 59 were HPV negative. The majority (17 out of 26, 65%) of HPV-positive patients were positive for HPV16. The others were positive for HPV45 (4 patients, 16.6%), HPV18 (2 patient, 8.3%), HPV35 (1 patient), HPV56 (1 patient), and HPV68 (1 patient). Human papillomavirus positivity was significantly correlated with strong p16 expression (p= 0.045). In all, 7 out of the 59 HPV-negative patients were negative for p16 immunostaining, while the remaining 83% showed varying expression: 39 out of 59 (60.9%) showed moderate or strong p16 expression.

## DISCUSSION

In this study, HPV status and the expression of molecular markers p16 was evaluated as prognostic markers in 90 patients diagnosed with PCV. The p16 expression correlated with histopathological grade, HPV status, and survival. Interestingly, both moderate and strong p16 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 expression and the presence of HPV DNA. It has previously been reported that HPV is a positive prognostic factor in PCV. 10 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 expression. In both studies, there was a clear correlation between p16 expression and HPV status [11].

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 [12]. Likewise, Brunner *et al* 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 patient's 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 [13].

Human papillomavirus positivity was detected in 65% of the patients with PCV in our study, which is slightly lower compared with previously reported data from meta-analyses and other studies, with a prevalence ranging between 51.4% and 81% [14,15]. 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 65% of patients, which is in accordance with previous case series of patients with PCV, as well as patients with cervical and vulvar cancer [16]. 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.

It has been demonstrated that tumours with strong p16 expression have a more favourable prognosis regardless of HPV status, which is why the authors suggested that p16 immunohistochemistry alone is the best test to use for risk stratification and for predicting response to radiotherapy in this type of cancer [17]. p16 expression has also been shown to predict improved survival after chemoradiation therapy

for advanced-stage invasive cervical carcinoma [18]. p16 is strongly expressed in HPV-related vulvar intraepithelial neoplasia but p16-negative vulvar intraepithelial neoplasia is not associated with HPV infection. Similarly, HPV positive invasive vulvar SCCs are p16 positive, whereas the more common non-HPV-related neoplasms are largely negative, or focally positive [19]. However, the prognostic and predictive value of p16 expression has never been investigated in vulvar carcinoma.

In the present study, we found that p16 expression correlated with survival and also with HPV positivity. However, we observed that 17 out of 25 of the HPV-negative PCVs were p16 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 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.

Our results are limited by some variation between studies in the definition of p16 positivity, however, this did not seem to heavily influence the reported overall p16 positivity in the individual studies, which ranged from 63% to 87%. Double positivity for HPV and p16 has been shown to be a stronger prognostic marker than either HPV or p16 alone in specific histological groups in head and neck cancer. To our knowledge, this has not been evaluated in vaginal cancer, however, this may become helpful for clinicians in order to plan treatment and follow-up for these patients.

## CONCLUSION

The vast majority of HPV positive vaginal cancers showed p16 overexpression, suggesting active involvement of HPV in the malignant transformation process. HPV vaccines will help prevent some of the primary female genital cancers associated with HPV type 16. More in-depth studies are needed to understand the molecular carcinogenesis pathway in these p16-negative tumors and to improve outcomes for this population.

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