

The Relationships between p16^{INK4a} Immunohistochemistry Expression with Histopathological Subtypes in Cervical Adenocarcinoma

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ABSTRACT

Background

Cervical adenocarcinoma is one of the most common types of epithelial cancer in the cervix after squamous cell carcinoma. The link between human papillomavirus (HPV) and squamous cell carcinoma is well-established, with HPV detected in nearly all cases of cervical squamous cell carcinoma. However, HPV prevalence in the various morphological subtypes of cervical adenocarcinoma has not been thoroughly studied, although it generally shows a significant association. This study aims to determine HPV status across different subtypes of cervical adenocarcinoma, using p16^{INK4a} as a marker for oncogenic HPV infection. Positive p16 expression indicates the presence of HPV.

Methods

This research was analyzed by performing p16^{INK4a} immunohistochemical staining on 34 cervical adenocarcinoma samples. Assessment of p16^{INK4a} expression was made based on analysis of the percentage of positive tumor cells and staining intensity. Score 0: (no positive tumor cells). Score +1: ($\leq 10\%$ positive tumor cells). Score +2: (11–50% positive tumor cells). Score +3: ($> 50\%$ positive tumor cells). The color intensity of p16^{INK4a} is categorized as follows: Score 0: (negative). Score 1: (weak). Score 2: (strong).

Results

The most common age was 40-49 years, The majority had a parity of ≥ 2 deliveries (82.4%), FIGO clinical stage was unknown (70.6%), LVI was negatif (85.3%). The most common histopathological subtype shows among HPV-associated adenocarcinomas (58.8%) had strong positive p16^{INK4a} expression. For HPV-independent adenocarcinomas (14.7%) had strong positive expression.

Conclusion

No significant association was found between p16^{INK4a} expression with histopathological subtype in cervical adenocarcinoma.

Keywords: Endocervical carcinoma, cervical adenocarcinoma, p16^{INK4a} immunohistochemistry, histopathology subtypes



INTRODUCTION

Cervical cancer is the most common malignancy in women. In 2020, it ranked as the fourth most common cancer and the fourth leading cause of cancer-related death among women worldwide, with 604,000 new cases and 342,000 deaths.¹ In Indonesia, cervical cancer was the second most common cancer after breast cancer in 2020, with the highest mortality rate reaching 36,633 deaths (17.2%).¹

Cervical adenocarcinoma is one of the epithelial malignancies frequently observed in the cervix, following squamous cell carcinoma. It accounts for 10-25% of all cervical cancer cases. However, in recent decades, there has been a rise in adenocarcinoma cases compared to squamous cell carcinoma in developed countries.² According to the World Health Organization (WHO) 2020 histopathological classification, cervical adenocarcinoma can be categorized into HPV-associated and HPV-independent types.³

Cervical cancer is caused by oncogenic human papillomavirus (HPV) infection. E6 and E7 are the main oncoproteins of HPV. HPV types 16 and 18 are the main types causing carcinogenesis and are responsible for 70% of cervical cancers. Oncoprotein E6 inhibits apoptosis by blocking p53, and oncoprotein E7 inhibits cell cycle arrest by blocking pRb. This virus infects the cervix through sexual contact and often presents without symptoms, even when the infection has caused precancerous lesions.⁴

Cyclin-dependent kinase inhibitor 2A protein, also known as p16^{INK4a}, is a tumor suppressor gene involved in regulating the cell cycle, aging, apoptosis, cell invasion, and angiogenesis. Detection of p16^{INK4a} protein in precancerous lesions is expected to be used as a biomarker to assist in early detection of cancer and as a marker of oncogenic HPV infection. Oncoprotein E7 causes disruption in the function of pRb. E7 will alter the binding between pRb and E2F (elongation 2 factor), hence the protein p16^{INK4a}, which functions to regulate E2F, will accumulate in the cell and experience overexpression. Protein p16^{INK4a} is a tumor suppressor protein that prevents the release of E2F from pRb by inhibiting the activity of the cyclin D1-cdk4 and cdk6 complexes in

phosphorylating pRb. The accumulation of E2F, released from pRb, will induce p16^{INK4a} activity and lead to overexpression of p16^{INK4a}.⁵⁻⁹

Precancerous epithelial lesions of the uterine cervix and invasive carcinoma, especially squamous cell carcinoma (SCC) with high-risk HPV infection, have a clear association. Research by Castellsagué et al, states that high-risk HPV infection also increases the risk factor for developing cervical adenocarcinoma. Compared to squamous carcinoma, research on HPV in cervical adenocarcinoma is much less, but it is generally assumed that most cervical adenocarcinomas are HPV-associated, although the percentage is lower compared to squamous carcinoma. Houghton et al, reported that the percentage of cervical adenocarcinoma cases found is highly variable among studies. It is suspected that this may be due to a lower viral load in glandular neoplasms compared to squamous lesions.

Prognostic factors related to the survival rate in cervical cancer cases include age, tumor size, lymph node status, FIGO stage, and lymphatic or vascular invasion.¹⁰ Based on the above explanation, there has been limited research, especially in North Sumatra, to evaluate the relationship between p16^{INK4a} expression and the histopathological subtype of cervical adenocarcinoma. Therefore, this study is deemed necessary and could be used as a molecular marker to determine diagnostic and prognostic outcomes.

METHODS

This research is analytical study with a cross-sectional approach. The population of this study consisted of all secondary data and paraffin blocks from patients who were histopathologically diagnosed with cervical adenocarcinoma and meet with inclusion and exclusion criteria at Haji Adam Malik central Hospital, Medan, Prof. Dr. Chairuddin Panusunan Lubis Hospital, North Sumatra University, and Putri Bidadari Hospital Langkat. The sample numbers in this study was 34 samples. The variables in this study were immunohistochemical expression of p16^{INK4a} as the independent variable and age, parity, subtypes, as the dependent variable.



Assessment of p16^{INK4a} expression was made based on analysis of the percentage of positive tumor cells and staining intensity. Score 0: (no staining). Score 1+: ($\leq 10\%$ positive tumor cells). Score 2+: (11–50% positive tumor cells). Score 3+: ($> 50\%$ positive tumor cells). The color intensity of p16^{INK4a} is categorized as follows Score 0: (no staining), Score 1: (weak staining), Score 2: (moderate staining), Score 3: (strong). The Staining Index (SI) is calculated by multiplying the intensity & proportion scores. SI score 0=negative expression, 1-3= as a tumor with weak expression and score 4-9 = as a tumor with strong expression.¹¹

The p16^{INK4a} expression using mouse monoclonal antibody with a dilution of 1:100 (Mob575: Diagnostic BioSystems). The assessment was carried out semiquantitatively based on the intensity and percentage of expressed tumor cells. The expression of p16^{INK4a} is characterized by a brownish color in the nuclei and/or cytoplasm of tumor cells, which is assessed in 4 fields of view of microscopic preparations with magnifications of 40X, 100X, and 400x.

The collected data will be analyzed using the SPSS v 20 statistical program. Data analysis will be carried out using the Somers'd test. A p-value < 0.05 is declared statistically no significant.

RESULTS

From this study, the highest percentage of cervical adenocarcinoma patients were aged 40-49 years (32.4%). The youngest patient was 25 years old, and the oldest was 74 years old. Most patients had a history of parity with $\geq 2x$ partus, accounting for 28 cases (82.4%), while 6 cases (17.6%) had a parity of $< 2x$ partus.

The most commonly found FIGO clinical stage was FIGO N/A (data not listed in medical records), with 24 cases (70.6%). This was followed by FIGO stage IIIB with 5 cases (14.7%), FIGO stage IVA with 3 cases (8.8%), and FIGO stages IIB and IVB, each with 1 case (2.9%). Vascular invasion by tumor cells (LVI) was found to be negative in 29 cases (85.3%) and positive in 5 cases (14.7%).

In this study, the HPV-associated adenocarcinoma subtype was found in 24 cases (70.6%), with the most common subtype being papillary adenocarcinoma, observed in 14 cases (41.2%), followed by villoglandular adenocarcinoma in 6 cases (17.6%), and the least common being mucinous NOS adenocarcinoma in 4 cases (11.8%). Meanwhile, in the HPV-independent adenocarcinoma subtype, 10 cases were found, with the most common subtype being clear cell adenocarcinoma in 5 cases (14.7%), followed by mesonephric adenocarcinoma in 4 cases (11.8%) and gastric adenocarcinoma in 1 case (2.9%).

Immunohistochemical examination of cervical adenocarcinoma patients in this study revealed positive p16^{INK4a} immunohistochemical expression in 27 cases (79.4%), with strong positive expression in 25 cases (73.5%) and weak positive expression in 2 cases (5.9%). Negative immunohistochemical expression was found in 7 cases (20.6%). The characteristics of the study samples are shown in Table 1.

In this study, strong positive immunohistochemical expression of p16^{INK4a} in the most common cervical adenocarcinoma subtype was found in papillary adenocarcinoma with 12 cases (35.3%), followed by villoglandular adenocarcinoma and mucinous NOS adenocarcinoma with the same number, each with 4 cases (11.8%). Next, clear cell adenocarcinoma had 3 cases (8.8%), and mesonephric adenocarcinoma had 2 cases (5.9%).

Weak positive expression was found in clear cell adenocarcinoma and mesonephric adenocarcinoma, each with 1 case (2.9%). Negative expression was found in papillary adenocarcinoma and villoglandular adenocarcinoma, each with 2 cases (5.9%), as well as in gastric adenocarcinoma, clear cell adenocarcinoma, and mesonephric adenocarcinoma, each with 1 case (2.9%). The distribution frequency of immunohistochemical expression can be seen in Table 2.



Table 1. Frequency Distribution of Cervical Adenocarcinoma Patient Characteristics.

Variable	Amount (n)	Percentage (%)
Age (years old)		
<30	1	2.9
30-39	6	17.6
40-49	11	32.4
50-59	10	29.4
≥60	6	17.6
Parity History		
Parity amount < 2	6	17.6
Parity amount ≥ 2	28	82.4
Clinical Stage		
FIGO stage IA	0	0.0
FIGO stage IB	0	0.0
FIGO stage IIA	0	0.0
FIGO stage IIB	1	2.9
FIGO stage IIIA	0	0.0
FIGO stage IIIB	5	14.7
FIGO stage IIIC	0	0.0
FIGO stage IVA	3	8.8
FIGO stage IVB	1	2.9
FIGO stage N/A	24	70.6
LVI		
Positive	5	14.7
Negative	29	85.3
Subtype		
Adenocarcinoma, HPV-associated	24	70.6
Papillary adenocarcinoma	14	41.2
Villoglandular adenocarcinoma	6	17.6
Micropapillary Adenocarcinoma	0	0.0
Mucinous NOS adenocarcinoma	4	11.8
Intestinal Adenocarcinoma	0	0.0
Signet-ring cell adenocarcinoma	0	0.0
Stratified mucin- producing carcinoma	0	0.0
Adenocarcinoma HPV-independent	10	29.4
Gastric adenocarcinoma	1	2.9
Clear cell adenocarcinoma	5	14.7
Mesonephric adenocarcinoma	4	11.8
Immunohistochemical Expression		
Strong positive	25	73.5
Weak positive	2	5.9
Negative	7	20.6



Table 2. Frequency distribution of p16^{INK4a} immunohistochemical expression based on subtype in patients with cervical adenocarcinoma

Subtype	p16 ^{INK4a} Expression					
	Strong positive		Weak positive		Negative	
	n	%	n	%	n	%
Papillary adenocarcinoma	12	35.3	0	0.0	2	5.9
Villoglandular adenocarcinoma	4	11.8	0	0.0	2	5.9
Micropapillary Adenocarcinoma	0	0.0	0	0.0	0	0.0
Mucinous NOS adenocarcinoma	4	11.8	0	0.0	0	0.0
Intestinal Adenocarcinoma	0	0.0	0	0.0	0	0.0
Signet-ring cell adenocarcinoma	0	0.0	0	0.0	0	0.0
Stratified mucin-producing carcinoma	0	0.0	0	0.0	0	0.0
Gastric adenocarcinoma	0	0.0	0	0.0	1	2.9
Clear cell adenocarcinoma	3	8.8	1	2.9	1	2.9
Mesonephric adenocarcinoma	2	5.9	1	2.9	1	2.9
Total	25	73.5	2	5.9	7	20.6

From the table above, it was found that the subtype of HPV-associated adenocarcinoma with negative p16^{INK4a} expression had 4 cases (11.8%), while strong positive staining results were observed in 20 cases (58.8%). For HPV-independent adenocarcinoma, the most common results were strong positive staining in 5 cases (14.7%), weak positive staining in 2 cases (5.9%), and negative staining in 3 cases (8.8%). This data was statistically analyzed

using the Somers'd test, yielding a p-value of 0.097 (p-value > 0.05, which is not significant). Therefore, it can be concluded statistically that there is no significant relationship between cervical adenocarcinoma subtypes and p16^{INK4a} immunohistochemistry. The relationship between p16^{INK4a} immunohistochemical expression and histopathological subtypes of cervical adenocarcinoma can be seen in Table 3.

Table 3. The relationship between p16^{INK4a} immunohistochemical expression and histopathological subtypes of

Subtype	Strong Positive		Weak Positive		Negative		Total		Value	P Value
	n	%	n	%	n	%	n	%		
Adenocarcinoma, HPV-associated	20	58,8	0	0,0	4	11,8	24	70,6	-3,300	0,097
Adenocarcinoma HPV-independent	5	14,7	2	5,9	3	8,8	10	29,4		
Total	25	73,5	2	5,9	7	20,6	34	100,0		

cervical adenocarcinoma
Somers d' test



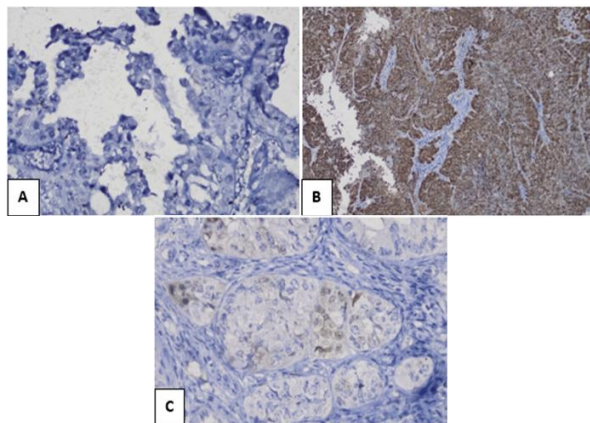


Figure 1. Image of p16^{INK4a} expression in cervical adenocarcinoma (original magnification 400 times) A. Negative B. weak expression. C. strong expression (H&E,400x).

DISCUSSION

In this study, the most common age group for cervical adenocarcinoma patients was 40-49 years, with 11 cases (32.4%). The youngest patient was 25 years old, and the oldest was 74 years old. This is consistent with Gao et al. (2013), which found the average age at diagnosis to be 49.2 years among 159 patients. The impact of age on cervical cancer survival remains unclear due to controversies in treatment outcomes related to age, compounded by factors such as age-related mortality risks, disease stage, treatment methods, and histological types of cancer.¹²

This study shows that parity of ≥ 2 was found in 28 cases (82.4%), while parity < 2 was found in 6 cases (17.6%). This aligns with Tekalegn et al. (2022), who link high parity with an increased risk of cervical cancer. This finding is supported by a multicenter case-control study by the International Agency for Research on Cancer (IARC), which reported that women who have never given birth (nulliparous) have a lower risk of cervical cancer, while the risk increases with the number of full-term pregnancies. Previous studies also show a positive relationship between parity and cervical cancer. This could be due to increased levels of estrogen and progesterone during pregnancy, which peak in the third trimester, potentially

causing changes in the transformation zone between squamous and columnar epithelium.¹³

Prognostic factors associated with the survival rate in cervical cancer incidence include age, tumor size, lymph node status, FIGO stage, and lymphatic or vascular invasion.¹⁴ This study's results show that FIGO stage IIIB was the most common, with 5 cases (14.7%), followed by stage IVA with 3 cases (8.8%), and stage IVB with 1 case (2.9%). This is consistent with the study by Ismail et al. (2023), which found that most patients were at advanced FIGO stages, with 22 cases (47.8%) in stage IIIB and 10 cases (21.7%) in stage IIB.¹⁵ However, it does not align with Glaze et al. (2019), which reported that nearly all patients were at early stages: 86.7% at stage I, 9.6% at stage II, and only 3.6% at stages III and IV.¹⁶ A limitation of this study was that 24 samples (70.6%) were not included in medical records, limiting the number of available samples for analysis.

The main treatment for cervical cancer is based on clinical, imaging, and pathological findings. However, there is no specific treatment based on histological type, genomic changes, or HPV status as outlined in current guidelines. Some studies report that patients with HPV-independent tumors have a worse prognosis compared to HPV-associated tumors. However, the clinical impact of HPV detection for determining treatment remains unclear. There is no prospective evidence evaluating the final outcomes for HPV-independent cervical cancer patients. Fernandes et al. (2022) state in their review that the first-line treatment proposed for early-stage or stage IA1 cervical cancer with lymphovascular invasion to IB2 and IIA1, according to FIGO 2018 is open radical hysterectomy with pelvic lymph node assessment. Additional treatment with chemoradiotherapy may be required based on pathological findings. For advanced stages or stages IB3, IIA2 to IVA, the standard treatment is concurrent platinum-based chemoradiotherapy, and for metastatic disease in stage IVB, platinum-based therapy with bevacizumab.¹⁷

The subtype of HPV-associated adenocarcinoma most frequently encountered in this case exhibited strong positive p16^{INK4a} expression in 20 cases (58.8%), with negative

expression found in 4 cases (11.8%). For HPV-independent adenocarcinoma subtypes, strong positive p16^{INK4a} expression was observed in 5 cases (14.7%), weak positive expression in 2 cases (5.9%), and negative expression in 3 cases (8.8%). A Somers'd test was performed on these variables, yielding a p-value of 0.097 (not significant), which aligns with the study by Houghton et al. (2010), where all usual types (HPV-associated) showed diffuse p16^{INK4a} immunoreactivity except for one case.⁸

Similarly, 11 out of 20 adenocarcinomas with unusual morphology (HPV-independent) showed immunoreactivity with 5 focal and 6 diffuse cases of p16^{INK4a}. Most cervical adenocarcinomas were p16^{INK4a} positive without HPV, indicating that diffuse p16^{INK4a} immunoreactivity is not a reliable surrogate marker for the presence of high-risk HPV in this neoplasm.⁸

It can be concluded that there is no strong correlation between p16^{INK4a} expression and the presence of oncogenic HPV in cervical adenocarcinoma. Overexpression of p16^{INK4a} in neoplastic cells occurs as an indirect phenomenon of the disrupted Rb functional pathway. In p16^{INK4a}-positive cervical adenocarcinomas without high-risk HPV presence, the Rb pathway dysfunction is due to mechanisms other than HPV infection.⁸

The most common subtype was papillary adenocarcinoma with strong diffuse p16^{INK4a} expression in 12 cases (35.3%), followed by villoglandular adenocarcinoma in 4 cases (11.8%), and mucinous NOS adenocarcinoma in 4 cases (11.8%). According to the International Endocervical Adenocarcinoma Criteria and Classification (IECC), these subtypes are classified as HPV-associated usual type (papillary and villoglandular) and mucinous type (mucinous adenocarcinoma NOS). Stolnicu et al. (2023) evaluated 297 endocervical adenocarcinomas, finding that 95% of usual type adenocarcinomas were HPV-positive, and 90% were positive for p16^{INK4a}. Mucinous carcinoma HPV-associated subtypes, including mucinous adenocarcinoma NOS, were 69% positive for p16^{INK4a}.¹⁸

p16^{INK4a} expression shows high specificity and sensitivity for HPV-associated adenocarcinoma. However, this study found that 2 cases (5.9%) of papillary and villoglandular

adenocarcinomas did not express p16^{INK4a}, raising questions about its diagnostic value. Literature and previous studies on this are limited. Rokutan-Kurata et al. (2020) analyzed diagnostic tissue samples from 5 SCC cases with negative p16^{INK4a} expression among 107 patients. This was attributed to unusual virus infections (low-risk HPV and EBV) and abnormalities in the CDKN2A gene (methylation and homozygous deletion of CDKN2A).¹⁹

Gastric adenocarcinoma, clear cell adenocarcinoma, and mesonephric adenocarcinoma are types of cervical adenocarcinoma not related to HPV. p16^{INK4a} immunohistochemistry is also a useful marker, with most of these subtypes showing negative expression. In this study, clear cell adenocarcinoma cases showed strong positive p16^{INK4a} expression in 3 cases (8.8%), weak positive in 1 case (2.9%), and negative in 1 case (2.9%). Gastric adenocarcinoma had negative p16^{INK4a} expression in 1 case (2.9%), while mesonephric adenocarcinoma showed varied expression, with 2 cases (5.9%) strong positive, 1 case (2.9%) weak positive, and 1 case (2.9%) negative.

These findings align with Ueno et al. (2013), who reported that 25% of clear cell adenocarcinoma cases were positive for p16^{INK4a}, with no HPV DNA detected in any case. Ueno noted that in clear cell adenocarcinoma, the Rb pathway becomes nonfunctional due to HPV-independent mechanisms. Thus, p16^{INK4a} immunohistochemistry is not an effective surrogate marker for HPV infection in clear cell carcinoma.²⁰

Park et al. (2011) also found that all gastric adenocarcinoma cases were negative for HPV DNA, and 5 cases were entirely negative for p16^{INK4a}, showing only focal staining in a few cells. Hence, p16^{INK4a} can be used as a negative immunomarker for these tumors.²¹

For mesonephric adenocarcinoma, p16^{INK4a} expression varied despite being HPV DNA-negative. Because p16^{INK4a} can show positive staining in some non-HPV-related tumors, it should be interpreted carefully, especially when used as a marker for HPV infection.²¹

Research on HPV in cervical adenocarcinoma is less extensive than in



squamous carcinoma, though most cervical adenocarcinomas are assumed to be HPV-related, just at a lower rate. This may be due to a lower viral load in glandular tumors compared to squamous ones. Unlike squamous lesions, which contain numerous episomal viral particles and integrated HPV, the glandular epithelium is less receptive to viral infection, and thus requiring sensitive detection methods. Sensitive HPV detection can be achieved through methods like in situ hybridization (ISH) for HPV DNA or polymerase chain reaction (PCR). Additionally, HPV E6/E7 RNA expression can be detected using quantitative reverse transcriptase-PCR (qRT-PCR).^{8,22,23}

CONCLUSION

16INK4a is a highly sensitive immunostaining method for detecting HPV-associated lesions. However, for HPV-independent lesions, this biomarker is not sufficiently specific, particularly for some subtypes. Therefore, p16^{INK4a} is often used in conjunction with other tests, such as HPV DNA PCR, which serves as a diagnostic confirmation. This was not performed in this study. Another limitation of this study is the potential for errors in determining the morphological classification of histopathological subtypes in cervical adenocarcinoma, which could affect the diagnostic significance of p16^{INK4a} expression. Therefore, no significant association was found between p16^{INK4a} expression with histopathological subtype in cervical adenocarcinoma.

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