ORIGINAL RESEARCH

p16 expression in patient with Loop Electrosurgical Excision Procedure (LEEP)

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ABSTRACT

Objective: The aim of this study was to reveal p16 expression with the clinicopathological characteristics of patients who underwent cervical biopsy using the Loop electrosurgical excision procedure (LEEP) at Udayana University Hospital, Bali, Indonesia, for the period 2020 – 2023.

Materials and Methods: The research was conducted at Anatomic Pathology Laboratory of Udayana University Hospital, Denpasar, Bali, Indonesia. The samples were selected based on inclusion criteria such as the formalin fixed paraffin embedded (FFPE) sample from positive IVA patient and continue for LEEP procedure. Otherwise, the exclusion criteria were moldy FFPE sample and incomplete clinical data. Then, p16 immunostaining procedure was carried out manually. The interpretation of p16 results was analyzed using SPSS software, version 25 by International Business Machines (IBM) Corporation.

Results: The positive p16 expression was revealed in 12 samples (38.7%), in contrast the negative staining appeared in 19 samples (61.3%). Unfortunately, p16 expression was not significant statistically based on age, parity, and contraceptive history, with p-values of 1.00, 0.45, and 0.65, respectively. Meanwhile, a statistically significant association was found between p16 expression and histopathologic diagnosis (p = 0.02, 95% CI 1.4 – 38.3). In addition, 22.2% of the variation of p16 expression based on multivariate analysis demonstrating a significant correlation (p = 0.01).

Conclusion: p16 expression with histopathology diagnostic characteristic in patient who underwent Loop electrosurgical excision procedure (LEEP) was found statistically significant. Moreover, clinical application of p16 in daily practice should be performed with consideration especially for pre-cancer lesion in LEEP biopsy specimen procedure and clinicopathological approach is essential.

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Highlights:

1. Clinicopathologic characteristic of patient with LEEP procedures dominated by women above 35 years old, with total parity less than 3 and without history of contraceptive usage.
2. The p16 expression analyzed by immunohistochemistry technique. The positive and negative reactivity of p16 ratio was 3:5 on LEEP specimen which microscopically appear as chronic cervicitis, low-grade and high-grade squamous intraepithelial lesion.

INTRODUCTION

In 2020, based on number of new cancer cases both sexes and all age group in Indonesia, cervical cancer (9.2%) ranked third after other cancer type (51.4%) and breast cancer (16.6%). Unfortunately, the age standardized (world) incidence and mortality rate placed the cervical cancer as the second most common cancer that has mortality rate reaches almost a quarter compare to breast cancer.[1] Furthermore, study research that conducted in Bali in the periods of 2017-2019, cervical cancer ranked second based on cancer topography data from private and public hospital in Bali.[2] Meanwhile, the screening program especially for cervical cancer has been purposed to detect it in the early phase, especially squamous intraepithelial lesions (SIL), because the epidemiology of SIL is linked to that of HPV infections, with a peak incidence observed in young women and a declining rate in the ensuing decades.[3] Importantly, HPV DNA is detectable in as many as 80% of women in their early 20s but falls to about 5% among women in their sixth decade of life.[3-4]

In clinical practice, p16 as the one high risk HPV and prognostic marker in cervical cancer, has not been routinely used due to some reason. Firstly, p16 antibodies was not available in daily practice which make it unreliable especially for prognostic marker, in fact challenges in diagnosis of high- or low-grade intraepithelial lesion due to undetermined morphology can be distinguished by p16 expression and the established research of p16 as a specific marker of high-risk oncogenic HPV infection, although some there is challenges of its clinical used especially in daily practice of cervical cancer diagnosis.[5] In other hand, the application of p16 immunohistochemistry is recommended by the Lower Anogenital Squamous Terminology (LAST) standardization project in three specific contexts: to aid in the distinction of HSIL from mimickers of precancer (immature metaplasia, atrophy, reparative changes, or tangential sectioning), to supplement morphological assessment for biopsy specimens interpreted as ≤ LSIL that are at high risk for missed high-grade disease based on the prior Pap or HPV testing result, and to inform the diagnosis of HSIL (CIN 2) versus LSIL in morphologically equivocal cases – block-type staining supports HSIL (CIN 2).6-7 For this reason, this study proposed to reveal the p16 expression with clinicopathologic data of patient who had positive IVA test and underwent LEEP procedure.

MATERIALS AND METHODS

This study received ethical clearance number 1268/UN14.2.2.VII.14/LT/2023 from the Research Ethics Committee of the Faculty of Medicine, Udayana University, on May 15, 2023. The study was conducted with analytical cross-sectional study design. Furthermore, sample was collected from January 2020 to July 2023, with predefined inclusion criteria like tissue samples are formalin fixed paraffin embedded (FFPE) from patient who are clinically IVA test positive and continued for Loop electrosurgical procedure (LEEP). In contrast, the exclusion criteria were moldy or deteriorated FFPE samples and incomplete clinical data. Moreover, clinical data of these patients were obtained from hospital medical records. Subsequently, the immunohistochemical staining of p16 was performed in eligible FFPE samples. The interpretation of p16 results was defined by positive and negative using scoring criteria.[8-9] The positive expression of P16 was divided into three categories and mainly expressed in the nucleus and cytoplasm with brown color. The positive 1 (+) p16 expression was limited to epithelial basal layer; when the p16 expression between 1/3-2/3 layer of the cervix squamous epithelium it was marked as positive 2 (++); In addition, when the expression beyond 2/3 to whole layer of the cervix squamous epithelium then it was marked as positive 3 (+++). On the contrary, if there was no brown color in the cell, or patchy or uneven staining it was marked as negative (-).[4,10-11] Data were analyzed using statistical IBM software SPSS, version 25.

RESULTS AND DISCUSSION

Squamous intraepithelial lesions (SILs) of the uterine cervix, also known as cervical intraepithelial neoplasia (CIN), are proliferations of squamous cells driven by HPV infection, showing maturation abnormalities and/or viral cytopathic changes that do not extend beyond the basement membrane. They are divided into low-grade SILs (LSILs) and high-grade SILs (HSILs).[4,12] In this study, samples were obtained from patients who were positive with acetic acid application (IVA test) from screening procedure at primary health care and continued for loop electrosurgical excision procedure (LEEP). Most of them were female above 35 years old (71%) and more than half of the patients were private employee. Furthermore, clinically most of the patients did not suffer any signs and symptoms. However, some of them suffer from vaginal discharge or vaginal spotting and lower abdominal pain (Table 1).

Theoretically, the epidemiology of SIL is linked to that of HPV infections, with a peak incidence observed in young women and a declining rate in the ensuing decades. HPV DNA is detectable in as many as 80% of women in their early 20s but falls to about 5% among women in their sixth decade of life.[4] In screened

population in high-income countries, LSIL has a cross-sectional prevalence of 5–10% and the prevalence of HSIL is 0.5–1%.[13,14] HSIL typically occurs at an older age than LSIL, although there is broad overlap and HSIL has been demonstrated within a year or two of HPV infection in adolescents. Importantly, the rate of HSIL regression is higher in adolescents and young women than in older populations.[4,12] In this study, based on microscopic findings with routine hematoxylin eosin staining, the histopathological diagnosis of HSIL was only 3% compared to LSIL 35% and chronic cervicitis (62%) was the most common findings.

Subsequently, the monoclonal antibody p16-INK4 clone MX007 was used in this study. As one of the cyclin dependent kinase inhibitors that inhibit cyclin-dependent kinases 4 and 6, p16INK4A is encoded by tumor suppressor gene CDKN2A.[15] The tumor suppressor p16 INK4A plays an important role in cell cycle regulation.[16-17] Beside in cervical cancer, p16 is implicated in several cancer such as breast cancer which specifically increased in hormonal estrogen receptor-positive tumor tissues ($p < 0.01$).[14] In contrast, there is no significant correlation was found between the p16 protein expression and the other clinicopathological features. In our study, p16 reactivity through immunohistochemistry procedure was tested on all samples of IVA test positive patient who underwent the LEEP procedure. Totally, negative staining of p16 was 61.3% and only 38.7% were positive. The rise of p16 expression is seen as the organism ages and reduces proliferation of stem cell. In addition, this depletion in the division and production of stem cell protects against cancer while increasing the risk associated with cellular senescence.[18-19]

Unfortunately, mutation in the p16 gene associated with loss or over expression of the protein are associated with increased risk of wide range of cancer and cancer precursor lesion. The immunohistochemical identification of p16 reactivity is shown in Figure 1. Moreover, p16 expression was grouped into two categories positive and negative staining. Positive staining is characterized by brown staining of the cell nuclei and cytoplasm of squamous epithelial cells limited in epithelial basal layer (+) or between 1/3-2/3 squamous epithelial layer (++) or beyond 2/3 of the basal cell layer or throughout the thickness of the metaplastic squamous epithelium (+++) (A and B). Meanwhile, negative staining is characterized by negative staining or a patchy appearance or uneven brownish colour on the cervical transitional zone (C and D).

Table 1. Clinicopathological characteristic of patients with LEEP Procedure

Parameters		Frequency	Percentage
Age	≤ 35 y.o	9	29%
	> 35 y.o	22	71%
Education level	Elementary school	4	13%
	Senior high school	17	55%
	Diploma	4	13%
	Bachelor degree	6	19%
Occupation	Health care worker	2	6%
	Private employee	21	68%
	Self-employed	1	3%
	Housewife	7	23%
Marital status	Married	28	90%
	Single	3	10%
Parity	≥ 3	20	65%
	< 3	11	35%
Clinical symptoms	No symptom	12	39%
	Vaginal spot	8	26%
	Vaginal discharge	8	26%
	Lower abdominal pain	3	9%
Contraception history	Yes	6	19%
	None	25	81%
Histopathological diagnosis	Chronic cervicitis	19	62%
	Low grade squamous intraepithelial lesion (L-SIL)	11	35%
	High grade squamous intraepithelial lesion (H-SIL)	1	3%

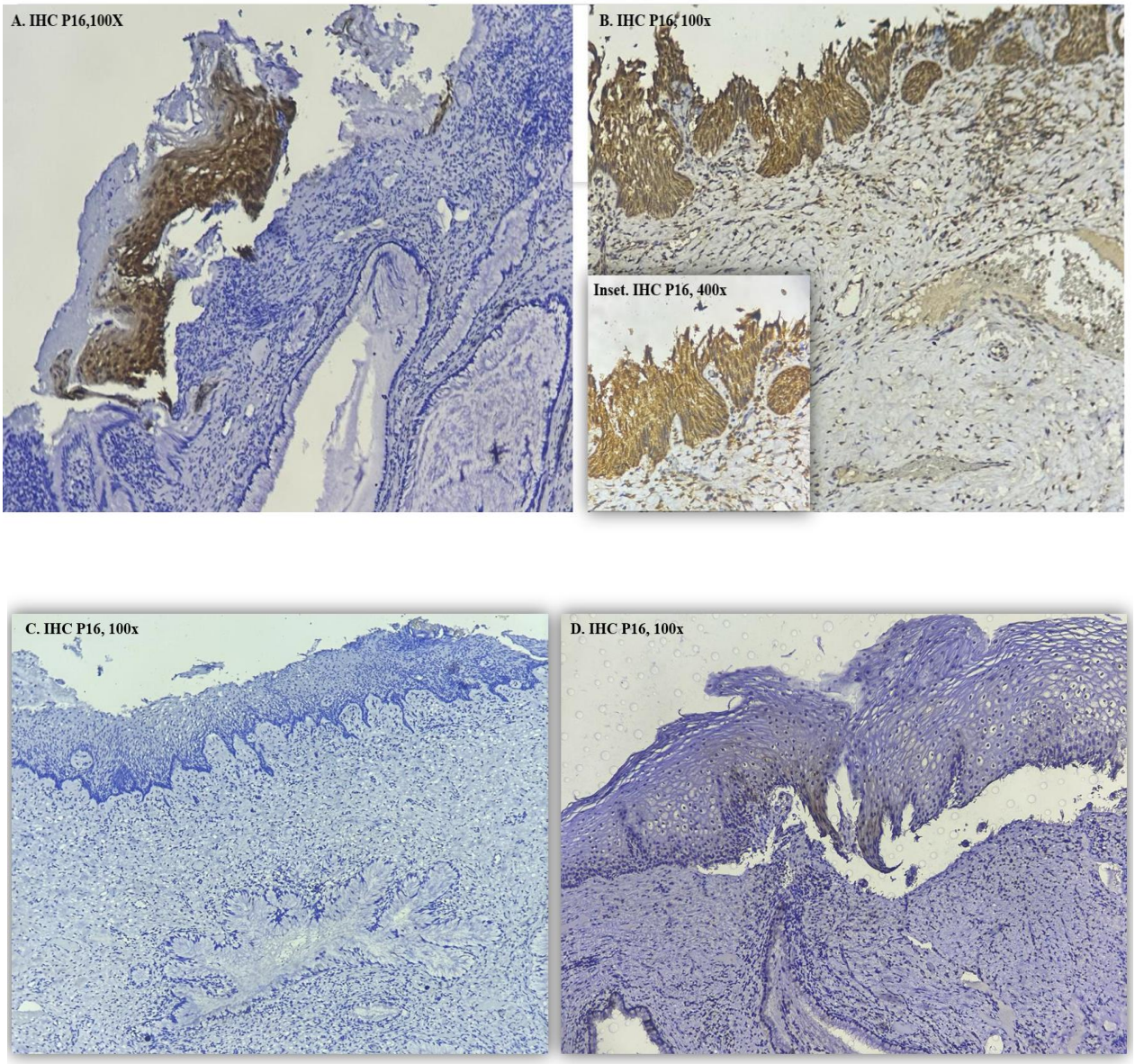

Figure 1. Immunohistochemistry of p16 reactivity result

A-B. Positive staining, the expression beyond 2/3 of the layer (A) and extends to whole layer (B) of the cervix squamous epithelium (IHC p16, 100x). C-D. Negative staining, there is no brown color in squamous epithelial layer (C), or patchy or uneven staining (D) (IHC p16,100x)

Table 2. Chi-square analysis of p16 expression with clinicopathological characteristic

Parameters	p16 expression		Total	Risk Estimate (CI 95%)	p*	R
	Positive	Negative				
Age						
≤ 35 y.o	4 (25%)	5 (31.6%)	9	0.3-7.0	1.00	0.01
> 35 y.o	14 (68.4%)	8 (75%)	22			
Total parity						
≤ 3	8 (58.3%)	13 (73.7%)	21	0.4-9.3	0.45	
> 3	4 (41.7%)	6 (26.3%)	10			
Contraception history						
Yes	10 (75%)	15 (84.2%)	25	0.3-10.7	0.65	
No	2 (60%)	4 (40%)	6			
Histopathological diagnostic						
Chronic cervicitis	4 (21%)	15 (79%)	19	1.4-38.3	0.02*	
Squamous intraepithelial lesion	8 (67%)	4 (33%)	12			

*Statistically significant if $p < 0.05$

Furthermore, to determine the proportion of p16 expression with clinicopathological parameters, the comparative test Chi-square analysis was performed and presented in Table 2. Based on the result, a statistically significant difference with p value <0.05 can be seen between the p16 expression variable and histopathological diagnosis. Unfortunately, the results of other clinicopathological characteristics such as age, number of parities and contraception history were not found to be statistically significant.

Based on the result, p16 expression in this study showed negative staining due to reparative changes of cells which mostly found in chronic cervicitis cases compared to squamous intraepithelial lesion with ratio 5:3. The diagnostic of HSIL in this study was only found in 1 LEEP sample, so that the comparative analysis of LSIL and HSIL become one category compared to chronic cervicitis. Some studies have emphasized the significance of using p16 immunostaining as marker for identifying dysplastic and neoplastic lesion caused by high-risk HPV.[13-15] The staining intensity of p16 varies based on amount of dysplasia cells. As the grade of dysplasia increased, the p16 expression increased as well. However, it should be emphasized that other reason for negative expression of p16 in squamous intraepithelial lesion especially LSIL due to a certain percentage is thought to be caused by low-risk HPV types.[19,20] Previous study indicated that viral oncoprotein of low risk HPV such as HPV-6 does not affect p16 because the affinity of HPV-6 E7 protein for cellular pRb is ten-fold lower than that of HPV-16 E7 for pRb.[20]

The main strength of our study lies in the inclusion of a sample of IVA-positive patients undergoing the LEEP procedure, where formalin-fixed, paraffin-embedded biopsy samples were evaluated for p16 reactivity. Additionally, this study is noteworthy as it is the first to analyze p16 in samples from the LEEP procedure, especially in Bali, providing foundational data for future research. However, we acknowledge that invasive carcinoma cases were not included, and the histopathological diagnoses from the LEEP procedure comprised chronic cervicitis, low-grade squamous intraepithelial lesion (L-SIL), and high-grade squamous intraepithelial lesion (H-SIL). Therefore, specimens from other procedures, such as conization and hysterectomy, should be considered. Challenges have also arisen due to LEEP crush artifacts, requiring careful evaluation. Another limitation is the small diagnostic sample size, as no positive cervical cancer cases were identified through the LEEP procedure. Despite these limitations, we believe our findings provide valuable baseline data for future studies and recommendations.

CONCLUSION

The study found that p16 expression, analyzed in relation to the histopathological diagnosis of patients who underwent cervical biopsy using the LEEP procedure, was statistically significant. Our findings contribute to knowledge by highlighting that p16 expression is most frequently observed in cases of chronic cervicitis, likely due to reparative processes, and that the intensity of p16 reactivity varies with the degree of dysplasia. As dysplasia grade increases, so does the p16 expression. However, it is important to note that p16 negativity in squamous intraepithelial lesions, particularly LSIL, may be attributed to the

presence of low-risk HPV types. These findings suggest that using p16 as a clinical marker in LEEP biopsy specimens, particularly for pre-cancerous lesions, requires careful consideration, and a combined clinicopathological approach is essential in routine practice.

DISCLOSURES

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Conflict of interest

The authors declare no conflict of interest.

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Author contribution

All authors have contributed to all processes in this research, including preparation, data gathering and analysis, drafting and approval for publication of this manuscript.

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