INTRODUCTION

Cervical cancer is one of the world’s third most common gynecologic malignant tumors, with a prevalence of 11.7%, mainly occurring in developing countries. This cancer is also the leading cause of cancer-related death, which ranks fifth most frequently in women, estimated at 50,000 cases per year.

This malignancy can be prevented because the cause is known, which is mostly caused by persistent high-risk human papillomavirus (HPV) infection. The Pap smear is a screening test that can reduce cancer incidence by about 75% but has limitations such as low sensitivity and false negative results. Cervical intraepithelial neoplasia (CIN) is a transitional lesion that transforms the normal cervix into squamous cell carcinoma (SCC). Low-grade CIN is often associated with non-carcinogenic HPV infection, which usually resolves without treatment. Some low-grade NIS can transform into high-grade CIN. High-grade CIN is a precancerous lesion often requiring surgical treatment to prevent SCC. Histomorphological diagnosis of cervical biopsy sometimes difficult to distinguish between low-grade and high-grade CIN. A rapid and effective method is needed to differentiate low-grade CIN, high-grade CIN and SCC, which is important for proper treatment planning.

The tumor suppressor gene p16, a member of the cyclin-dependent kinase inhibitor (CDK) 4 family (INK4a), is a biological marker that can be used as a surrogate marker to improve diagnostic agreement for high-risk HPV infection. Expression of p16 indicates the presence of high-risk HPV infection and the integration of the viral genome with the host. Cervical cancers arising without HPV infection are p16 negative and have a more aggressive prognosis.

The incidence of cervical cancer in young women is increasing yearly, indicating an active transformation of precancerous lesions in the younger group.
of women.\textsuperscript{1} p16 can be used for screening or early diagnosis because cervical cancer with p16 expression has a better prognosis.\textsuperscript{1,2,6} p16 is also an indicator for radiosensitive therapy, so it can be considered a targeted chemotherapy for cervical cancer.\textsuperscript{1}

**RESEARCH DESIGN AND METHODS**

**Specimen collection**

The research design was a cross-sectional analytic observational study that was conducted during the year 2022, with a total sample of 40 which was taken from biopsy or surgery specimens from patients with cervical intraepithelial neoplasia (CIN) and cervical squamous cell carcinoma, whose tissues were examined at the Anatomical Pathology Laboratory Faculty of Medicine Universitas Udayana/ Prof. dr. I G.N.G. Ngoerah General Hospital. The samples must correspond to inclusion and exclusion criteria. Namely, new patients who have never received chemotherapy or radiotherapy were included in this study. The specimens containing a lot of necrotic and hemorrhaged tissue and damaged paraffin blocks were excluded. Samples were collected on a consecutive basis until the required sample size was met following the calculation of the sample. This study has been permitted by the ethical committee of the Faculty of Medicine, Universitas Udayana, with letter number No. 1258/UN14.2.2.VII.14/LT/2022.

**Histopathological and Immunohistochemical of p16 Examination**

The biopsy or surgery specimens from patients with CIN and cervical squamous cell carcinoma were diagnosed histopathologically at the Anatomical Pathology Department Faculty of Medicine Universitas Udayana/ Prof. dr. I G.N.G. Ngoerah General Hospital. Histopathological diagnostic of low-grade CIN, high-grade CIN and SCC according to WHO criteria,\textsuperscript{7} and stained with Hematoxylin and Eosin (H & E) staining. Expression of p16 was evaluated immunohistochemically from paraffin-embedded tissues from biopsy or surgery tissue of patients diagnosed histopathologically with low-grade CIN, high-grade CIN and SCC. The Gene Ab Monoclonal Mouse Anti-Human p16\textsuperscript{INK4a} Antibody GenomeMe was used. Expression of p16 is classified according to Lower Anogenital Squamous Terminology (LAST) criteria, namely block positivity, ambiguous and negative. Block positivity, if stained strongly, continuously, on the nucleus, with or without staining on the cytoplasm, extends from the basal layer upwards to at least 1/3 of the thickness of the epithelium (basal & parabasal layers), which is then graded as 1/3, 2/3, and more than 2/3, and laterally at significant distances with the diffuse appearance of >25% cells. Ambiguous staining if the staining is strong, basal, diffuse, and continuous (involving only the lower 1/3 with no upward extension), or the staining is weak, diffuse and discontinuous (involving at least 2/3 of the epithelium), or the staining is strong, focal and discontinuous (located at any epithelial level). Negative staining is if it is not completely stained or lightly stained, focal and discontinuous, or only stained in the cytoplasm. Furthermore, p16 expression is grouped into 2 categories, namely block positive; and negative, which consists of negative and ambiguous.\textsuperscript{8}

**Statistical Analysis**

The characteristics of the sample were analyzed descriptively. The results were analyzed using the chi-square test to prove differences in p16 expression in various age groups, and the Kruskal-Wallis Test followed by the Mann-Whitney Test to prove differences in p16 expression in low-grade CIN, high-grade CIN and SCC. Statistical significance was set at 2-sided of 0.05 levels with a 95% confidence interval (CIs).

**RESULTS**

The results of the 40 samples are shown in Tables 1 - Table 4, which show the distribution of samples based on age group following WHO in 2015, which is grouped into groups 25-44 years, >44-60 years and >60-75 years, and p16 expression of low-grade CIN, high-grade CIN and squamous cell carcinoma.\textsuperscript{9} Table 1 shows the distribution of samples based on age group, CIN grade and squamous cell carcinoma.

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**Table 1. Sample Distribution Based on Age Group, CIN grade and Squamous Cell Carcinoma**

<table>
<thead>
<tr>
<th>Age Group (Year)</th>
<th>Low-grade CIN</th>
<th>High-grade CIN</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>25-44</td>
<td>7</td>
<td>53,8</td>
<td>6</td>
</tr>
<tr>
<td>&gt;44-60</td>
<td>6</td>
<td>46,2</td>
<td>6</td>
</tr>
<tr>
<td>&gt;60-75</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>100</td>
<td>13</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>45,5±8,44</td>
<td>46,1±9,11</td>
<td>50,9±10,80</td>
</tr>
<tr>
<td>Total Mean ± SD</td>
<td>47,6±9,62</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2. Sample Distribution Based on Age Group, p16 Expression, CIN grade and Squamous Cell Carcinoma**

<table>
<thead>
<tr>
<th>Age Group (Year)</th>
<th>Low-grade CIN</th>
<th>High-grade CIN</th>
<th>SCC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p16 Expression</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>Block-positive</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td>n(%)</td>
<td>n(%)</td>
<td>n(%)</td>
</tr>
<tr>
<td>25-44</td>
<td>6(46,15)</td>
<td>1(7,7)</td>
<td>2(15,4)</td>
</tr>
<tr>
<td>&gt;44-60</td>
<td>6(46,15)</td>
<td>0</td>
<td>3(23,1)</td>
</tr>
<tr>
<td>&gt;60-75</td>
<td>0</td>
<td>0</td>
<td>1(7,7)</td>
</tr>
<tr>
<td>Total</td>
<td>12(92,3)</td>
<td>1(7,7)</td>
<td>6(46,2)</td>
</tr>
<tr>
<td>p-value</td>
<td>0,34</td>
<td>0,45</td>
<td>0,28</td>
</tr>
</tbody>
</table>
The age range of the research sample for low-grade CIN was between 32-58 years, high-grade CIN was between 33-63 years, and SCC was between 36-70 years. The highest distribution of low-grade CIN was found in the 25-44 years group, namely 7 samples (53.8%) and no samples aged >60 years. The highest distribution of high-grade CIN was found in the 25-44 year and >44-60 years group, namely 6 samples (46.15%) each. The highest distribution of squamous cell carcinoma was found in the >44-60 years group, namely 6 samples (42.9%). The mean age of low-grade CIN is younger than that of high-grade CIN, namely 45.5±8.44 compared to 46.1±9.11. The mean age of low-grade and high-grade CIN is also younger than the mean age of SCC, which is 50.9±10.80. The mean age for all samples was 47.6±9.62, with an age range between 32-70 years.

In low-grade CIN, most negative p16 expressions were found in the 25-60 age group (92.3%), but 1 sample (7.7%) showed block-positive p16 expression in the 25-44 years age group. In high-grade CIN, block-positive p16 expression was more than negative expression, namely 53.8% compared to 46.2%. The highest expression of p16 block-positive was in the 25-44 years group with 4 samples (30.7%). In SCC, block-positive p16 expression was more than negative expression, namely 57.1% compared to 42.9%. The highest expression of p16 block-positive was in the 25-44 years group with 4 samples (28.5%).

A chi-square test was performed on each sample group, and no significant difference was found in the expression of p16 in the various age groups, with a p-value for low-grade CIN of 0.34, for high-grade CIN of 0.45 and SCC of 0.28.

Negative p16 expression was found in 12 samples (30%) of low-grade CIN, and 6 samples (15%) of high-grade CIN and SCC, respectively. Block-positive p16 expression was found in 1 sample (2.5%) of low-grade CIN, 7 samples (17.5%) of high-grade CIN and 8 samples (20%) of SCC. The most negative expression of p16 was found in low-grade NIS. The highest expression of block-positive p16 was found in SCC. The Kruskal-Wallis Test was carried out, and a significant difference was found in the expression of p16 in low-grade CIN, high-grade CIN and SCC (p = 0.011).

Mann-Whitney Test was performed to determine the difference in expression between variables. There was a significant difference in the expression of p16 in low-grade CIN with high-grade CIN (p = 0.012). There was a significant difference in the expression of p16 in low-grade CIN with SCC (p = 0.008). There was no significant difference in p16 expression in high-grade CIN and SCC (p = 0.866).

The sample with H & E stain on low-grade CIN, high-grade CIN and SCC is shown in Figure 1. Outcome results with p16 immunohistochemical stain are shown in Figure 2 - Figure 4.

DISCUSSION

Cervical cancer is one of the most common gynecologic malignant tumors in the world, especially in developing countries, and its incidence in young women is also
Cervical cancer ranks third, with a global prevalence of 11.7% and an estimated incidence of 500,000 cases annually. Cervical cancer is the leading cause of cancer-related death in women. In Indonesia, cervical carcinoma is the most common cancer, accounting for 28.6% of all female cancers in Indonesia. The incidence of cervical cancer is estimated at 25-40/100,000 women. Cervical cancer develops from precancerous lesions, namely intraepithelial neoplasia, which takes about 5-15 years to become invasive cancer.

Cervical cancer is a malignant tumor with a known and preventable cause, persistent infection with high-risk Human Papilloma Virus (HPV). Infection with high-risk human papillomavirus (HPV) is the main cause or major risk factor for cervical cancer. HPV is a double-stranded DNA virus, of which about 120 types have been identified so far and are classified as low-risk HPV and high-risk HPV. Minor risk factors are multiple sexual activities with multiple partners, sexually transmitted diseases, early age at first pregnancy, multiparity, low socioeconomic status, smoking, immunosuppression disorders, vitamin deficiencies, and oral contraceptive.

Cervical intraepithelial neoplasia (CIN), which is classified into low-grade NIS and high-grade CIN, is an important transitional stage from normal cervical tissue to transform into cervical squamous cell carcinoma (SCC). Low-grade CIN usually heals on its own without therapy. In contrast, high-grade CIN can develop into SCC. Some low-grade CIN can transform into high-grade CIN, which can then develop into SCC. HPV screening is an important examination to determine whether there is HPV infection in cervical lesions. It is very important to find a diagnostic method that can indicate the extension of cervical lesions. It can quickly and effectively differentiate low-grade CIN, high-grade CIN and SCC, which are clinically important for planning therapy.

Low-grade CIN is usually followed up without therapy, because about 80% will experience spontaneous regression. Only 10% will progress towards high-grade CIN or cervical cancer, whereas high-grade CIN requires excision therapy.

A pap smear is a screening method that can reduce the incidence of cervical cancer by about 75%, especially in developing countries. However, the pap smear has several limitations: low sensitivity, false negative results and low reproducibility. The agreement of histomorphological diagnosis from a cervical biopsy can differ, resulting in under or over-therapy.

The diagnostic marker p16 can be used as an alternative and as a surrogate marker for cervical cancer, which can increase diagnostic agreement, because it can correctly identify CIN and cervical cancer from biopsy tissue. p16 can predict the extent of cervical lesions. The criteria for p16 immunoreactivity according to Lower anogenital squamous terminology (LAST) provide standard guidelines for determining p16 expression to reduce interobserver variation and improve accuracy.

In this study, differences in the age range of the study sample were obtained, namely those with low-grade CIN tended to be younger than those with high-grade CIN and SCC. The youngest ages of low-grade CIN, high-grade CIN and SCC are 32 years, 33 years and 36 years, respectively. The oldest ages of low-grade CIN, high-grade CIN and SCC are 58 years, 63 years and 70 years, respectively. The mean age of low-grade CIN is younger than that of high-grade CIN, namely 45±8.44 compared to 46±9.11. The mean age of low-grade and high-grade CIN is also younger than the mean age of SCC, which is 50±10.80. Research conducted by Kalyani et al. found the age of cervical cancer to be between 30-80 years, with a mean age of 54.3±12.0.

In this study, the sample distribution based on age group and p16 expression showed that in low-grade CIN, most negative p16 expressions were found in the 25-60 years old group (92.3%), but there was 1 sample (7.7%) which showed expression of p16 block-positive in the age group of 25-44 years. In high-grade CIN, the highest block-positive p16 expression was in the age group of 25-44 years, with a mean age of 4 (30.7%). In SCC, the highest block-positive p16 expression was in the age group of 25-44 years, with 4 samples (28.5%). It appears that block-positive expression of p16 tends to occur at a younger age, indicating the active transformation of precancerous...
lesions in the younger age group, so p16 as a surrogate marker can be used as an early screening marker of cervical cancer. A study by Kalyani et al. showed that the expression of block-positive p16 in pre-and perimenopausal patients compared to post-menopause was not significantly different, nor was the association between p16 expression and age at marriage.\(^1\) p16 examination can be used for screening or early diagnosis because cervical cancer with p16 expression has a better prognosis.\(^{1,2,4,6}\) In this study, there were no significant differences in the expression of p16 in various age groups in low-grade CIN (p=0.34), high-grade CIN (p=0.45) and SCC (p=0.28).

In this study, 1 sample (7.7%) showing block-positive p16 expression in the age group of 25-44 years requires attention in considering other therapy because low-grade CIN generally indicates non-carcinogenic HPV infection that resolves without therapy. In patients with block-positive p16 expression results, high-grade precancerous lesions may require surgical intervention to prevent progression to SCC. The results of p16 staining in high-grade CIN are important for patient management.\(^2,3\)

Previous studies on SCC showed positive, ambiguous and negative p16 block expressions of 89.3%, 6.6%, and 4%, respectively.\(^1\) In this study, p16 block positive expression in low-grade CIN, high-grade CIN and SCC were 2.5%, 17.5% and 20%, respectively. The most negative p16 expression was found in low-grade CIN. The highest block-positive p16 expression was found in SCC. Kruskal-Wallis Test was performed, and there were significant differences in p16 expression in low-grade CIN, high-grade CIN and SCC (p=0.011). The next test using the Mann-Whitney Test showed a significant difference in the expression of p16 in low-grade CIN with high-grade CIN (p=0.012), a significant difference in p16 expression in low-grade CIN with SCC (p=0.008), but there was no significant difference in the expression of p16 in high-grade CIN with SCC (p=0.866). Negative p16 expression in SCC could be caused by the absence of HPV infection, inappropriate immunohistochemistry technique, mutations in the promoter region, epigenetic mechanisms and hypermethylation.\(^3\)

Persistent infection with high-risk HPV can induce cervical cancer through an uncontrolled G1-S transition. The E6 and E7 proteins of high-risk HPV inhibit the p53 and pRb proteins, which are cell cycle regulatory proteins that control the G1-S transition. The p16 protein is a family of cyclin-dependent kinase (CDK) 4 inhibitors (INK4a family), which can inhibit CDK-induced pRb phosphorylation. Phosphorylation of pRb will induce the release of transcription factor E2F from its binding to pRb so that the release of E2F will cause the G1-S transition. The E7 protein of HPV can bind to pRb, thereby inducing E2F release. Inactivation of pRb will lead to overexpression of p16 due to negative feedback.\(^{4,5,8,10}\) p16, as a tumor suppressor gene, as the main target of HPV, cannot express its suppressive effect after an interaction between E7 and Rb protein through the ubiquitin-dependent proteasome system.\(^3\) p16 expression indicates an infection of high-risk HPV and integration of the viral genome with the host genome.\(^{1,2,4,5,10}\) Overexpression of p16 is rare in patients infected with low-risk HPV because E7 of low-risk HPV has low affinity for pRb.\(^1,5\) Infection with high-risk HPV can also cause amplification and mutation in the p16 gene.\(^1\)

Research conducted by Huang et al. showed a significant association between p16 expression and increased disease-free survival and 5-year survival rates. Expression of p16 may predict a better prognosis in cervical cancer patients.\(^1,4,7\) p16 is often used to classify cervical lesions because cancers that arise via the HPV-independent pathway are p16 negative and are more aggressive. In contrast, tumors that develop from HPV-associated will show strong overexpression of p16 and have a better prognosis.\(^5,7\)

The analysis of p16\(^{INK4a}\) expression is a useful diagnostic tool. Its expression is related to the grade of histologic dysplasia, which is a prognostic and predictive marker for managing cervical neoplasia.\(^1,6\) The anti-cancer activity of p16 supports p16 to develop as a cervical cancer target, a sensitive indicator.\(^1\)

Further research is needed on a larger population and a combination of p16 and Ki-67 as cell proliferation markers to detect the cell cycle in the non-G0 phase. In normal tissues, there is rarely concomitant overexpression of p16 and Ki-67. Research by Shi et al. showed a significant positive correlation between the levels and intensity of protein expression of p16 and Ki-67 and the grade of cervical lesions, namely chronic cervicitis, low-grade CIN, high-grade CIN, and SCC.\(^2,5\)

CONCLUSION

p16 expression can be used as a screening method for diagnostic markers of high-risk HPV infection and a reference for planning therapy.

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.

CONFLICT OF INTEREST

The authors declare no conflict of interest regarding the publication of this article.

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REFERENCE


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