

## The Correlation of P16 Expression with Degree of Differentiation and Tumor Stage in Cervical Squamous Cell Carcinoma (CSCC)

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### ABSTRACT

#### Background

Cervical squamous cell carcinoma (CSCC) represents 70-80% of all cases of cervical cancer. This cancer ranks 4th and the 4th most common cause of death due to malignancy in women in the world. Cervical SCC patients were found at an older age and were detected at an advanced stage. High risk human papillomavirus (HPV) infection is the main cause of SCC. The protein cyclin-dependent kinase inhibitor 2A also called p16 is a well-known tumor suppressor protein involved in cell cycle regulation, senescence, apoptosis, cell invasion and angiogenesis. This p16 protein can be used as a surrogate marker for HPV infection. From several previous studies, there were differences in the results between the relationship of p16 expression with the degree of differentiation and stage of cervical SCC tumor. Research on the relationship between p16 expression and the degree of differentiation and tumor stage of cervical SCC patients has never been conducted in West Sumatra.

#### Methods

This research was a retrospective observational cross sectional study with 35 cases of cervical SCC that have been diagnosed at the Anatomical Pathology Laboratory, RSUP Dr. M. Djamil Padang period January 2018-December 2019. Samples were obtained by simple random sampling from paraffin blocks derived from tumor tissue. The histopathological slide was reassessed to assess the degree of differentiation and tumor stage data were obtained from the patient's medical record. Expression of p16 in tumor cells was seen by immunohistochemical examination. To determine the correlation, bivariate statistical analysis was performed using the chi-square test with a significance level of  $p < 0.05$ .

#### Results

p16 high expression was found in 22 cases, tended to be higher in the degree of well differentiation (40.9%) and moderate differentiation (50.0%). The p16 high expression was higher at an advanced stage (90.9%). Statistically, there is no correlation between the expression of p16 and the degree of differentiation with  $p$  value=0.138. There was no correlation between p16 expression and tumor stage with  $p$  value=0.337.

#### Conclusions

This study concluded that p16 expression was not associated with the degree of differentiation and tumor stage in cervical SCC.

**Keywords:** cervical SCC, p16 expression, degree of differentiation, tumor stage.

## INTRODUCTION

Cervical cancer is one of the most common malignancies in women. This cancer ranks 4<sup>th</sup> and is the 4<sup>th</sup> most common cause of death due to malignancy in women in the world, with 604,000 new cases and 342,000 deaths worldwide in 2020.<sup>1</sup> According to The American Cancer Society's invasive cervical cancer in the United States in 2021 is estimated around 14,480 new cases and around 4,290 women will die from this disease.<sup>2</sup> The incidence of cervical cancer in Southeast Asian countries was found to be the highest in Indonesia.<sup>3</sup> The incidence of cervical cancer in 2020 in Indonesia ranks 2<sup>nd</sup> after breast cancer with the number of cases 36,633 (17.2 %).<sup>4</sup> Based on data from the Cancer Registration Agency of the Association of Indonesian Anatomical Pathology Specialists, cervical cancer in West Sumatra ranks 3<sup>rd</sup> with a total of 108 cases in 2014.<sup>5</sup>

The most common histological type of cervical cancer is cervical squamous cell carcinoma (CSCC), representing 70-80% of all cervical cancer cases. The 5 years survival rate for KSS is around 57-67%.<sup>6</sup> This cancer originates from the squamous cells that line the outside of the cervix. Most tumors originate from the transformation zone (squamocolumnar junction).<sup>7</sup>

High risk human papillomavirus (HPV) infection is the main cause of SCC. The pathogenesis of cervical SCC through HPV infection is a slow, multi-stage process. In 99.7% of cases of high risk HPV infection, the virus will survive in cells undergoing division causing high grade squamous intraepithelial lesions (HSIL). These tumor cells can pass through the epithelial basement membrane to become invasive carcinoma after going through several processes. This cancer is often asymptomatic in the early stages and is often detected at an advanced stage.<sup>6</sup> Half of cervical SCC patients who are treated with surgery and chemoradiotherapy die from this disease. Therefore, correct diagnosis is essential for appropriate clinical management.<sup>6</sup>

According to the WHO female genital tract book, fifth edition in 2020, cervical squamous cell carcinoma is divided into 2 groups, namely squamous cell carcinoma, HPV-related and squamous cell carcinoma, not HPV-

related. The majority of cervical squamous cell carcinomas (>90-95%) are associated with HPV. Approximately 5-7% of cases of cervical squamous cell carcinoma are negative for HPV testing even on screening techniques that are highly sensitive for HPV.<sup>8</sup> Patients with non-HPV-related tumors had a higher rate of lymph node invasion (67% vs. 36%,  $p<0.01$ ) and worse survival (59.8 vs 132.2 months,  $p<0.01$ ) compared with patients with HPV-associated tumors.<sup>9</sup>

Cyclin-dependent kinase inhibitor 2A protein, also called p16, is a protein that is well known as a tumor suppressor protein that is involved in cell cycle regulation, aging, apoptosis, cell invasion and angiogenesis. This p16 protein can be used as a surrogate marker for HPV infection.<sup>6</sup> Although the association of positive p16 expression with high-risk HPV-related cases of cervical SCC is now a recognized fact, there are also p16-negative cases. According to research by Damata et al (2021) p16 negative tumors are more common at older ages and at advanced stages.<sup>10</sup> Research conducted by Horba S et al (2021) in cervical SCC, a relationship was found between tumor grade and p16 staining intensity, but there was no relationship between tumor stage and p16 expression intensity.<sup>11</sup>

Prognostic factors related to survival rates in cervical cancer are age, tumor size, lymph node status, FIGO stage, degree of tumor differentiation, depth of invasion and lymphatic or vascular invasion.<sup>7</sup> Research on the relationship between p16 expression and the degree of differentiation and stage of the patient's tumor Cervical SCC has never been done in West Sumatra. Based on the background above, the author is interested in conducting research on the relationship between p16 expression and the degree of differentiation and tumor stage in cervical SCC.

## METHOD

This research is an observational study with a cross sectional design. The population in this study were all cases of cervical squamous cell carcinoma that had been diagnosed in the Anatomic Pathology laboratory at Dr. RSUP. M.Djamil Padang for the period January 2018-December 2019. The research samples were all

cases diagnosed with cervical squamous cell carcinoma by biopsy and hysterectomy, in the form of complete status and data, slides and paraffin blocks were available. 35 samples were taken using simple random sampling. Re-evaluation of HE slides was carried out in the form of degree of differentiation and tumor stage data obtained from medical records (secondary data). Assessment of the degree of differentiation of cervical squamous cell carcinoma was divided according to WHO 2014 criteria, divided into 3 groups, well, moderate and poor differentiation. Reevaluation of the Hematoxylin-Eosin (HE) SCC of the cervix slides was carried out by looking at the degree of differentiation, cell pleomorphism and mitotic activity.

Paraffin blocks of research samples were re-cut for immunohistochemical staining with primary antibody p16INK4A (JC2) at a dilution of 1:100 (Cell Marque, mouse monoclonal antibody). The immunohistochemical staining method used is Avidin Biotin Complex (ABC), which is carried out using an automatic procedure in the Anatomic Pathology laboratory at Dr. RSUP. M. Djamil Padang. Interpretation of p16 immunohistochemical smears was assessed semiquantitatively, observed using an Olympus CX23 binocular light microscope in one field of view with magnification from 40 times to 400 times. Points were awarded according to the intensity of staining and the proportion of cells stained. The intensity of staining was categorized as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining). According to the proportion of stained cells in the IHC score as follows: 0 (no staining), 1 (<1%), 2 (1-10%), 3 (11-33%), 4 (34-66%), 5 (>66% cells). The total score was calculated by adding the score for intensity and the score for the proportion of cells stained with the p16INK4a immunostain. The results of measuring low expression, if the total score is 0-5 and over expression, if the total score is 6-8.13 IHC assessment is carried out by 1 person, because assessing p16 expression is quite easy to do with guidance.

Univariate analysis consisted of descriptive data on the characteristics of cervical SCC, were age, subtype, type of operation, tumor stage, degree of differentiation and p16 expression. The statistical test used to determine the relationship between p16 expression and the

degree of differentiation and tumor stage in cervical SCC is Chi-square. A statistical test is considered significant if the p value <0.05.

## RESULTS

The most cases of cervical SCC were found in the 41-50 years old age group, amount of 12 cases. Univariate analysis consisted of descriptive data on the characteristics of cervical SCC, were age, subtype, type of operation, tumor stage, degree of differentiation and p16 expression. The statistical test used to determine the relationship between p16 expression and the degree of differentiation and tumor stage in cervical SCC is Chi-square. Statistical tests are considered significant if the p-value is <0.05 (34.3%), with an average age of 50.60 years old and an age interval between 28-80 years old. The most common subtype found in this study was non-keratinized SCC with 24 cases. (68.6%). The most common type of surgery in this study was biopsy with 27 cases (77.1%). The most common tumor stage in this study was advanced stage with 30 cases (85.7%). The characteristics of the research sample can be seen in Table 1.

Table 1. Clinicopathological characteristics of cervical SCC.

Variable	f=35	%	X ± SD
Age (years)			50.60±12.973
<31 years old	3	8.6	
31-40 years old	4	11.4	
41-50 years old	12	34.3	
51-60 years old	9	25.7	
61-70 years old	5	14.3	
>70 years old	2	5.7	
Minimum	28		
Maximum	80		
Subtype			
Keratinized SCC	11	31.4	
Non-Keratinized SCC	24	68.6	
Type of operation			
Biopsy	27	77.1	
Hysterectomy	8	22.9	
Tumor stage			
Early	5	14.3	
Advanced	30	85.7	
Degree of differentiation			
Well Differentiation	11	31.4	
Moderate differentiation	18	51.4	
Poor differentiation	6	17.1	
p16 expression			
Low expression	13	37.1	
High expression	22	62.9	

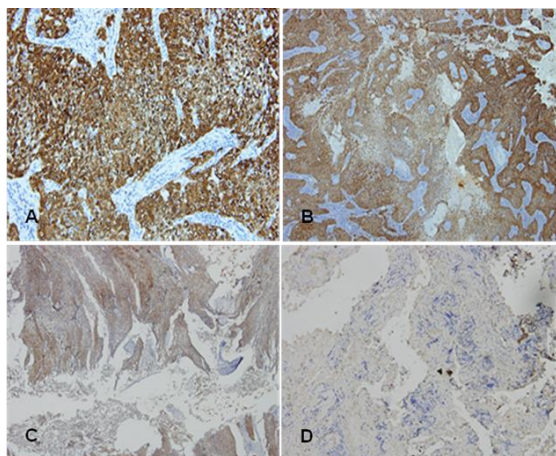


Figure 1. Image of p16 expression in cervical SCC (original magnification 200 times). A. High expression (score 3+5=8). B. High expression (score 2+5=7). C. Low expression (score 1+4=5). D. Low expression (score 0+0=0).

Table 2 shows the percentage of well differentiation degrees in cervical SCC, which is higher in high p16 expression compared to low p16 expression (40.9%:15.4%). Statistically there is no relationship between p16 expression and the degree of differentiation with a value of  $p=0.138$ . The results of p16 immunohistochemical staining can be seen in Figure 1.

Based on Table 3, it can be seen that the percentage of advanced tumor stages in cervical SCC is higher for p16 high expression compared to p16 low expression (90.9%:76.9%). Statistically, there is no relationship between p16 expression and tumor stage with a value of  $p=0.337$ .

Table 2. Relationship between p16 expression and the degree of differentiation in cervical SCC.

p16 expression	Degree of differentiation						Total		p-value
	Well		Moderate		Poor				
	f	%	f	%	f	%	f	%	
Low expression	2	15.4	7	53.8	4	30.8	13	100	0.138
High expression	9	40.9	11	50.0	2	9.1	22	100	

Table 3. Correlation of p16 expression with tumor stage in cervical SCC.

p16 expression	Tumor Stage							Total	p-value	
	Early		Advanced		Poor					
	f	%	f	%	f	%	f			
Low expression	3	23.1	10		Low expression	3	23.1	10	Low expression	3
High expression	2	9.1	20		High expression	2	9.1	20	High expression	2

## DISCUSSION

In this study, it was found that the largest age group of cervical SCC sufferers was the 41-50 years old age group, were 34.3% with the youngest age being 28 years old (non-keratinized SCC with poor differentiation) and the oldest age was 80 years old (non-keratinized SCC with moderate differentiation). The average age of the entire sample in this study was  $50.60 \pm 12.973$  years old. Although there have not been many epidemiological studies of cervical SCC in Indonesia, in general the average age of cervical SCC patients in this study is not much different. Research conducted by Dina et al (2019). in Palembang reported that the majority of cervical SCC sufferers in Palembang are 51-60 years old.<sup>14</sup> HPV infection is the beginning of the process of SCC carcinogenesis, but there are

other factors such as HPV infection cofactors, which cause reaches decades changes to become precancerous lesions, even invasive cancer. This process takes a long time to arrive, therefore the highest incidence of KSS occurs at ages >50 years old.<sup>12,15</sup>

In this study, cases of non-keratinized SCC were reported as the most common cases with 24 cases (68.6%) and 11 cases of keratinized SCC (31.4%). Research conducted by Kalyani et al, (2020) Keratinized KSS was found to be the largest number of cases at 90.6% and non-keratinized KSS was 9.2%.<sup>16</sup> In the research of Dina et al (2019), reported keratinized and non-keratinized cases as the most common KSS cases, with a distribution of 6 cases (50%) and 5 cases (41.7%) respectively.<sup>14</sup> According to the WHO book female genital tumor (2020) the

histopathological subtypes are non-keratinized and basaloid occurs in the majority of cases of HPV-related SCC. Meanwhile, the keratinized histopathological subtype constitutes the majority of SCC cases not related to HPV.<sup>8</sup>

The majority of samples in this study were obtained by biopsy as much as 77.1% compared to hysterectomy as much as 22.9%. The same thing in the research of Dina et al (2019) found that the largest number of samples obtained by biopsy was 75% compared to 25% by hysterectomy.<sup>14</sup> In the research of Anu et al (2018), obtained the largest number of samples from hysterectomy, 96 cases and from biopsy, 34 cases.<sup>17</sup>

The most common tumor stages found in this study were advanced stages with 30 cases (85.7%), followed by early stages with 5 cases (14.3%). According to research by Sarbashis et al (2021), found that advanced stages were the most cases, namely 83.0%.<sup>18</sup> The same thing was also found in research conducted by Inmaculada et al (2020), was the advanced stage with the highest number of cases at 60%.<sup>19</sup> Generally, cervical cancer patients are diagnosed at an advanced stage due to the lack of implementation of the cervical cancer screening program.<sup>20</sup> The pap smear test as a screening test has helped reduce the incidence of cervical cancer by 75%, especially in developed countries. However, this test has several limitations such as low sensitivity, false negative results and low reproducibility. To overcome this, the p16 biomarker has been used as an alternative and as a surrogate marker for in-situ and advanced stage cervical cancer.<sup>16</sup>

In this study, p16 high expression was found in 22 cases (62.9%) and p16 low expression in 13 cases (17.1%). In research conducted by Priyatharsini et al (2020) found 100% of cases with p16 high expression.<sup>13</sup> p16 high expression was significantly associated with longer survival in cervical SCC cases, regardless of other clinical parameters such as age, stage and vascular invasion. However, several reports have linked p16 overexpression to poor prognosis. It is thought that HR-HPV associated tumors are less genetically influenced, and therefore tend to respond better to therapy and have better outcomes.<sup>6</sup>

The percentage of well differentiation degrees in cervical SCC was higher in high p16 expression compared to low p16 expression (40.9%:15.4%). Statistically there is no relationship between p16 expression and the degree of differentiation with a value of  $p=0.138$ . According to research by Kalyani et al (2020), there was no significant relationship between histopathological grade and p16 expression ( $p=0.877$ ). However, the majority of cases of well differentiated squamous cell carcinoma WDSCC (88.0%) and moderately differentiated squamous cell carcinoma MDSCC (93.7%) showed a positive p16 expression block, although there was no statistical significance.<sup>16</sup> According to research by Vatsala et al (2017), p16 high expression is found in 75% of cervical cancer cases.<sup>21</sup>

The intensity of p16 staining increased with increasing degree of differentiation ( $p=0.0002$ ). This is consistent with the predilection of high-risk HPV as the etiology of relatively undifferentiated cervical squamous cell carcinoma according to the study of Sarbashis et al.<sup>19</sup> The expression of p16INK4A increases progressively with increasing severity of cervical dysplasia. Significant positive expression for p16INK4A and giving a high expression score indicates the association of HPV with most cases of cervical carcinoma.<sup>21</sup>

The difference in results obtained between this study and several previous studies could possibly caused by the assessment of the degree of differentiation between several researchers also varying based on the researcher's subjectivity. Another thing could also be because the interpretation of p16 from various studies varies. As in all immunohistochemical techniques, other factors that may influence the results must be considered, including the fixative used for the tissue, duration of fixation, size of the tissue being fixed, method of antigen retrieval.

Based on tumor grade, p16INK4a was diffusely expressed in 60% of CIN1 (low-grade lesions) and was mostly associated with HR-HPV genotype, but the presence of HR-HPV was detected in p16INK4a negative tumors. In addition, a high incidence of HR-HPV has been observed among young women and most of these infections are transient and spontaneously regress. Therefore, it can be concluded that

p16INK4a overexpression is already an indication of advanced viral interference that progresses towards invasive cancer. Apart from active viral replication, there are significant morphological changes occurring at the histological, cellular and molecular levels that can be assessed visually. However, in invasive cervical cancer, HPV protein expression is not detected by IHC and HPV genotyping in formalin fixed paraffin embedded (FFPE) tumor samples.<sup>20</sup>

Overexpression of p16 in cervical SCC, thought to be due to inhibition of the retinoblastoma protein (Rb) by the HPV E7 protein triggers a negative feedback loop leading to over-expression of p16, in an effort to stop cell proliferation. HR-HPV-associated tumors are less genetically altered, and therefore less likely to respond to therapy better and get better results.<sup>6</sup>

Abnormal status of CDKN2A is one of the reasons p16 is negative. The p16 protein is encoded by CDKN2A which is a tumor suppressor gene. The p16 product inhibits G1–S progression by preventing phosphorylation of the RB protein and preventing activation of the transcription factor, E2F1, leading to uncontrolled cell cycle. Therefore, loss of CDKN2A function due to hypermethylation or deletion of the promoter results in RB protein phosphorylation and carcinogenesis.<sup>22</sup>

Cervical cancer with p16 expression has a better prognosis. p16 high expression in cervical cancer was reported to have high five-year overall survival and statistically significant disease-free survival (DFS). Five-years overall survival in high and p16 low expression was 62.0% and 35.2%, respectively. DFS in high and p16 low expression was 60.0% and 31.2%, respectively. The p16 protein is also an indicator of radiosensitivity. Because of its anti-cancer activity, p16 can be utilized for the development of targeted chemotherapy for cervical cancer.<sup>16</sup>

The percentage of advanced tumor stages in cervical SCC was higher for p16 high expression compared to p16 low expression (90.9%:76.9%). Statistically, there is no relationship between p16 expression and tumor stage with a value of  $p=0.337$ .

The p16 biomarker was significantly expressed in cervical cancers from relatively younger age groups and in early-stage disease.

No significant relationship between p16 expression and age at marriage ( $p=0.951$ ), age at menopause ( $p=0.311$ ), parity ( $p=0.554$ ), clinical symptoms, disease stage ( $p=0.28$ ), or histopathological grade was found. in the research of Kalyani et al (2020).<sup>16</sup> Differences in the results obtained in this study from previous studies could be due to variations in the number of cases or different p16 assessment methods and also differences in tumor stage assessment.

The clinical significance of p16INK4a overexpression in cervical cancer has been reported by many researchers. However, the results of these reports are still conflicting. Research by Lin et al showed that low p16INK4a expression indicated a worse prognosis for patients diagnosed with cervical cancer than p16INK4a overexpression. These findings support the theory that HR-HPV is a triggering factor in the development of cervical cancer, but simultaneously induces p16INK4a-mediated protective mechanisms. The reasons remain unclear at present, but it is possible that p16INK4a overexpression can be recognized by the immune system as a low-expressing protein antigen. This p16INK4a protein can ultimately initiate an antitumor response in cervical cancer patients.<sup>11,23</sup> Cervical cancer cells with lower P16INK4A expression have a higher self-renewal ability, an essential ability of cancer stem cells. Furthermore, repression of P16INK4A expression increases cell chemoresistance and radioresistance.<sup>24</sup>

However, the lack of a standard cutoff for positive expression could underestimate the prognostic significance of p16INK4a overexpression. Unclear guidance is available regarding its use in routine practice. Thus, predictive evaluation of the scoring system to reach a consensus on the positive threshold is necessary.<sup>11</sup>

This research has several limitations. Most samples in this study were obtained by biopsy so the pathological stage could not be determined. A further limitation is that only p16INK4A expression was examined. The addition of HPV PCR or IHC analysis may be helpful in clarifying the precise relationship between p16INK4A expression and HPV infection status. Because of the difficulty in correctly and easily determining whether a tumor

has HPV infection, only p16INK4A expression was considered.

## CONCLUSION

p16 expression showed no significant relationship with the degree of differentiation and tumor stage in cervical SCC.

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