

The Relationships between P16^{INK4a} Immunohistochemistry Expression with Subtypes and Histopathological Grading in Squamous Cell Carcinoma of The Penis

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ABSTRACT

Background

Penile squamous cell carcinoma is a malignancy of the epithelial cells of the prepuce or glans penis, occupying 95% of all malignancies in the penis and around 60% caused by Human Papilloma Virus. The average patient age is 50 until 70 years. The p16^{INK4a} immunohistochemistry is a cyclin dependent kinase inhibitor and can be expressed by human papilloma virus. Several studies have tried to relate this expression to eradicate this virus.

Methods

This study use cross-sectional analytical study with 30 samples of histopathological preparations diagnosed as penile squamous cell carcinoma at H. Adam Malik Hospital Medan. 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 and 60% of tumors were located in more than one region. Based on subtypes, 70% displayed a HPV-associated while p16^{INK4a} positive weak in 43,3% and positive strong in 26,7% of all cases. Based on histopathological grading, 50% displayed grade 1 while positive weak in 16,7% and positive strong in 16,7% of all cases. Subtypes correlated well with p16^{INK4a} positivity (p-value $<0,005$) but uncorrelated with histopathological grading (p-value $>0,005$).

Conclusion

There is a significant relationship between p16^{INK4a} and histopathological subtypes, but there was no significant with the grading of penile squamous cell carcinoma.

Keywords: p16^{INK4a}, penile squamous cell carcinoma, penile cancer



INTRODUCTION

Penile cancer is a rare malignancy and still has a high mortality rate in the world,^{1,2} which is the most common type is squamous cell carcinoma.^{3,4,5} Penile cancer has a high incidence and mortality rate in Western countries.^{1,2} In Indonesia, at Sardjito Hospital from 2016 to 2013 there were 35 cases found with an average age of 56 ± 14.4 years.⁶ In Haji Adam Malik Hospital Medan, the incidence of penile cancer over the last four years (2012 – 2015) was 35 patients.^{7,8}

Penile squamous cell carcinoma is closely related to high-risk Human Papilloma Virus (HPV) infections and is the most common cause, it is known that strains 16 and 18 are the most common.³ The evolution of penile squamous cell carcinoma involves two pathways, namely HPV related and HPV unrelated.^{9,10} More than 20% of penile cancer sufferers have positive results for HPV infection.²

The HPV virus can be grouped into high risk and low risk. Integration of high risk types-HPV (hr-HPV) in host cell DNA is associated with malignancy causing high expression of genes produced by HPV, namely oncoproteins E6 and E7, which has a significant carcinogenesis regulation in penile squamous cell carcinoma.¹¹

P16^{INK4a} is a tumor suppressor protein that works as a cyclin-dependent kinase inhibitor and works to regulate the cell cycle. Tumor lesions that are specifically regulated by oncogenic viruses can increase the expression levels of p16^{INK4a}, which is associated with the HPV virus. Immunohistochemical expression of p16^{INK4a} may arise as a result of functional inactivation of the retinoblastoma protein (pRb) against the HPV protein E7 gene. This is what causes increased regulation of the p16 protein. This is specific for squamous cell carcinoma lesions in the head and neck organs, but still needs to be studied for other organs, including the penis. In addition, the immunohistochemical function of p16^{INK4a} is used to evaluate anogenital lesions associated with HPV associated and alternative markers for high-oncogenic risk HPV in cervical squamous cell carcinoma organs as well as in other organs.^{12,13}

According to Ardhaputri KA,et al (2021) study, p16 protein is a tumor suppressor protein that is important in normal cell cycle regulation. The infection process caused by HPV will disrupt cell cycle regulation resulting in a buildup of p16 protein in the cell nucleus. Accumulation will cause uncontrolled cell proliferation and will significantly trigger transformation into malignancy. It is recommended to use p16 immunohistochemistry as a stain for HPV infection and determine histological classification.¹¹

Grading assessment is an assessment of the degree of cellular differentiation which is important as a predictive factor for metastases to lymph nodes, also known as pathological grading. What is assessed is core pleomorphism consisting of well-differentiated to poorly-differentiated and also keratin production. Grading predicts the presence of lymph node metastasis, where regional lymph node metastasis is an important risk factor in predicting survival in penile squamous cell carcinoma.¹⁴ According to Sali et al, the tumor infiltrating growth pattern can be correlated with tumor grading and also tumor depth.¹⁵

Several studies have linked the immunohistochemical expression of p16^{INK4a} as a prognostic factor in breast and esophageal organs but still needs more data. According to Langendijk and Psyrri (2010), in cases of oropharyngeal squamous cell carcinoma, they concluded that patients with p16^{INK4a} overexpression had a better prognosis and clinical condition.¹⁶ Eradication of HPV early in the development of the disease is important in preventing disease progression and the spread of tumor lesions to the lymph nodes affects therapy.^{5,17} Without effective and adequate treatment, mortality will increase with complications due to local lesion growth and metastasis.¹⁷ This study aims to see how the immunohistochemical expression of p16^{INK4a} is related to subtypes and histopathological grading in penile squamous cell carcinoma.

METHODS

This research is analytical study with a cross-sectional approach. The research was taken in the Haji Adam Malik Central Hospital, Medan. The population of this study consisted of



all secondary data and paraffin blocks from patients who were histopathologically diagnosed with penile squamous cell carcinoma and meet with inclusion and exclusion criteria in the Department of Anatomical Pathology, Faculty of Medicine, University of North Sumatra and the Anatomical Pathology Unit of Haji Adam Malik Hospital, Medan. The sample numbers in this study was 30 samples. The variables in this study were immunohistochemical expression of p16^{INK4a} as the independent variable and age, tumor region, subtypes, grading, 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 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). The Staining Index (SI) is calculated by multiplying the intensity & proportion scores. SI score 0=negative, 1-3= as a tumor with weak expression and score 4-9 =as a tumor with weak expression.^{11,18}

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.^{11,18}

The data obtained were analyzed using the SPSS program, with the chi-square test or Fisher's exact test. The p value < 0.005 , is declared statistically significant.

RESULTS

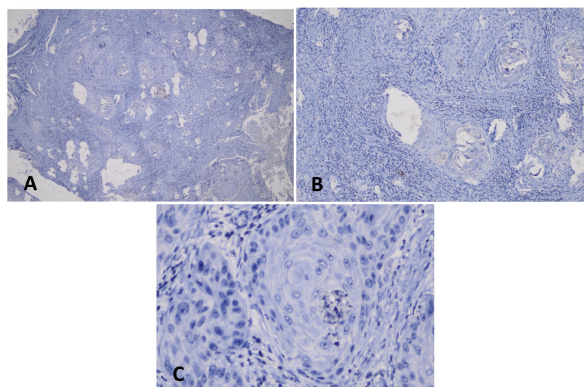
This study using 30 samples of penile squamous cell carcinoma from Pathology Laboratory, Faculty of Medicine, University of North Sumatra, and the Anatomic Pathology Unit, H. Adam Malik Hospital, Medan.

Table 1. Characteristics of samples of patients with penile squamous carcinoma.

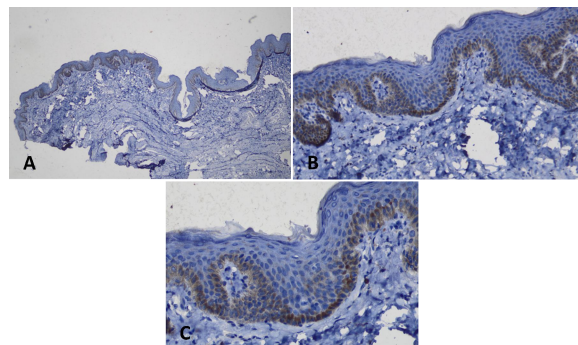
Variable (n=30)	Frequency (f)	Percent %
Age (years old)		
30-39	3	10.0
40-49	13	43.3
50-59	7	23.3
60-70	6	20.0
>70	1	3.3
Subtype		
HPV-Associated	9	30.0
HPV-Independent	21	70.0
Tumor region		
Glans	12	40.0
Sulcus coronal	0	0.0
Corpus penis	0	0.0
>1 region	18	60.0
p16 ^{INK4a} expression		
Negative	9	30.0
Weak positive	13	43.3
Strong positive	8	26.7
Grading		
Grade 1	15	50.0
Grade 2	6	20.0
Grade 3	9	30.0

From this study, the highest percentage of penile squamous cell carcinoma sufferers were aged 40-49 years (43.3%), while the youngest age is between 30-39 (10.0%) years and the oldest age is ≥ 70 years (3.3%). The most histopathological subtype was HPV-associated SCC of the penis namely 21 cases with a percentage of 70%, while HPV-independent SCC of the penis was found in 9 cases with a percentage of 30%. There were 18 cases (60%) more than one region location, meanwhile in the region of the coronal sulcus and corpus of the penis it was not found, whereas in the region of the glans it was found 12 cases (40%). The most common grading is grade 1 with 15 cases (50%), then grade 3 with 9 cases (30%), then the least is grade 2 with 6 cases (20%). From the table found the diffusely weak positive expression of p16^{INK4a} was 13 cases (43.3%), followed by no expression (0%) namely 9 cases (30.0%), then only 8 cases appeared strong positive expression (26.7%).

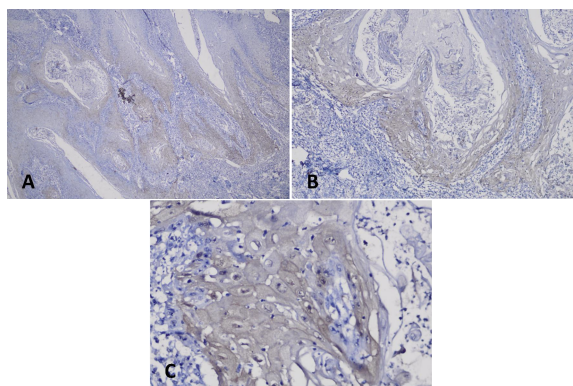




Picture 1. (A,B,C) Negative p16^{INK4a} expression staining (40 times magnifications, 100 times magnifications, 200 times magnifications)



Picture 3. (A,B,C) Strong positive p16^{INK4a} expression staining (40 times magnifications, 100 times magnifications, 200 times magnifications)



Picture 2. (A,B,C) Weak positive p16^{INK4a} expression staining (40 times magnifications, 100 times magnifications, 200 times magnifications)

Tabel 2. The relationships between p16^{INK4a} immunohistochemical expression and penile squamous cell carcinoma histological subtypes.

p16 ^{INK4a} ekspresion										value	p-value
Subtype	Negative		Weak positive		Strong positive		Total				
	n	%	n	%	1,00	%	n	%			
Independent	9	30.0	0	0.0	0	0.0	9	30	1.00	0.00	
Associated	0	0.0	13	43.3	8	26.7	21	70			
Total	9	30.0	13	43.3	8	26.7	30	100			

Somers'd test

Tabel 3. The relationships between p16^{INK4a} immunohistochemical expression and penile squamous cell carcinoma histological grading.

p16 ^{INK4a} ekspresion										value	p-value
Grading	Negative		Weak positive		Strong positive		Total				
	n	%	n	%	n	%	n	%			
Grade 1	5	16.7	5	16.7	5	16.7	15	50.0	-0,051	0,749	
Grade 2	2	6.7	2	6.7	2	6.7	6	20.0			
Grade 3	2	6.7	6	20.0	1	3.3	9	30.0			
Total	9	30.0	13	43.3	8	26.7	30	100			

Somers'd test

From the table above, it was obtained that the subtype of HPV-independent SCC of the penis with negative p16^{INK4a} expression namely 9 cases (30.0%), while for weak positive p16^{INK4a} expression namely 13 cases (43.3%) and the results of a few strong positive stain were 8 cases (26.7%). This data was analyzed statistically using the Somers'd test, obtaining a p-value=0.000 (significant p-value<0.005). From the statistic results can be concluded that there is a significant relationship between penile squamous cell carcinoma subtype and p16^{INK4a} immunohistochemistry. The most common histopathological grading was grade 3 with weak p16^{INK4a} expression in 6 cases (20,0%), followed by grade 1 with negative, weakly and strong positive p16^{INK4a} expression in 5 cases (16.7%) and grade 2 with negative, weakly and strong positive p16^{INK4a} expression in 2 cases (6.7%). This data was analyzed statistically using the Somers'd test, obtained p-value=0.749 (p-value>0.005 significant). So statistically it can be concluded that there is no relationship between the histopathological grading of penile squamous cell carcinoma and p16^{INK4a} immunohistochemistry.

DISCUSSION

The number of penile squamous cell carcinoma samples that met the inclusion and exclusion criteria in the Anatomical Pathology Unit of Haji Adam Malik Hospital, Medan, was 30 samples, with a highest age group was 40-49 years old. According to Ertoy Baydar et al (2020) that the average age at diagnosis of penile squamous cell carcinoma is 51-83 years old. Patients who are older, especially those over the age of >50 years old, are at high risk of developing penile squamous cell carcinoma.¹⁹ In Brazil, the incidence of this disease was found to be 2.8-6.8 per 100,000 men.² The age group that most often suffers from this malignancy is 50 to 70 years with the location most often found in the glans region of the penis.^{2,3,5,6,18,20} There are several factors that support the relationship between age and the onset of cancer, including cellular damage, cellular senescence, and decreased immune function.²¹ With the increasing medical care and living conditions of a person in developing countries, the health and life expectancy of a

person from the age of 50 to the age of 80 is also increasing.²²

Squamous cell carcinoma of penis is an epithelial malignancy that originates from the preputium and glans epithelial.⁶ In this study, the largest number location of lesion is in more than one locations (mix), namely 18 cases (60%). Materson et al (2017) in his study claim that glans and prepuce are the most common location, related to the ease of metastases to the lymph nodes so that the prognosis of the disease becomes poor.²³ Primary penile squamous cell carcinoma can attack any part of the penis. Usually it affects the glans region most often, but it can also affect the prepuce region, both the prepuce and glans parts at once, the coronal sulcus, and the shaft.²⁴

Penile squamous cell carcinoma is closely related to HPV virus infection, where more than 20% of all penile cancers are related to HPV infection.^{2,3} The pathogenesis of HPV in infecting the human body has several processes. In the squamous epithelial layer, HPV infects in two ways, namely as viral infection and viral-associated precancerous lesions.²⁴ In this study, the subtypes of invasive penile squamous cell carcinoma under study were HPV-associated squamous cell carcinoma and HPV-independent squamous cell carcinoma.⁵ Penile malignancies induced by HPV infection develop through precursor lesions of penile intraepithelial neoplasia (PeIN), after inactivation of the host cell-cycle regulatory protein with binding of the E7 oncoprotein of the high-risk HPV genotype to hypophosphorylated retinoblastoma protein and binding of the E6 oncoprotein of the high-risk HPV genotype to p53. The subsequent proliferation of autonomous cells causes nuclear accumulation of the cyclin-dependent kinase inhibitor p16 (p16^{INK4a}).^{5,25}

In this study, HPV-associated SCC is the most common subtype, namely 21 cases (70%), with the most immunohistochemical expression of p16^{INK4a} being weakly positive, namely 13 cases. Santos RS et al (2022) said that it was found that 53.2% of penile SCC sufferers were at high risk of HPV. HPV infection is not associated with metastasis to lymph nodes, it is systemic and does not affect locoregional recurrence. However, it is related to long-term changes in DNA methylation that



induce carcinogenesis in penile cancer.²⁶ Ardhaputri K et al (2021) said that from 142 cases of penile squamous cell carcinoma from her research, 68 cases were found to be positive for p16 protein expression (47.9%). She explain that p16 protein is a tumor suppressor protein which is important in normal cell cycle regulation, the infection process caused by HPV will disrupt cell cycle regulation resulting in a buildup of p16 protein in the cell nucleus. It is recommended to use p16 immunohistochemistry as a strtain for HPV infection and determine histological classification.¹¹

This research is also in line with Olesen TB (2018), of the 4199 cases studied, half of them were related to HPV DNA virus infection. The most common subtype variants associated with the HPV virus are basaloid carcinoma and warty carcinoma. A total of 79.6% had p16^{INK4a} immunohistochemical expression, which was due to regulation by the HPV oncogene. It is also necessary to examine HPV DNA together with p16^{INK4a} to determine whether the patient has an HPV virus infection or not. He also stated that HPV16 is the most dominant type of HPV strain in penile squamous cell carcinoma and penile intraepithelial neoplasia.²⁵

Several recent studies have also revealed that the HPV virus has no association with any histological prognostic parameters, except with basal histological tumor subtype. According to Ferrandiz-Pulido et al (2013), usual high risk HPV-related penile carcinoma appears to have a good prognosis when compared with non-related HPV. Where cases with HPV-independent have higher survival compared with HPV-associated SCC of the penis. There are two pathways that support carcinogenesis, namely dependent HPV and independent HPV. HPV dependent is said to be biologically different and has a fairly good response to therapy.²⁷

Immunohistochemistry is an important clinical support in the service of pathological examination of a disease. This examination is useful in supporting the accuracy of a diagnosis, in some cases helping to determine therapy and also predict prognosis.²⁸ Determination of grading is an important prognostic factor and also a predictive factor for metastasis. The presence of spread to the lymph nodes is a

prognostic factor related to the survival of penile squamous cell carcinoma patients. Histological grading and primary tumor stage are relevant criteria in selecting patients for inguinal lymphadenectomy.²⁹ p16 is a tumor suppressor protein and is a cyclin-dependent kinase inhibitor. p16 is essential in cell cycle regulation, where it works by inactivating cyclin-dependent kinase which phosphorylates Rb, so that p16 can accelerate the cell cycle. In infections caused by HPV, the HPV oncogenes E6 and E7 inactivate pRb resulting in overexpression of p16. In addition, overexpression of p16 is a surrogate biomarker of HPV virus infection (most of which are high-risk types of HPV).¹²

The p16 protein was identified as a low molecular weight protein bound to cyclin dependent kinase 4 and 6 (CDK4 and CDK6). By binding p16^{INK4a} to CDK4 or CDK6 it will disrupt or damage its binding to cyclin D1, D2 or D3 which is very useful as a catalyst.^{13,30,31} With the activation of this catalyst complex, it initiates phosphorylation of RB-tumor suppressor in the early/middle phase of G1 in the cell cycle. RB protein negatively regulates the activity of E2F-transcription factor, this controls the expression of genes needed in nucleotide biosynthesis, DNA synthesis, mitotic activity progression, DNA damage repair activities.^{13,30,31}

High levels of p16^{INK4a} are also found in some pre-malignant lesions, which some researchers suggest contributes to stopping progression of the lesions.¹³ The p16 immunohistochemical smear uses a ready-to-use p16 monoclonal primary antibody for humans and is positive in the nucleus and/or cytoplasm of tumor cells.^{11,18} Patients with squamous cell carcinoma of the penis, whether they have had a partial penectomy or total penectomy, also need to consider removing the lymph nodes, because it is thought to have metastasized and spread to the lymph nodes so it can be used as a reference for determining the primary tumor.³²

From the research results, it was found that the highest grade was grade 1 with p16^{INK4a} immunohistochemical stained being the same for those that were negative stain, those that were stained weakly positive and those that were strongly positive. This is not in line with research by Laksmi et al (2022), where the



highest grade found was grade 2 with 15 cases, followed by grade 3. Tumor grade can predict the presence of lymph node metastases and is related to survival rates.¹⁴ This is also not in line with Sali AP et al (2019), in his research they found that the highest grade was grade 2.¹⁵ However, this result is in line with Chaux A, et al (2015), found 20 cases (54%) grade 1.³³

Grading assessment is an assessment of the degree of cellular differentiation which is important as a predictive factor for metastases to lymph node nodes, also known as pathological grading. What is assessed is core pleomorphism consisting of well-differentiated to poorly-differentiated and also keratin production.¹⁴ Grading predicts the presence of lymph node metastasis, where regional lymph node metastasis is an important risk factor in predicting survival in penile squamous cell carcinoma.^{14,34} In addition, 15%-53% of penile squamous cell carcinoma patients who show lymph node nodules that can be palpated during physical examination may indicate underlying inflammatory pathology.¹⁵ According to Sali et al, the growth pattern of infiltrating tumors can be correlated with tumor grading and also the depth of the tumor, meanwhile there is also an infiltrating pattern at grade 1, namely the appearance of cords and trabeculae.¹⁵ Low-grade infiltrating tumors, such as verrucous carcinoma, have a wide spread. low to the lymph nodes.³³

CONCLUSION

The age of most sufferers is in the 40-49 year age range. Most tumor locations are more than one region. The most common histopathological subtype is HPV-associated SCC of the penis. The most common histopathological grading is grade 1. The most negative p16^{INK4a} immunohistochemical expression was in the HPV-independent subtype, the most weakly positive expression were in the HPV-associated subtype, the most strongly positive expression were in the HPV-associated subtype. There was a significant association between p16^{INK4a} immunohistochemistry and subtype. There is a significant relationship between penile squamous cell carcinoma subtype and p16^{INK4a} immunohistochemistry but there is no relationship between

the histopathological grading of penile squamous cell carcinoma and p16^{INK4a} immunohistochemistry.

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