RESEARCH ARTICLE

Over-expression of Ki-67 as a predictor of lymph node metastasis in penile cancer patients [version 1; peer review: 2 approved with reservations]

Syah Mirsya Warli , Ginanda Putra Siregar

Department of Urology, Universitas Sumatera Utara Hospital, Universitas Sumatera Utara Hospital, Jl. Dr. T. Mansyur no. 66, Medan, 20155, Indonesia

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Abstract

Introduction: Penile cancer is a moderately common malignancy in developing countries. Metastasis to regional lymph nodes is an essential factor in a patient's prognosis, as its occurrence predicts poor patient prognosis. As micro-metastasis occurs in more than 25% of cases, the need for more accessible diagnostic tools is necessary. Ki-67 is commonly used as a marker of proliferation associated with tumor grade and lymph node metastasis.

Methods: Samples were taken from penile cancer patients between 2013 to 2018, in the form of *formalin-fixed paraffin-embedded* (FFPE) blocks were analyzed. Patient demographic data, current and pre-cancer condition, cancer staging, outcomes, and other results of adjuncts and treatment modalities were obtained from medical records. Immunohistochemistry analysis was carried out on FFPE preparations. Under 20% of nuclei stained was considered as low-expression and more than 20% of nuclei stained was considered as Ki-67 over-expression. Data processing and analysis were carried out using SPSS software.

Results: In total, 48 FFPE samples were analyzed, with a mean patient age of 50.79 (± 9.51 SD). For all patients, the type of pathology was squamous cell carcinoma. Node metastasis was positive in 34 patients (70.8%) and negative in 14 patients (29.2%). Statistical analysis was carried out using the Chi-Square test, resulting in a significant correlation between the expression of Ki-67 and lymph node metastasis in penile squamous cell carcinoma ($p=0.045$).

Conclusion: Over-expression of Ki-67 were found in penile cancer patients with lymph node metastasis. Therefore, Ki-67 might be useful in predicting lymph node metastasis in penile cancer patients.

Keywords

Penile Cancer, Lymph Node Metastasis, Ki-67, Molecular Marker, IHC

Open Peer Review

Approval Status ? ?

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1. Juan Chipollini , University of Arizona, Tucson, USA		
2. Yudhistira Pradnyan Klopang , Universitas Airlangga, Surabaya, Indonesia		
Wahjoe Djatisoesanto , Universitas Airlangga, Surabaya, Indonesia		

Any reports and responses or comments on the article can be found at the end of the article.

This article is included in the **Oncology** gateway.

Corresponding author: Syah Mirsya Warli (war_uro@yahoo.com)

Author roles: **Warli SM:** Conceptualization, Data Curation, Funding Acquisition, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Siregar GP:** Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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Introduction

Penile cancer is one of the rare malignancies in men in developed countries. In the UK, there are 600 new cases each year, and the incidence rate has remained below 1 per 100 000 men over the years. Incidence rates are 2 - 3%, and the frequency is higher in old to middle-aged men, with a peak incidence in the sixth decade of life¹. Penile cancer is a moderately common malignancy in the developing countries, specifically squamous cell carcinoma of penis, which accounts for 95% of all cases^{2,3}. Risk factors for penile cancer include uncircumcision, phimosis, cigarette smoking, and human papillomavirus (HPV) infection⁴.

Examination of primary lesions, regional lymph nodes, and distant metastasis is beneficial in determining the management of penile cancer. Moreover, regional lymph node involvement can also be an essential factor in patient prognosis, as its occurrence is a predictor of poor prognosis. Lymphatic metastasis in penile cancer follows the anatomical drainage route, which originates from the inguinal lymphatic nodes, then drains to both superficial and deep and pelvic lymphatic nodes.

As it holds an important role in predicting patient's prognosis and survival, diagnosis and treatment of lymph node metastasis is essential. Physical examination is sometimes inaccurate and cannot be a reliable predictor. Patients who have a palpated inguinal lymph node may experience micro-metastasis, which occurs in more than 25% cases. To increase diagnostic accuracy and prevent unnecessary lymphadenectomy, several biomarkers have been investigated for easier diagnostics⁵.

The Ki-67 protein is a marker for predicting prognosis in cancers. The Ki-67 protein is a nuclear antigen that is expressed in the cell division phase, especially the G2/M phase and controls the timing of the mitosis process⁶. Because Ki-67 is expressed during the active phase of the cell cycle, it considered as a useful marker for cell division activity. Ki-67 is widely used as a proliferation marker related to tumor grade and lymph node metastasis in penile cancer^{7,8}. In this study, we will look at the association of Ki-67 with lymph node metastasis in patients with penile cancer.

Methods

Ethical statement

Before penile cancer samples were analyzed, the research team informed patients and all patients consented to participate. The study was explained verbally, and written consent was obtained in the form of a patient signature. For patients who could not read or write, we obtained the signature of their family member as a witness or the patient's finger print. Ethical clearance was obtained from Experimental Research Ethical Committee of University of Sumatera Utara (455/TGL/KEPK FK USU-RSUP HAM/2019).

Participants

Samples were collected from penile cancer patients between 2013 and 2018. Research was conducted between August to November 2019. The samples were taken using total sampling

of all penile cancer patients during this period. All of the samples included in this study were from men who fulfilled the eligibility criteria: penile cancer patients prior to any surgical intervention, chemotherapy, or radiotherapy for penile cancer; those who had an excisional biopsy sample; and those who agreed to participate in the study. The exclusion criteria for this study was a history of other malignancies.

Sample and data collection

The samples were collected as a part of routine medical care, and the additional analysis of Ki-67 was based on the histopathology sample availability. The samples were taken directly from the lesion of the patients using frozen section excisional biopsy. A urologist performed the excisional biopsy using a standard No. 20 blade under local anesthesia using 4 cc of 2% lidocaine HCl. After that, the samples were immediately fixed with 10% formalin buffer. After fixation, the tissue was dehydrated by soaking in alcohol from a low to a high concentration (70% → 95% → 100% → 100% → 100%). The next stage was purification by soaking tissue into a xylol solution twice, once for 60 minutes and second for 120 minutes, so that a transparent/clear tissue is obtained. The next step is impregnation of paraffin wax by infiltration of paraffin wax into the tissue so that the tissue becomes dense and stiff. The tissue containing paraffin wax is put in hot paraffin wax liquid, which is provided in small boxes. When this cassette is cooled, a paraffin block is formed, which contains the tissue inside. The next step is to cut the paraffin block containing tissue using a microtome with a tissue thickness of 4μ. Paraffin embedded tissue section was enhanced by pretreatment with citrate plus (ScyTek catalog# CPL500) or 10mM citrate buffer, pH 6.0 (ScyTec catalog# CBB500). Samples in the form of formalin-fixed paraffin-embedded (FFPE) blocks were analyzed in the Pathology Department, University of Sumatera Utara.

Patient demographic data, current and pre-cancer condition, cancer staging, outcomes and other results of adjuncts and treatment modalities were obtained from medical records. Lymph node invasion had been assessed from either physical examination, or imaging modalities such as multi-slice computed tomography (MSCT) with intravenous contrast, and the outcome was extracted from medical records for analysis in this study.

Sample processing

Immunohistochemistry examination was carried out on FFPE preparations. Every sample slice was warmed to a temperature of 58°C and afterwards, Xylol was used for deparaffinization (ethanol-xylene) 25% (I), 50% (II) and 70% (III) for 10 minutes each. The sample was then rehydrated with antibody solution (primary antibody was optimized in solution for use with the recommended Diagnostic BioSystems Detection System, catalog# A00095, and did not need further dilution). The primary KI-67 antibody used was rabbit polyclonal antibody (Diagnostic BioSystems, catalog# A00095). For fluorescent IHC staining of paraffin-embedded tissue sections using the KI-67 antibody, the antibody was incubated with the tissue overnight at 2–8 °C. This incubation regime optimizes

specific binding of antibodies to tissue targets and reduces non-specific background staining. Antibody was incubated for 30 - 60 minutes at room temperature. From this step forward, samples were protected from light. After that, samples underwent heat-induced epitope retrieval using a boiler at 125°C at pH 6 and were cooled at 90°C for 2-10 minutes. Enzyme blocking was endogenously carried out using H₂O₂ 0.3% and ethanol 95%. After that, the specimen was counterstained with hematoxylin. Samples were then dehydrated, cleaned (using Xylol I [25%], II [50%], III [70%]), and placed on a slide. Inspection was carried out visually using light microscopy by two examiners. Microscopic calculations were carried out in 10 visual fields containing a minimum of 500 cells using ImageJ version 1.50⁹ application at 40x magnification. Expression of the Ki-67 protein was considered positive if brown overstaining appeared in the nucleus and cytoplasm of the cell. Expression in under 20% of stained nuclei was considered as low-expression while more than 20% of stained nuclei was considered as Ki-67 over-expression.

Statistical analysis

Data processing and analysis were carried out using SPSS Software version 25. Because the data we collected is categorical, we used the chi-square categorical test using SPSS.

Results

Samples were taken from 48 men, with a mean age of 50.79 (± 9.51 SD). The pathology of all patients was squamous cell carcinoma. Ki-67 expression level was divided into two groups; more or less than 20%. Ki-67 expression ranged from 0% to 100%, with a median value of 4.0%¹⁰. From 48 samples, 30 patients (62.5%) were found to have Ki-67 expression equal to or less than 20%, while in 18 patients (37.5%), expression of Ki-67 was more than 20%. Patient's characteristics are shown in Table 1.

Node metastasis was found to be positive in 34 patients (70.8%) and negative in 14 patients (29.2%). Statistical analysis using the chi-square test resulted in a significant correlation between expression of Ki-67 and lymph node metastasis in penile squamous cell carcinoma ($p=0.045$) Table 2.

Discussion

The prognosis of penile squamous cell carcinoma is heavily influenced by the incidence and severity of local lymph node metastasis¹¹. It is characterized by the slow growth of the tumor. However, advanced regional tumor stages are often observed. Due to the low occurrence of penile squamous cell carcinoma, limited information on molecular markers for its occurrence is available^{12,13}.

Lymphadenectomy is currently the gold standard in lymph node metastasis, and it has a strong therapeutic effect if performed early. However, there is a high complication risk associated with inguinal lymphadenectomy such as surgical site infection, flap necrosis, lymphocele formation, and lymphedema that correlate with wide excision of lymphatics, devascularization

Table 1. Patient characteristics.

Variable	
Samples	48
Age (Mean $\pm$ SD)	50.79 $\pm$ 9.51
Staging	
T1	3 (6.3)
T2	11 (22.9)
T3-T4	34 (70.8)
Nodal metastasis	
N+ (n%)	34 (70.8)
N0 (n%)	14 (29.2)
Ki-67 expression	
$\leq 20\%$ (n%)	30 (62.5)
$> 20\%$ (n%)	18 (37.5)
Median (min-max)	4.5 (0-100)

Table 2. Expression of Ki-67 and lymph node metastasis.

Ki-67 expression	Nodal metastasis		p-value
	N0	N+	
$\leq 20\%$	11	16	0.045 ¹
$> 20\%$	3	18	

¹ Chi-Square test

of skin flaps, and interruption of collateral vessels and lymphatics¹⁴.

Hence, finding a valid marker for the incidence of metastasis of the lymph node can lead to a significant improvement in penile squamous cell carcinoma therapy, especially to avoid overtreatment. In this research, we examined the association of Ki-67 labeling with related clinicopathological variables and patient survival in primary penile cancer.

During all phases of the cell cycle except G0, Ki-67, which is a non-histone protein in the nuclear matrix, is expressed. The Ki-67 antigen can be found within the nucleus during interphase, whereas most of the protein is transferred to the chromosome surface in mitosis. The fact that the Ki-67 is majorly expressed during the active phase of the cell cycle makes it an excellent marker for evaluating a given cell population's so-called growth fraction¹⁵.

To our knowledge, the association between penile Ki-67 expression and clinicopathological variables for squamous cell carcinoma has not been reported in the past. Studies show that there is a relationship between Ki-67 proliferation frequency and tumor dedifferentiation, nodal involvement, or progression

of disease for head and neck squamous cell carcinomas. The lack of evidence for these variables could be due to too few patients^{16–18}.

Some previous studies have examined whether there is an association between the frequency of lymph node metastasis, the extent of invasion, and lymphatic and venous embolism, but with contradictory results^{12,19,20}. Emerson *et al.*¹⁹ found that in patients with penile squamous cell carcinoma, the extent of stromal and vascular tumor invasion was predictive of cancer progression.

High levels of Ki-67 expression has been reported to be of the prognostic predictors in several squamous cell carcinomas, such as the larynx or cervix uteri, but there is little data on Ki-67 expression in penile squamous cell carcinoma^{8,21,22}. Penile cancer itself has three different sub-types histologically. Protzel *et al.*²² stated that there is a strong correlation between Ki-67 expression with the basaloid sub-type of penile cancer, while verrucous and warty types showed only medium or weak Ki-67 expression.

Stankiewicz *et al.*²³, however, showed that in primary penile squamous cell carcinoma, high Ki-67 expression is associated with lymph node metastasis. This is consistent with previous studies that indicate a similar trend, but lack statistical significance⁸. With statistical analysis, a strong correlation was found between the expression of Ki-67 and metastasis of the lymph node (Chi-square $p=0.005$) and Ki-67 expression and remote metastasis (Chi-square $p=0.026$)⁷. These results are similar to those of oral squamous cell carcinomas and medullary thyroid carcinomas, which show a correlation between

high-level Ki-67 expression and lymph node metastasis^{24,25}. Similar to results of this study, Ki-67 was upregulated in patients with lymph node metastasis.

Ki-67 expression in penile squamous cell carcinoma is correlated with the metastasis of lymph nodes, so the staining of Ki-67 could help identify patients with a high risk of metastasis of the lymph node. In addition to that, a significant prognostic effect of Ki-67 on survival rates in uterine cervix carcinomas and other malignancies has been found²⁵.

Data from studies suggest Ki-67 as a useful parameter for selecting patients who may benefit from inguinal lymphadenectomy, especially in conventional laparoscopic cholecystectomy, although the number of post-surgical complications in our sample is relatively small. For this relatively rare cancer, further investigation of molecular markers are required to predict tumor activity in penile cancer, using cohorts with more cases. This may be helpful in identifying patients who would benefit from inguinal lymphadenectomy, in addition to known prognostic factors such as pT stage and level. Further investigations will explain the effect of Ki-67 expression in unusual subtypes.

Data availability

Underlying data

Zenodo: Over-expression of Ki-67 as a predictor of lymph node metastasis in penile cancer patients. <https://doi.org/10.5281/zenodo.3692088>¹⁰

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Yudhistira Pradnyan Kloping

Department of Urology, Dr. Soetomo General Academic Hospital, Universitas Airlangga, Surabaya, Indonesia

Wahjoe Djatisoesanto

Department of Urology, Dr. Soetomo General Academic Hospital, Universitas Airlangga, Surabaya, Indonesia

Thank you for submitting this interesting article. However, there are several issues that need to be addressed before the article can be recommended for publication.

1. There are several grammatical errors that need to be revised.
2. A few references are out of date. Please refrain from using references that are more than 7 years old, unless they are necessary. A few old references were necessary, however, there are too many in this.
3. The study design and sample size are massive limitations in this study. The authors have explained the limitations and future suggestions for this study, however, these should be elaborated further at the end of the discussion section.
4. It is too early to draw a conclusion that Ki-67 may be useful as a predictor. I suggest changing the title and conclusion in the abstract.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Urology, Oncology-Urology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Reviewer Report 19 October 2020

<https://doi.org/10.5256/f1000research.23762.r69645>

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Juan Chipollini

Division of Urology, University of Arizona, Tucson, AZ, USA

The authors performed a retrospective, immunohistochemistry analysis of 48 penile cancer patients and the association of Ki-67 expression with lymph node (LN) status outcomes. They found Ki-67 was significantly associated with LN metastasis for these patients. Of note was that >70% of patients had already locally advanced disease so unclear if the proportion of patient with LN metastasis was based more on local histopathological risk features rather than Ki-67 expression alone. Likely an adjusted analysis (considering T stage, LVI, PNI, etc..) would be needed to further evaluate the impact of Ki-67 expression as a prognosticator of LN metastasis.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Urologic Oncology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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