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Association Between LMP-1 and p16 Expression with Prognostic Factor of Nasopharyngeal Carcinoma

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Abstract

Background: Latent Membrane Protein-1 and p16 expression are potential biomarkers for nasopharyngeal prognostic factors. Epstein Barr Virus interferes with intracellular signaling processes related to cell cycle regulation and LMP-1, inactivating p16 expression and increasing cell proliferation. LMP-1 has also been reported to remove the promoter of p16 via methylation, and inactivating the p16 gene leads to the uncontrolled proliferation of cell tumors.

Objective: To analyze the association between LMP-1 and p16 expression with prognostic factors of nasopharyngeal carcinoma

Methods: This cross-sectional design analyzed the LMP-1 and p16 expression based on immunostaining of nasopharyngeal carcinoma. The patient clinical data used as prognostic factors are histopathological type, quality of life, TNM, and tumor stage. Statistical analysis used chi-square and spearman correlation.

Results: Of the 61 patients, 49 were non-keratinizing carcinoma, undifferentiated type (80,3%). About 54 (88.5%) of the 61 samples are LMP-1 positive, 47 of which were non-keratinizing carcinoma. We determined the significant association between the p16 expression with lymph node involvement (p=0.026) and staging (p = 0.196) in nasopharyngeal carcinoma. No correlation between LMP-1 and p16 Expression (p=0,134) (r=-0,194).

Conclusion: p16 overexpression is associated with lymph node involvement and advanced-stage nasopharyngeal carcinoma.

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a type of cancer originating from the nasopharynx's mucosal lining, most commonly located in the *Rosenmüller fossa*.[1] According to global cancer statistics (GLOBOCAN) 2018, 129.079 cases of nasopharyngeal cancer were found world-wide, mainly in men (70%). Although the incidence rate is below 1 per 100,000 persons per year for both genders, it is common in certain regions, especially Southeast Asia.[2] According to data, NPC also occurs in many countries, such as Malaysia, Singapore, Indonesia, and Vietnam in Southeast Asia and Micronesia, Algeria, and Kenya in North Africa.[3] In Indonesia, the recorded mean prevalence is 5.66 per 100,000 population or 1,0,00 new cases monthly, which reflects a significant health problem in Indonesia.[4]

Most patients were in advanced stages because of the subtle initial signs and symptoms.[5] Histopathological type of NPC is considered insufficient for predicting therapy outcome, so an indicator is needed that can explain a more accurate prediction of therapeutic response.[6] Therefore, it is required to identify better biomarkers that correlate with tumor growth, metastasis, and treatment outcome in patients with NPC to assist in the molecular therapeutic targets.[5] The identification of prognostic factors has been the need to estimate the effect of treatment accurately. TNM stage (tumor, node, and metastatic) are the most important prognostic factors.[7] Although recent studies have shown there is variability in the same category indicating that other factors, such as molecular or ethnic variables, may also affect prognosis.[8]

The most common causative agent for NPC other than genetics is Epstein-Barr Virus (EBV) which contributes to the malignant phenotype and plays a significant role in the development, metastasis, and invasion of NPC.[9] Viral infection causes carcinogenesis to occur faster with the proliferation of cancer cells and reduces apoptosis, while food and environment contribute to the neoplastic process and cancer development.[10] EBV infection is reflected by the Expression of Several EBV latent genes, including Latent Membrane Protein 1 (LMP-1)[11.] LMP-1 is an integrated membrane protein that acts as a tumor necrosis factor (TNF) receptor and activates many signaling pathways. It promotes invasion, proliferation, and migration of the NPC cells and regulates squamous epithelial differentiation.[9] LMP1 expression is part of the molecular treatment option for nasophary-ngeal carcinoma.[6]

p16 is an inhibiting factor in cell proliferation. The absence of p16 may result in increased cellular proliferation. A study has shown that negative expression of the p16 protein was much higher in nasopharyngeal cancer compared to standard or chronic inflammation of nasopharyngeal mucosa.[12] The absence of p16 was reported to be associated with the presence of EBV and pRb. LMP-1 can block the induction of p16 INK4a expression and inhibit downstream effectors from promoting cellular proliferation and increasing the effect of p16 gene mutations. There is the possibility that p16 expression is related to aggressive behavior in EBV- positive NPC.[13,14]

Study on LMP-1 and p16 as prognostic factors of NPC in RSUP. Dr. Kariadi has never been done, while NPC cases in this hospital are

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pretty high. Several studies on LMP-1 and p16 in nasopharyngeal carcinoma and the reported prognostic role in NPC patients show inconsistency. The previous study shows that LMP1 expression and inactivation of p16 are related to a more aggressive clinical course and poorer clinical prognosis, especially its metastatic potential.[5] In contrast with other studies that reported no significant association between p16 protein expression and prognosis and no relationship between the expression of LMP-1 and the progression of the tumor. [12,15] This study aims to analyze the association between lmp1 and p16 expression with a prognostic factor of nasopharyngeal carcinoma.

METHODS

This research was conducted using a cross-sectional design. Sixty-one patients were diagnosed with Nasopharyngeal carcinoma between January 2017 and June 2020 by a specialist in anatomical pathology at Dr. Kariadi Hospital Semarang. The data collected included age, sex, histological, type, and TNM staging. LMP-1 Expression, p16 Expression, and TNM staging are established as the variables in the study. The analysis for the histopathology sample was done by two observers on hematoxylin and eosin sections and classification using the WHO system. Expression of LMP-1 and p16 was evaluated by immunostaining. This research will be conducted after obtaining ethical clearance from the Health Research Ethics Commission (KEPK) RSUP Dr. Kariadi (license number: 815/EC/KEPK-RSDK/ 2021).

Paraffin blocks from each patient were collected, then cut using a microtome to produce tissue bands/tissue ribbons with a thickness of 3-4 µm—antigen retrieval with pH 6 for 40 minutes in a microwave at 96°C. The slides were then washed with phosphate buffer saline (PBS). Elimination of endogenous peroxidase using H2O2 peroxidase for 20-30 minutes. Rinse in running water for 10 minutes and wash with PBS. The slide is dripped with a sniper background for 10 minutes. The primary antibodies were diluted in antibody diluent according to the following concentrations: Mouse Anti-Human Epstein-Barr virus, Latent Membrane Protein-1 (Novocastra), 1:100; p16 INK4a [BC42], mouse monoclonal antibody (Biocare Medical).

The LMP1 and p16 were quantitated by cytoplasmic and nuclear staining, respectively. All stained slides will be assessed under a microscope at 400x magnification for a maximum of 5 HPF. The percentage of tumor cells stained was scored as 0 (negative), 1+ (staining in <10% of the neoplastic cells) 2+ (staining in >50% of the neoplastic cells). Staining intensity was evaluated semi-quantitatively: 0 (negative), 1+ (weak), and 2+ (strong). The two individual parameters were added, resulting in ≥4 as high expression and cases with IRS <4 as low expression.[5] Statistical analysis of the data using the Statistical Package for Social Sciences (SPSS) version 21 program (IBM Chicago, IL). The relation between the research variables, the Chi-square test, and different tests was performed, with a degree of significance of $p < 0.05$, and Spearman's rank test for correlation between Expression of LMP-1 and p16.

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RESULTS

Table 1. Clinicopathological characteristics of the patient

Variables	F	%
Ages		
> 46	38	62,3
≤ 46	23	37,7
Genders		
Male	38	62,3
Female	23	37,7
Primary tumor (T)		
T1	5	8,2
T2	12	19,7
T3	15	24,6
T4	29	47,5
Nodal stastus (N)		
N0	6	9,8
N1	14	23,0
N2	37	60,7
N3	4	6,6
Metasatasis (M)		
M0	58	95,1
M1	3	4,9
Stage		
I	3	4,9
II	3	4,9
III	23	37,7
IVA	24	39,3
IVB	4	6,6
IV C	4	6,6
Histopathological type		
K-SCC	7	11,5
NK-SCC, DIFF	5	8,2
NK-SCC, UNDIFF	49	80,3
P16		
Negative	42	68,9
Low	15	24,6
High	4	6,6
LMP-1		
Negative	7	11,5
Low	32	52,5
High	22	36,1

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Table 1. shows clinicopathological characteristics. Most of the nasopharyngeal carcinoma patients were diagnosed at age > 46 years (62,3%) with a median age of 61 years (range 19-85 years). There is a male predominance with a male-to-female ratio of 1,6:1. Non-keratinizing squamous cell carci-noma was the most common histopathological type, found in 49 samples (80.3%), with the majority being stage IVA (24; 39.3%). LMP1 expression was found in 54 samples (88.6%) and p16 expression in 19 samples (31,1%).

There was a statistical significance in the relationship between p16 expression with lymph node involvement and staging in nasopharyngeal carcinoma with a p-value <0.05, while the tumor size, metastasis, and histopathological type variables did not have a statistical significance. (Tables 2 and 3). There is no significant difference between LMP-1 with TNM stage (tumor, node, and metastatic) and histopathology type (Table 4). Spearman's rank test results show no significant linear correlation between LMP1 expression and p16 Expression (Table 5).

Table 2. Test results for the relation between p16 and prognostic factors in nasopharyngeal carcinoma

Variables	p16			p¥
	Negative	Low	High	
Primary tumor (T)				
T1				
T2	4 (80%)	1 (20%)	0 (0%)	0,253
T3	9 (75%)	3 (25%)	0 (0%)	
T4	12 (80%)	2 (13,3%)	1 (6,7%)	
Nodal stastus (N)	17 (58,6%)	9 (31%)	3 (10%)	
N0				
N1	3 (50%)	2 (33,3%)	1 (16,7%)	0,026*
N2	7 (50%)	6 (42,9%)	1 (7,1%)	
N3	29 (78,4%)	7 (18,9%)	1 (2,7%)	
Metasatasis (M)	3 (75%)	0 (0%)	1 (25%)	
M0				
M1	41 (70,7%)	13 (22,4%)	4 (6,9%)	0,226
Stage	1 (33,3%)	2 (66,7%)	0 (0%)	
I				
II	2 (66,7%)	1 (33,3%)	0 (0%)	0,196
III	1 (33,3%)	2 (66,7%)	0 (0%)	
IVA	20 (87%)	2 (8,7%)	1 (4,3%)	
IVB	15 (62,5%)	7 (29,2%)	2 (8,3%)	
IV C	3 (75%)	0 (0%)	1 (25%)	
Histopathological type				
K-SCC	1 (25%)	3 (75%)	0 (0%)	
NK-SCC, DIFF	3 (42,9%)	3 (42,9%)	1 (14,3%)	0,292
NK-SCC, UNDIFF	4 (80%)	1 (20%)	0 (0%)	

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Table 3. Multivariate multiple linear regression between p16 and prognostic factors in nasopharyngeal carcinoma.

Variables	B	P
Constanta	1,128	0,000
Nodal status (N)	-0,301	0,012*
Metastasis (M)	-0,224	0,595
Staging	0,211	0,028*

Table 4. Test results for the relation between LMP-1 and prognostic factors in nasopharyngeal carcinoma.

Variables	LMP-1			p¥
	Negative	Low	High	
Primary tumor (T)				
1	0 (0%)	2 (40%)	3 (60%)	0,388
2	3 (25%)	4 (33,3%)	5 (41,7%)	
3	2 (13,3%)	7 (46,7%)	6 (40%)	
4	2 (6,9%)	19 (65,5%)	8 (27,6%)	
Nodal status (N)				
0	0 (0%)	3 (50%)	3 (50%)	0,491
1	2 (14,3%)	9 (64,3%)	3 (21,4%)	
2	5 (13,5%)	19 (51,4%)	13 (35,1%)	
3	0 (0%)	1 (25%)	3 (75%)	
Metastasis (M)				
0	6 (10,3%)	30 (51,7%)	22 (37,9%)	0,254
1	1 (33,3%)	2 (66,7%)	0 (0%)	
Stage				
I	0 (0%)	1 (33,3%)	2 (66,7%)	0,325
II	1 (33,3%)	2 (66,7%)	0 (0%)	
III	3 (13%)	9 (39,1%)	11 (47,8%)	
IVA	2 (8,3%)	16 (66,7%)	6 (25%)	
IVB	0 (0%)	1 (25%)	3 (75%)	
IVC	1 (25%)	3 (75%)	0 (0%)	
Histopathological type				
K-SCC	0 (0%)	6 (85,7%)	1 (14,3%)	0,108
NK-SCC, DIFF	0 (0%)	4 (80%)	1 (20%)	
NK-SCC, UNDIFF	7 (14,3%)	22 (44,9%)	20 (40,8%)	

Table 5. Results for Spearman's correlation test between LMP-1 and P16.

LMP-1	P16			p	R
	Negative	Low	High		
Negative	4 (57,1%)	3 (42,9%)	0 (0%)	0,134	-0,194
Low	20 (62,5%)	9 (28,1%)	3 (9,4%)		
High	18 (81,8%)	3 (13,6%)	1 (4,5%)		

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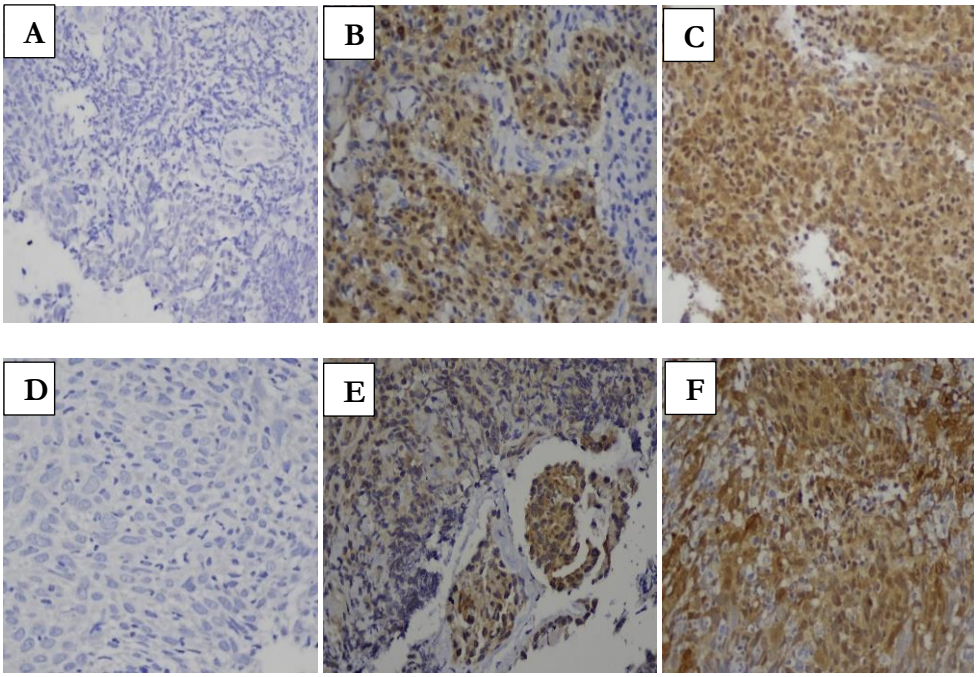

Figure 1. LMP-1 expression (400x). A. Negative. B. Low expression C. High expression. P16 expression (400x) D. Negative. E. Low expression F. High expression.

DISCUSSION

Consistent with the literature, which reports that symptoms of NPC are not specific, pT4 (47,5%), pN2 (60.7%), pM0 (95,1%), and S-IVa (39,3%) were most frequently observed in our study.[4] Patients with nasopharyngeal carcinoma come to the clinicians at a late stage because of symptoms such as headache and other symptoms caused by cranial nerve damage that are signs of a tumor with intracranial extension and/or involvement of cranial nerves (T4) and the presence of a cervical lymph node enlargement.[6]

Our study showed a negative p16 in 42 samples (68.9%) of nasopharyngeal carcinoma patients. Previous studies have found that the lack of p16 expression in the tissue of nasopharynx cancer has a statistically significant association between the overall survival rate and a higher local recurrence rate in NPC patients.[12] Other studies report that p16 expression of nasopharynx cancer is higher than usual or chronic inflammation of nasopharyngeal tissue.[16]

In the current study, univariate and multi-variate analyses revealed that the association between p16 expression with nodal status/pN and staging/ S were significantly correlated (P< 0.05). A previous study found that p16 regulates nodal spread via integrin. The study showed a growth advantage in the low p16 expression setting.[17] That the knockout of the p16 gene affected the growth of the cell, so there was an association between p16 protein expression and larger tumor size.[16] In this study, there was no association of p16 expression with tumor size/ pT in naso-pharyngeal carcinoma patients. Although the association between p16 status and tumor size T was insignificant, a trend of larger size and

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low expression of p16 was seen. 57 of 61 patients (93.4%) with T3/T4 showed p16 negative and low expression. We found the possibility that p16 expression did not affect distant metastases. In contrast, previous studies reported that p16 expression was significantly associated with the risk of distant metastases and that p16 regulates cell invasion and metastatic potential by impairing angiogenesis.[17] These findings are inconsistent with reports published by others[18], probably due to the small number of patients with metastases (3 out of 61 patients).

In the present analysis, 54 patients (88,6%) with Nasopharyngeal carcinoma had LMP-1-positive tumors. These findings are consistent with reports that found positive LMP-1 in NPC cases is higher than in healthy individuals, where positive LMP-1 is positively related (X_2 = 24.95, P = 0.00) with NPC.[19] Our study of the relationship between LMP-1 Expression and tumor size/pT, nodal status/pN, metastasis/pM, and staging/S are not statistically significant. The T4 group in our study showed more LMP-1 expression than the other groups, as much as 36%, and stages III and IV showed positive LMP-1 expression in 61 cases out of 42 cases (80.3%). The results are inconsistent with the results of the study revealed a significant association of LMP1 expression with the TNM clinical stage (p=0.001); however, they did not use LMP-1 but used plasma EBV DNA levels in patients.[20] This difference may be due to LMP-1 immunochemistry not being as effective as EBER-ISH in detecting EBV in NPC, while plasma EBV DNA concentrations are a useful molecular marker for detecting EBV. LMP-1 immuno-histochemical staining is a rapid, cheaper, and effective method of EBV detection but is often not detectable even in obvious EBER-positive[21].

The undifferentiated type of NPC is 100% related to Epstein-Barr virus (EBV) infection.[4] EBV infection often occurs during early childhood and is the cause of mutations in several genes that can cause gene inactivation, one of which is the p16 gene.[14] p16 negativity showed a higher association with non-keratinizing in association with EBV via the p16 promoter methylation from the production of EBV.[16] In this study, there was no correlation between LMP-1 and p16 expression, which has a negative correlation. Although there were 38 cases (62.2%), LMP-1 positive showed p16 negative expression. We also found that the undifferentiated non-keratinizing squamous cell carcinoma type showed p16 expression in 35 cases (57.3%), while positive LMP-1 expression in 42 cases (68.8%), more than other histopathological types, but this difference was not statistically significant (p=0,292). Because this may be due to the low incidence of keratinizing NPC in this study (11,4%).

The limitations of this study are that it is a retrospective study from a single institution, the number of subjects is limited, and we did not include data regarding post-treatment. Further research needs to continue to evaluate the prognostic value of NPC patients post-treatment in a multi-institutional study.

CONCLUSION

p16 lack of expression is associated with lymph node involvement and advanced stage in nasopharyngeal carcinoma but without a statistically significant difference between p16

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and tumor size and metastasis. There was no association between tumor size, nodal status, metastasis, and staging with LMP1 expression.

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