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Correlation of LMP-1 Expression with BCL11B in Undifferentiated Type Nasopharyngeal Carcinoma

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ABSTRACT

Background: Nasopharyngeal carcinoma (NPC) is an endemic malignancy in Asia and in Indonesia with a high incidence and mortality. The high mortality rate of patients with NPC is caused by recurrence and metastases early in the disease process, even though they have been given a combination of standard NPC therapy, namely radiotherapy and chemotherapy. Recurrences generally occur after the initial modalities of radiotherapy. Toxicity due to therapy given over a long time can also increase mortality. This study aims to determine the expression of BCL11B in undifferentiated NPC and its correlation with LMP-1 so that it can provide an overview of the nature of CSC in NPC, which is thought to cause recurrence and metastases and provide a poor prognosis in patients with NPC. **Methods:** This study was an analytical observational study using a cross-sectional approach using 30 samples from nasopharyngeal biopsy tissue diagnosed with undifferentiated NPC at the anatomical pathology installation of Dr. Saiful Anwar General Hospital, Malang, Indonesia, between 2018-2020. LMP-1 and BCL11B expression was examined using the immunohistochemical method. Data analysis was performed univariate and bivariate with the help of SPSS software. **Results:** Statistical analysis using the Spearman correlation test between LMP-1 and BCL11B expression in undifferentiated NPC biopsy tissue showed a significant correlation ($p=0.004$) with a correlation coefficient of $r=-0.511$. **Conclusion:** The higher the expression of LMP-1, the lower the expression of BCL11B in nasopharyngeal biopsies of patients with undifferentiated.

1. Introduction

Nasopharyngeal carcinoma (NPC) is a squamous cell carcinoma that occurs in the epithelial layer of the nasopharynx.¹ Nasopharyngeal carcinoma is an endemic malignancy in certain areas such as Southeast Asia, including Indonesia.^{2,3} The incidence of NPC is quite high, with a high mortality rate. In 2020, there were 133,354 new cases of NPC in the world, with a mortality rate of 80,008 cases. The highest incidence came from Asia, which amounted to 113,659 cases or 85.2% of cases in the world.^{2,3} The number of NPC incidents in Indonesia was 19,943

cases, with a mortality rate of 13,399 cases.^{4,3} Based on a retrospective study at Dr. Saiful Anwar General Hospital Malang, in 2018-2020 found, 256 cases of NPC.⁵ In the world and in Indonesia, NPC is more common in men, with a peak incidence at the age of 40-60 years.¹³

The standard combination therapy for NPC is radiotherapy and chemotherapy, but recurrence and early metastases are still common causes of death even though patients have received standard therapy.⁶ Recurrent NPC occurs in 10-20% of patients after the

initial treatment modality of radiation therapy. This is one of the main causes of death among NPC patients.⁷ Patients without distant metastases have a better prognosis, but repeated treatments often carry toxicity or higher risk.⁷ Cancer stem-like cells (CSC) have been identified in several cancer cells, including EBV-mediated. This group of cells has the ability to initiate tumor growth, maintain self-renewal and facilitate resistance. The recurrence is thought to be caused by a failure to eradicate all persisting CSCs, and CSCs then re-form the tumor both locally and in distant locations.⁸⁻¹⁰

Epstein-Barr Virus (EBV) is believed to be the etiology of nasopharyngeal carcinoma in endemic areas. The type of NPC that is closely related to EBV infection is undifferentiated.¹¹ Latent Membrane Protein-1 (LMP-1) is an oncoprotein encoded by EBV that is expressed in the latent phase. The latent phase of EBV infection can induce epigenetic changes in epithelial cells involving DNA methylation and histone modification. These epigenetic changes also affect the host epigenome, which changes cellular gene expression, which is able to create a microenvironment that supports viral replication until the formation of tumors.¹² The BCL11B protein is a zinc finger protein that is associated with B cell malignancy. BCL11B also functions as a tumor suppressor gene that represses transcription, which is thought to also function in the p53.¹³⁻¹⁵ BCL11B is identified as a marker of CSC in Head and Neck Squamous Cell Carcinoma.¹⁶ Previous studies identified BCL11B as a novel tumor suppressor with an impressive capacity to resist cancer stem cells (CSCs).¹⁷ This study aims to determine the expression of BCL11B in undifferentiated NPC and its correlation with LMP-1 so that it can provide an overview of the nature of CSC in NPC, which is thought to cause recurrence and metastases and provide a poor prognosis in patients with NPC.

2. Methods

This study was an analytical observational study with a cross-sectional that analyzed the correlation of

LMP-1 protein expression with BCL11B protein expression in nasopharyngeal carcinoma. This research was approved by the Health Research Ethics Committee of Dr. Saiful Anwar General Hospital Malang Number: 400/205/K.3/102.7/2022. This study used secondary data in the form of a collection of paraffin blocks from nasopharyngeal biopsies in the anatomical pathology laboratory at Dr. Saiful Anwar General Hospital Malang, Indonesia. A total of 30 nasopharyngeal paraffin biopsy block collections that met the inclusion criteria were included in this study. The inclusion criteria were: paraffin block nasopharyngeal biopsy of patients diagnosed with histopathologically undifferentiated NPC between 2018-2020, and the condition of the paraffin block was good, and there was no damage.

The paraffin block cut (5um thickness) was placed on a glass slide that had been coated with poly-L-Lysine, then deparaffinized by inserting the glass slide into xylol and alcohol solution. Then soaked peroxide block solution was for 25 minutes, followed by the DIVA solution, before being put into a decloaking chamber at 90° for 45 minutes. Staining was continued by immersing in PBS solution for 5 minutes, then incubating the primary antibody for 60 minutes, then administering the polymer. The next step was the administration of DAB chromogen and counterstaining using hematoxylin for 2 minutes and lithium carbonate. The next process is clearing into a xylol solution which is then followed by mounting. To see LMP-1 expression, Epstein-Barr Virus (EBV) antibody was used with a ratio of 1:50 (Biocare Medical, APA111 AA), and to see BCL11B expression, BCL11B antibody was used with a ratio of 1:200 (Cell Signaling Technology, BCL11B (D6F1) XP Rabbit mAb).

LMP-1 and BCL11B expression was calculated using a light microscope. The cells counted for LMP-1 expression were tumor cells stained brown in the cell membrane and/or cytoplasm, whereas, for BCL11B expression, the cells counted were tumor cells stained brown in the nucleus. A total of 500 cells were counted from 5 large visual fields (400x) with 100 cells in each

field of view. The number of cells stained was positively divided by 100 cells in each field of view, then expressed as a percentage. The control for LMP-1 immunohistochemical staining used nasopharyngeal carcinoma tissue that proved to be EBER positive, while the control for BCL11B used infiltrative type basal cell carcinoma tissue.

Data analysis was performed with the help of SPSS software version 25. Univariate analysis was performed to display the frequency distribution of the study subject's characteristic data. Bivariate analysis was performed to determine the correlation between

the test variables using the Spearman correlation test with $p < 0.05$.

3. Results

A total of 30 samples of undifferentiated NPC were taken in the period from 2018 to 2020 at the anatomical pathology laboratory of Dr. Saiful Anwar General Hospital Malang. The sample characteristics are shown in Table 1. The oldest in the male patient sample group was 76 years old, and the youngest was 38 years old. The oldest age of the female patient group was 61 years, while the youngest age was 15 years.

Table 1. Characteristics of the study sample.

Parameters	Nasopharyngeal carcinoma Frequency (%)
Gender	
Male	21(70%)
Female	9(30%)
Age	
0-10 years	-
11-20 years	2(6.67%)
21-30 years	-
31-40 years	3 (10%)
41-50 years	5 (16.67%)
51-60 years	5 (16.67%)
61-70 years	2 (6.67%)
71-80 years	1 (3,33%)
81-90 years	-

The average expression of LMP-1 in the Nasopharyngeal Carcinoma Biopsy Network was $87.0\% \pm 15.9$, with the lowest percentage value being 41% and the highest percentage value being 100%.

The average expression of BCL11B in the Nasopharyngeal Carcinoma Biopsy Network was $0.4\% \pm 0.4$, with the lowest percentage being 0% and the highest being 1.6%.

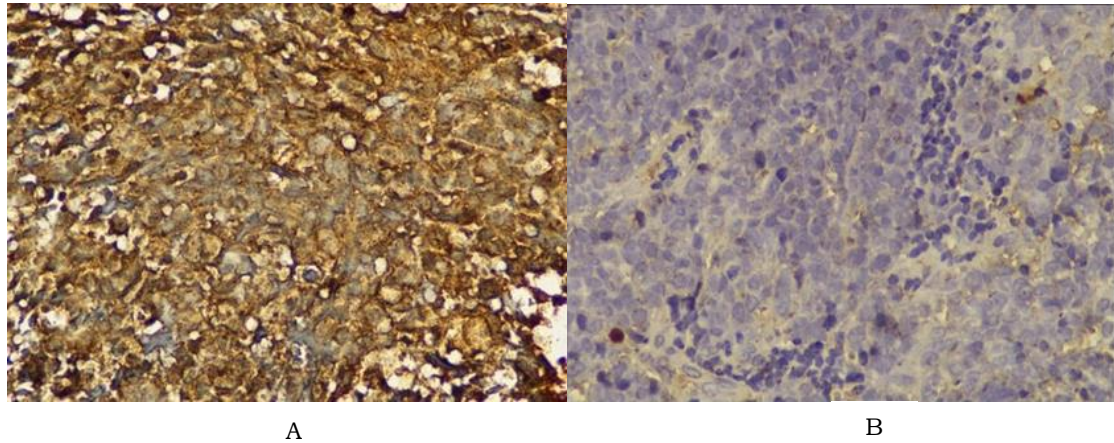


Figure 1. A. LMP-1 expression. The tumor cell membrane and cytoplasm were stained positively, magnification of 400x. B. BCL11B expression. The nucleus of tumor cells stained negatively, magnification 400x.

Spearman's correlation test results show a correlation coefficient of -0.511, which means that the correlation between the two variables is obtained with moderate strength. The correlation between the two variables is negative, which means that an increase in the percentage of LMP-1 expression will be followed by a decrease in BCL11B expression. The relationship between these two variables is statistically significant because a significance value (p-value) of 0.004 is obtained, which is less than 0.05.

4. Discussion

The results of this study are consistent with the incidence of NPC in the world and in Indonesia according to WHO data and in accordance with the NPC clinicopathology profile study conducted at Dr. Saiful Anwar General Hospital Malang previously showed that out of 244 cases of NPC, 68.35% or 175 cases came from male patients.^{3,5} Meanwhile, according to age distribution, as much as 55.85% or 43 cases of NPC occurred at the age of over 50 years.⁵ The results of other studies also showed that the risk of getting NPC increased 2.813 times in respondents aged ≥ 40 years compared to respondents aged < 40 years.¹⁸ The incidence of KNF increases in high-risk populations, i.e., at the age of over 30 years, with the peak incidence at the age of 40-60 years.¹ The results of this study are in accordance with the description of the distribution of NPC patients in endemic areas.

Research in Hong Kong showed that between 1983-2008 there were more men with NPC than women, but at the age of over 55 years, the incidence became the same. Mutations on the X chromosome that make males more susceptible to EBV infection are the initial suspected cause. However, this was considered insufficient. Diet and smoking factors are also thought to support the occurrence of NPC. In addition, working in an environment with high pollution also supports the occurrence of NPC in men because most of the breadwinners working in this sector are men. But this has not been studied before.¹⁹

The results of this study showed positive LMP-1 expression with an average percentage of 87.007%, the lowest percentage was 81%, and the highest percentage was 92%. These results are consistent with the results of previous studies, which showed that LMP-1 was generally detected in NPC biopsies but indeed with large variations in expression between samples.²⁰ Based on reports from various parts of the world, approximately 50-60% of nasopharyngeal biopsies in NPC patients show positive LMP-1 immunohistochemistry. LMP-1, which is expressed in NPC samples, is thought to play a role not only in carcinogenesis but also in maintaining the latent properties of the virus.²⁰ The results of this study are in line with previous studies showing that increased LMP-1 expression in NPC cells is related to overexpression of Id1 (inhibitor of DNA

binding/differentiation) in cancer cells and contributes to cell proliferation, invasion, and angiogenesis. Therefore, its role is important in tumorigenesis.²¹ results of this study indicate that EBV infection is associated with NPC found at Dr. Saiful Anwar General Hospital Malang, characterized by high LMP-1 expression in the undifferentiated examination.

The results of this study showed very low BCL11B expression to be negative. The average percentage of BCL11B expression was 0.35%, with the lowest percentage being 0% or a negative result and the highest percentage being 1.6%. This study is the first study to observe BCL11B expression in NPC in Indonesia. No studies have examined BCL11B expression in the normal nasopharyngeal epithelium or nasopharyngeal carcinoma. According to the Human Protein Atlas (HPA), BCL11B expression is found specifically in brain tissue, lymphoid tissue, and skin, with its expression located in the cell nucleus. Meanwhile, BCL11B expression in the respiratory system, especially the nasopharynx, was recorded to be low. In normal nasopharyngeal epithelium, an 82-year-old male in HPA showed an expression quantity of <25% with moderate staining in the nuclei of nasopharyngeal epithelial cells.²² gene B-cell leukemia/lymphoma (BCL11B) is a member of the BCL family that plays an important role in the development, proliferation, differentiation, and survival of T cells. Changes in the BCL11B gene are associated with the transformation of T cell malignancy. The BCL11B gene is responsible for the regulation of the apoptotic process and cell proliferation.²³ The function of BCL11B is as a key regulator of T-lymphocyte differentiation and survival during thymocyte development in mammals. In addition, BCL11B has an important role in controlling the response of hematopoietic stem cells to chemotactic signals by modulating the expression of CCR7 and CCR9 receptors, which direct the movement of progenitor cells from the bone marrow to the thymus.¹³ This protein is a regulator of the IL2 promoter and increases IL2 expression in activated

CD4(+) T lymphocytes.¹⁵ Low BCL11B expression is an independent indicator for overall survival (OS) and time to recurrence (TTR) for Hepatocellular Carcinoma (HCC) patients treated by surgical resection. In vitro and in vivo experiments confirmed BCL11B as a tumor suppressor in HCC with inhibitory effects on proliferation, cell cycle progression, apoptosis, and mobility. Furthermore, BCL11B can suppress CSC properties in HCC, as evidenced by reduced tumor formation, self-renewal potential, and drug resistance.¹⁷ Previous studies on BCL11B expression used many cell culture preparations with observation methods other than immunohistochemistry, such as western blot or qRT-PCR. This study is the first study on BCL11B expression in nasopharyngeal biopsy tissue.

The correlation between LMP-1 expression and BCL11B expression in the WHO Type III NPC biopsy tissue was analyzed using the Spearman correlation test. The results obtained a correlation coefficient of -0.511 with a significance value (p) of 0.004, which is smaller than alpha 0.05, so it can be concluded that there is a significant correlation between LMP-1 expression and BCL11B expression in nasopharyngeal biopsy tissue of patients with undifferentiated NPC (WHO). Type III) with a negative correlation direction and moderate strength, which means that the higher the LMP-1 expression, the lower the BCL11B expression. It is estimated that 15-20% of all cancers are related to viral infections. Tumors associated with viral infections are the result of the interaction of several physiological factors, including immunosuppression, specific gene mutations, chronic inflammation, and long-term virus-host interactions. The viruses associated with human cancer share similar characteristics. Generally, viruses cause persistent latent or pseudo-latent infection, and lytic replication is decreased or inhibited. Based on the results of previous studies, it was known that the roles of LMP-1 in the pathogenesis of NPC, it can be concluded that LMP-1 has an important role in this process.²⁴ The LMP-1 oncoprotein can block DNA damage and IRF5-mediated apoptosis that benefits

EBV. Besides that, this protein plays a role in the NF κ B and STAT 3 signaling pathways in order to create a tumor microenvironment so that the survival of tumor cells is greater.^{25,26} BCL11B functions as a tumor suppressor repressing transcription that is thought to also function in the P53 signaling pathway.¹³⁻¹⁵

The results of previous studies regarding the role of oncoproteins and their relationship with BCL11B were shown in the research by Permatasari et al. regarding Tax, which is an oncoprotein from HTLV-1 that is able to bind BCL11B thereby increasing the proteasomal degradation of BCL11B. In the early stages of HTLV-1-mediated leukemogenesis, Tax plays an important role in the growth of HTLV-1-infected T cells. Although the detailed mechanism for Tax-mediated proteasomal degradation of BCL11B remains unclear, our results suggest that Tax directly binds to tumor suppressors and cell cycle regulators, one of which is BCL11B, to enhance its protein degradation, resulting in increased cell proliferation that may contribute to Tax-driven leukemogenesis.²⁷ Based on these studies, it is suspected that LMP-1 as an oncoprotein of EBV, like Tax in HTLV-1, can interfere with the function of BCL11B. Although so far, the function of BCL11B has been well established in T cells, BCL11B has also been found in other tissues, including the nasopharyngeal epithelium. Given its function as a tumor suppressor and transcription factors that are repressors that are useful in checkpoints during the cell cycle, inhibition of BCL11B function by LMP-1 can occur and contribute to the pathogenesis of NPC. Even so, there is not enough evidence to show that LMP1 affects BCL11B through a particular mechanism and how big its role is in the pathogenesis of NPC, but the results of the correlation test show that there is a negative correlation with significant moderate strength between the two variables cannot be ignored. This study has several limitations, including the minimum number of samples and no previous studies on BCL11B expression in normal nasopharyngeal tissue and NPC, so other studies are needed to determine the role of BCL11B in the pathogenesis of NPC. In this study, we

demonstrated that BCL11B expression was decreased until it was not expressed in NPC. Decreased BCL11B expression correlated with LMP-1 expression in undifferentiated. Our findings provide new insights about BCL11B in NPC. The alleged role of BCL11B in tumorigenesis of NPC related to EBV infection still needs further research. We suggest examination of BCL11B in normal nasopharyngeal tissue, analyze the relationship between BCL11B and NPC at various stages and explore methods of BCL11B examination other than immunohistochemistry.

5. Conclusion

There is a negative correlation between LMP-1 expression and BCL11B expression in undifferentiated nasopharyngeal carcinoma ($r = -0,511$; $\text{sig} = 0.004$; $\alpha = 0.05$).

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