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Correlation of Cancer Stage with Expression of LMP-1 and BCL-2 in Patients with Undifferentiated Nasopharyngeal Cancer

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Staging is one of the considerations in treatment and prognosis of nasopharyngeal cancer (NPC). Previous studies have found an increased expression of latent membrane protein-1, LMP-1, which is an oncoprotein of Epstein-Barr Virus in NPC. BCL-2 is an anti-apoptosis protein which may be related to the stage of NPC. However, the correlation between NPC stage and the expression of LMP-1 and BCL-2 remains unclear. The aim of this study was to investigate the correlation between the stage of undifferentiated NPC and the expression of protein LMP-1 and BCL-2 in forty-four patients, aged between 20–79 years old, who received treatment in West Nusa Tenggara General Hospital, Indonesia. Most of the cases were found in the advanced stage (stages III and IV). The presence of BCL-2 and LMP-1 proteins was detected by immunohistochemical (IHC) staining. Approximately 34% of the biopsies showed positive LMP-1 expression in tumour cells and 66% were positive for BCL-2 expression. While staging was moderately correlated with LMP-1 expression (r = 0.421, p = 0.004), it was strongly correlated with BCL-2 expression (r = 0.610, p = 0.001). In conclusion, staging is very important in the treatment of NPC, it may be affected by the expression of LMP-1 and BCL-2.

Keywords: NPC; staging; LMP-1; BCL-2; IHC

I. INTRODUCTION

Nasopharyngeal carcinoma (NPC) is the malignancy of epithelium lining the hidden nasopharynx cavity. This leads to difficulty in early diagnosis, and most cases of NPC when found are in the advanced stage (Adham, 2018). Cancer could be labelled in stages from I to IV which indicate the increasingly severe extent of cancerous cell growth and spread. According to American Joint Committee on Cancer (AJCC, 2018), the stage of NPC is dependent on three factors: T (the size of primary tumour), N (the involvement of regional lymph nodes), and M (the presence of distant metastases). The stage of NPC could be used as one of the considerations in deciding the therapy to be delivered and also for prognosis prediction (Adham, 2018).

NPC is one of the most prevalent malignancies in the Southern China and Southeast Asia region. Approximately 50,000 new cases were reported every year in these regions (Lo *et al.*, 2003). Indonesia is considered to have a medium prevalence rate with 5.6 cases per 100,000 individuals. The cases were predominant in male with a ratio of 2–3:1 (Adham *et al.*, 2012). Based on the Global Cancer Network 2018 database, the incidence of NPC worldwide is estimated at 129,079 with the mortality rate of 72,987 per year (Bray *et al.*, 2018). World Health Organization (WHO) classifies NPC into three histologic types, including type I (keratinising), type II (non-keratinising), and type III

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(undifferentiated). According to studies in several countries, undifferentiated carcinoma (WHO type III) was the most common type of nasopharyngeal cancer (Adham *et. al.,* 2012; Fendri *et. al.,* 2011; Lo *et al.,* 2003).

NPC is one of the classic malignancies associated with Epstein-Barr virus (EBV) infection, where such association has been shown in several studies that detected in NPC cells the expression of latent membrane protein-1 or LMP-1, an oncoprotein encoded by EBV. Osman *et al.* (2012) found a correlation between NPC and EBV with immunohistochemistry (IHC). According to their study, 90% of the patients with NPC were infected by EBV. Adham *et al.* (2012) found 75% of the patients with NPC were LMP-1 positive. Similarly, the expression of LMP-1 in 94.4% of the patients with NPC was observed in a study in India (Borthakur *et al.,* 2010).

B-cell lymphoma-2 or BCL-2 is a human proto-oncoprotein located in the nuclear membrane, endoplasmic reticulum, and outer layer of mitochondrial membrane. The oncogenic effect of BCL-2 is exerted through inhibition of cell apoptosis. Therefore, the presence of BCL-2 has been considered as one of the prognostic factors of NPC. Overexpression of BCL-2 has been reported to correlate with aggressive traits in NPC, such as lymph node infiltration, metastasis, recurrence and poor survival rate (Tulalamba & Janvilisri, 2012).

The aim of this study was to investigate the correlation between the stages of NPC and the expression of LMP-1 and BCL-2, proteins which may have prognosis value for NPC, in patients with undifferentiated squamous cell carcinoma (WHO type III) NPC in Lombok. Lombok is a part of the West Nusa Tenggara Province which ranked 29th out of the 34 provinces in Indonesia based on the human development index in 2018 (Central Bureau of Statistics, 2018). We were interested to examine if there is a correlation between the stages of NPC and expression of LMP-1 and BCL-2 proteins in Lombok due to the region's relatively poor environmental conditions and personal hygiene status which caused its population to be more susceptible to EBV infection (Adham *et al.*, 2012).

II. METHODS

A. Subjects

Biopsies and medical records of patients with NPC, aged more than 20 years old and treated in the West Nusa Tenggara General Hospital were consecutively collected between 2012 to 2016. All cases were histopathologically classified using the WHO standard criteria. Cases of NPC classified as undifferentiated carcinomas (WHO type III) were enrolled as subjects in this study. This study has been approved by the ethics committee in Faculty of Medicine, Mataram University, Indonesia.

B. Staging of NPC

To establish the stage of NPC, staging system developed by the American Joint Committee on Cancer (2018) was used as a guideline. Cancer was divided into 4 stages according to the TNM system, with scores after each letter describing the extent of cancer development: stage I (T1 N0 M0), stage II (T0-T1 N1 M0 or T2 N0-N1 M0), stage III (T0-T2 N2 M0 or T3 N0-N2 M0), stage IVA (T4 N0-N2 M0 or T0-T4 N3 M0), and IVB (T0-T4 N0-3 M1). The status of the primary tumour (T) can be defined as absent (T0) or present depending on the size and tumour extension (T1, T2, T3, T4). The status of regional lymph nodes (N) is characterised by the absence (N0) or presence (N1, N2, N3) of metastases depending on the number of nodes with cancer or which nodal groups have cancer, while distant metastases are either absent (M0) or present (M1).

C. Detection of LMP-1 and BCL-2

IHC was used to detect the expression of LMP-1 and BCL-2 by pathologists. Tissue samples were obtained from the paraffin block bank in anatomical pathology laboratory, West Nusa Tenggara General Hospital. The paraffin-embedded tissues were then sliced into sections of 4 μm using microtome and placed on different glass slides for detecting LMP-1 and BCL-2. After heating at 60°C, tissue sections were dewaxed in toluene, rehydrated in ethanol and rinsed in distilled water for 5 min. For both proteins, endogenous peroxidase was quenched with hydrogen peroxide for 10 min and non-specific binding was blocked with protein blocker for 5 min. The sections were then incubated with primary antibody for 30 min. The monoclonal antibodies against LMP-1 (Thermo Fisher Scientific) and BCL-2 (Biocare Medical) were each used at dilution of 1:50 and 1:100 according to the manufacturer's instructions. The proteins were detected using the Mach 4 Universal HRP-Polymer detection system (Biocare

Medical). After incubation with primary antibody, the sections were incubated with the secondary probe for 10 min at room temperature, followed by incubation with polymer reagent for 20 min. The antibody/polymer conjugate was visualised by staining the sections with DAB for 5 min. The sections were counterstained with haematoxylin for enhanced visualisation. The expression of LMP-1 was classified as positive or negative depending on the colour staining cells, with cells staining blue scored as negative expression and those staining light brown as positive expression (Tabyaoui *et al.,* 2013). Expression of BCL-2 was scored by staining intensity as follows: less than 1% of the cells stained brown (-), weak with 1-9% of the cells stained brown (+), moderate with 10-50% staining (++), strong with more than 50% staining (+++) (Vera-Sempere *et al.,* 1997).

D. Statistical Analysis

Pearson's correlation was performed to establish the relationship between the stages of undifferentiated NPC and the expression of LMP-1 and BCL-2. The correlation coefficient (r) at 5% significance level was calculated using the SPSS programme.

III. RESULTS

During the study period, forty-four patients were enrolled in this study. NPC occurred mainly in the age range of 40-59 years old (49.9%). The proportion of patients with NPC above 40 years old was about twice as much as those below 40 years old. There were more male patients diagnosed with NPC than the females (Table 1).

The cases in this study were mostly in the advanced stage (III and IV) with a frequency of more than 75%. A detailed distribution of cases at different stages of NPC by gender was shown in Table 2.

According to the IHC staining of sections prepared from the paraffin blocks of 44 NPC patients, 29 of them (65.9%) were scored as LMP-1 negative and 15 (34.1%) were LMP-1 positive. In contrast, 15 patients (34.1%) were scored as BCL-2 negative, while 13 (29.5%), 1 (2.2%) and 15 (34.1%) NPC patients were scored with BCL-2 positivity of (+), (++) and (+++), respectively. The stages of undifferentiated NPC and LMP-1 expression were significantly correlated (r = 0.421, p = 0.004). A significant correlation was also observed between the stages of undifferentiated NPC and BCL-2 expression (r = 0.610, p = 0.001) (Table 3).

Table 1. Age and gender distribution of undifferentiated NPC patients

Age category (year)	Number of patients with NPC (%)		
	Male	Female	Total
20-39	10 (22.7)	7 (15.9)	17 (38.6)
40-59	16 (36.4)	6 (13.6)	22 (49.9)
60-79	3 (6.8)	2 (4.5)	5 (11.3)
Total	29 (65.9)	15 (34.1)	44 (100)

Table 2. Undifferentiated NPC stage distribution by gender

Gender	Number of patients with NPC (%)			
	Stage I	Stage II	Stage III	Stage IV
Male	1 (2.3)	8 (18.2)	8 (18.2)	12 (27.3)
Female	0 (0.0)	2 (4.5)	8 (18.2)	5 (11.4)
Total	1 (2.3)	10 (22.7)	16 (36.4)	17 (38.7)

IV. DISCUSSION

Undifferentiated squamous cell carcinoma (WHO type III) is the most common type of NPC. A previous study revealed that 80% of the NPC cases in Indonesia were undifferentiated squamous cell carcinoma (Adham *et al.*, 2012). A similar pattern of undifferentiated carcinoma

Table 3. Correlations between undifferentiated NPC stages and the expression of LMP-1 and BCL-2

NPC Stage	Number of patients (%)					
	LMP-1 expression		BCL-2 expression			
	(-)	(+)	(-)	(+)	(++)	(+++)
I	1 (2.3)	0 (0.0)	1 (2.3)	0 (0.0)	0 (0.0)	0 (0.0)
II	9 (20.5)	1 (2.3)	7 (15.9)	3 (6.8)	0 (0.0)	0 (0.0)
III	12 (27.3)	4 (9.1)	6 (13.6)	5 (11.4)	1 (2.3)	4 (9.1)
IV	7 (15.9)	10 (22.7)	1 (2.3)	5 (11.4)	0 (0.0)	11 (25.0)
Pearson correlation coefficient (r)	0.421				0.610	
p-value	0.004				0.001	

forming the majority cases of NPC was observed in other parts of the world (Abdullah *et. al.*, 2011; El Taher *et. al.*, 2017; Haleshappa *et. al.*, 2017; Osman *et. al.*, 2012; Sharma *et al.*, 2011). The characteristics of subjects with NPC in our study were similar to those reported in other previous studies worldwide, with more male patients than females, and more cases involving subjects above 40 years old (Adham *et. al.*, 2012; Borthakur *et. al.*, 2016; Cao *et. al.*, 2011; Fendri *et al.*, 2011).

Gender susceptibility may be caused by alcohol consumption and smoking (Adham *et. al.*, 2012; Osman *et. al.*, 2012; Wu *et al.*, 2018). Consumption of salted fish and exposure to carcinogenic agents have been suggested as the main contributor for the occurrence of NPC (Wu *et al.,* 2018). Salted fish is commonly consumed by the locals in Lombok, as it is one of the common fishery products on the market at the island which is home to many fishermen.

In this study, LMP-1 expression was positive in 34% of the cases, whereas BCL-2 expression was positive in 66% of the cases. The expression of LMP-1 in NPC varied among several IHC studies. Li *et al.* (2007) detected 43% LMP-1 positive expression (24 of 56 patients), while Bar-Sela *et al.* (2004) reported only 6 out of 40 patients (15%) with positive LMP-1 expression. More recent studies found higher frequency of NPC patients with LMP-1 positive expression. Adham *et al.* (2012) reported a 75% (160 patients) positive expression of LMP-1 among the NPC patients in their study, and Borthakur *et al.* (2016) found 17 patients (94.4%) with undifferentiated NPC to have the LMP-1 protein expressed. According to Yip *et al.* (2006), BCL-2 was more commonly observed in Asian/Chinese people (67%, or 33 of 49 cases were positive for BCL-2 expression) compared to a lower frequency in African/Caucasian patients (48%, or 15 of 31 cases). A study found that higher mRNA expression of BCL2L12, a member of the apoptosis-related BCL-2 gene family, was correlated with shorter disease-free survival (p = 0.017) and higher risk of relapse in NPC with a hazard risk of 6.82 (Fendri *et al.*, 2011). On the other hand, Kontos *et al.* (2013) in their study on the BCL-2 associated X gene (BAX), which is a pro-apoptotic member of the BCL-2 gene family, found that the mRNA expression of BAX was associated with longer disease-free survival in NPC patients (p = 0.016).

Previous study reported that LMP-1 of the EBV could inhibit the apoptosis in B lymphocytes by triggering expression of BCL-2 (Adham, 2018). LMP-1 can promote cell motility, invasion and metastasis as well as lymphangiogenesis (Nakanishi *et al.,* 2017). Although the association between BCL-2 and EBV is still debated, our recent study indicated a strong correlation between the LMP-1 and BCL-2 expression, which could be important for devising future strategy in NPC treatment, especially at specific regions such as Lombok which has low human development index (ranked 29th of the 34 provinces in Indonesia) (Central Bureau of Statistics, 2018).

This study explored the association between the stages of NPC with undifferentiated carcinoma (WHO type III) and the expression of LMP-1, the major oncogene of EBV which is the most important cause of NPC. Furthermore, this study also analysed the correlation between the stages of NPC with undifferentiated carcinoma (WHO type III) and the expression of BCL-2, an anti-apoptosis gene which may promote the progress of cancer. In line with the potential prognosis value of LMP-1 and BCL-2 expression in NPC, a moderate correlation between NPC stages and LMP-1 expression, as well as a strong correlation between NPC stages and the expression of BCL-2 were found in this study. The weaker correlation between NPC stages and the expression of LMP-1 was probably a result of the lack of preserved samples in our laboratory for further confirmation using other methods, as well as a limitation of the retrospective methods used in this study.

Staging is very important in the initial diagnosis to identify the extent of cancer. The cancer stage will be used as a starting point to plan for treatment and follow-up with patients, including to predict the prognosis. For instance, patients with early stage cancer (stages I-II) could be treated by radiotherapy alone. On the other hand, advanced stage cancer (stages III-IV) should be treated with the combination of radiotherapy and chemotherapy (Lee *et. al.,* 2015; Nakanishi *et al.,* 2017). Furthermore, if there was a relapse or partial response to radiotherapy or chemoradiotherapy, other modalities of treatment such as brachytherapy, photodynamic therapy or neck dissection could be given (Adham, 2018; Indrasari *et. al.,* 2016; Lee *et. al.*, 2015; Yan *et al.*, 2017).

Prognosis is better for patients in the early stage of cancer than those in the advanced stage. In the early stage, the overall 5-year survival could reach 95%, while that in the advanced stage is only 70-80% with intensity modulated radiotherapy (Lee *et al.*, 2015). A previous study found a better prognosis for NPC in patients with positive expression of LMP-1 compared to those with negative expression with an odds ratio of 9.28 (p = 0.003) based on the 24-month survival rate (Hariwiyanto *et al.,* 2010). On the other hand, the expression of BCL-2 was not significant for overall survival in a 10-year survival analysis (Yip *et al.,* 2006).

BCL-2 and LMP-1 may affect the staging of NPC since the correlations between the NPC stages and the expression of both proteins were significant in this study. A study by Kouvidou *et al.* (1995) found that BCL-2 was associated with the extent of tumour cell differentiation and the increase in tumour cell survival that led to the more advanced stage. The BCL-2 expression in EBV-positive NPC was higher than in EBV-negative NPC. Furthermore, BCL-2 could work synergistically with LMP-1 to promote more rapid cell growth in NPC (Tulalamba & Janvilisri, 2012). As such, the presence of BCL-2 and LMP-1 would affect the staging of NPC.

In conclusion, staging of undifferentiated NPC may be affected by the expression of LMP-1 and BCL-2. However, the independent effect of LMP-1 and BCL-2 on staging remained unclear. Further research should address the effect of the expression LMP-1 and BCL-2 on staging, as well as treatment and prognosis for NPC.

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