

# The Differences of Programmed Death Ligand 1 Expression Between the Early Stages and Advanced Stages of *Nasopharyngeal Carcinoma* in DR. M Djamil Padang Hospital

Nadya Dwi Karsa<sup>1,2\*</sup>, Sukri Rahman<sup>1,2</sup>, Al Hafiz<sup>1,2</sup>, Hirowati Ali<sup>3</sup>,  
and Hafni Bachtiar<sup>4</sup>

<sup>1</sup>Department of Otorhinolaryngology Head & Neck Surgery of Faculty Medicine Andalas University, Indonesia

<sup>2</sup>Dr. M. Djamil General Hospital Padang, Indonesia

<sup>3</sup>Department of Biochemical Science of Faculty Medicine Andalas University, Indonesia

<sup>4</sup>Department of Public Health of Faculty Medicine Andalas University, Indonesia

\*Corresponding author. Email: nadyadwikarsa@gmail.com

## ABSTRACT

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from the nasopharyngeal epithelial cells. Cell-mediated immunity has an important role in growth and development of cancer. Many studies have been conducted to predict the existence of an important relationship between immune checkpoints Programmed Death-1 (PD-1) and Programmed Death Ligand-1(PD-L1) with NPC. The research of checkpoint blocking antibodies against PD-1 and PD-L1 shows promising results for cancer immunotherapy. An analytical cross sectional study was conducted on 30 biological preparations for NPC patients consisting of 15 biological preparations for NPC patients with early stage and 15 biological preparations for NPC patients with advanced stages. Molecular examinations are carried out to see the expression of PD-L1 by the immunohistochemical (IHC) method on each of biological preparations,. Data were statistically analyzed with computer programs and were stated to be significant if  $p < 0.05$ . In this study, a high PD-L1 expression was obtained in 3 of early stage NPC patients and 4 of advanced stage NPC patients using the Tumor Proportion Score assessment. Meanwhile, using the Immunoreactive Score assessment, a high PD-L1 expression was obtained in 1 of early stage NPC patient and 4 of advanced stage NPC patients. Statistically, there was no significant difference in PD-L1 expression between early stage and advanced stage at NPC at Dr. M Djamil Padang Hospital with  $p > 0.05$ . A High PD-L1 expression was found to be more prevalent in the advanced stage of NPC patient but there was no significant difference in PD-L1 expression between early stage and advanced stage at NPC at Dr. M Djamil Padang Hospital

**Keywords:** *Nasopharyngeal carcinoma, PD-L1, PD-1, Immune checkpoint, immunotherapy.*

## 1. INTRODUCTION

Nasopharyngeal carcinoma (NPC) is one of the head and neck malignancies that has a specific etiology, racial and geographic distribution so that it is different from other head and neck cancers. NPC is a squamous epithelial carcinoma originating from the surface of the superior wall and lateral nasopharynx. Environmental

factors, genetics and Epstein-Barr virus infection closely interact with the pathogenesis of NPC. In South China, the highest incidence of NPC is found, but the incidence is rare in western countries [1,2].

Often NPC patients are diagnosed at an advanced stage, accompanied by lymph node metastases, distant metastases and recurrence are still obstacles to

increasing the survival rate of NPC patients. Therefore, increasing the survival rate of NPC patients with effective treatment is urgently needed. Current treatment for NPC is only radiation or chemoradiation [2].

The process of formation and development of NPCs is influenced by cell-mediated immunity, namely between immune checkpoints, PD-1 or PD-L1 and cancer [3,4]. Impaired effector function (cytokine production and cytotoxic efficacy against tumor cells) and poor results in some tumor types which associated with PD-1/PD-L1 expression by tumor-infiltrating lymphocytes (TILs) have been tested in many studies. Increased expression of PD-L1 can predict a poor prognosis for NPC has been shown in several studies, but increased expression of PD-L1 was only found in 25% (26 of 104 NPC patients) in the study of Lee et al [5]. However, it was found a positive correlation between PD-L1 expression with progression-free survival (PFS) and improved loco-regional failure-free survival (LRFFS). While overall survival (OS) and progression-free survival (PFS) not significantly correlate with PD-L1 expression in 161 NPC patients reported in the study of Chan et al. as cited by Zheng [6].

Promising cancer treatments have been reported through research developments on assays of blocking antibodies against PD-1 and PD-L, recently [3]. The use of *Pembrolizumab*, a monoclonal anti-PD-1 for the treatment of non-small cell lung cancer, has been approved by the United States Food and Drug Administration (US FDA), since then, *Pembrolizumab* has been tested for the treatment of several other tumor types, such as NPC [7]. Therefore, it is necessary to further investigate the role of PD-L1 expression in NPC.

## 2. METHOD

An observational analytic study with a cross-sectional approach used in this study. This research was conducted after obtaining ethical clearance, by taking paraffin blocks for examination at Anatomical Pathology Laboratory Faculty of Medicine Andalas University. Ethical clearance was obtained from the Ethics Committee of Dr. M Djamil Hospital (No:248/KEPK/2020). There was 30 samples NPC consist of fifteen early stages (stages I and II) and fifteen advanced stages (stages III and IV) were taken from patients who were biopsied and staging in the Department of Otorhinolaryngology Head & Neck Surgery Dr. M. Djamil Hospital Padang since January

1st, 2017. Anatomical pathology results based on the patient's status in the medical record was collected.

Immunohistochemistry was used to evaluate PD-L1 expression in tumor cells. Paraffin blocks were cut and pasted on Poly-L-Lysine-coated glass. Dewaxing in the oven at 60°C for 1 hour. Deparaffinization using xylene I, II, and II (5 minutes each). Rehydrate with Ethanol 100%, 90%, and 70% for 5 minutes each. Rinse with Aquadest for 5 minutes. Heat epitope was taken by microwave in citrate buffer pH 6.0 for 10 minutes at 90°C, then cool for 20 minutes. Rinse with PBS 3 times (5 minutes each). Endogenous peroxidase block with H2O2 3% in PBS for 3 minutes followed by 0.3% H2O2 in PBS for 30 minutes. Rinse with PBS 3 times (5 minutes each). Non-specific protein blocking with 2% normal goat serum in PBS for 20 minutes. Anti PD-L1 Clone 22c3 DAKO produced by Dako North America Inc, US was using to detect PD-L1 protein. Microscopic assessment using an Olympus BX 51 light microscope at magnification and 400x (objective 40x).

PD-L1 expression was evaluated by 2 scoring systems, Tumor Proportion Score (TPS) and Immuno Reactive Score (IRS). TPS only assessed the proportion of stained cells, negative (<1% stained cells), low (1- <50% stained cells), high (≥50% stained cells) [8] Negative and low staining are grouped into low expressions (underexpression) and high staining are grouped into high expressions (overexpression). IRS assesses 2 components that the calculation of the score for the ratio of cells that are positively stained and the assessment of the staining intensity score. The score for the ratio of positive cells is assessed by calculating the percentage of stained cells versus all cells observed, and is reported in percent. The proportion score is grouped into 5 levels; no staining found; score 0, score 1 for less of 10%; score 2 for 10-50%; score 3 for 51-80% ; and score 4 for more than 80%. The score of staining intensity was reported by semiquantitatively assessing the intensity of brown color in the epithelial cytoplasm in four levels from no staining reaction; 0, low ; 1, medium; 2, and high; 3. IRS is the product of the cell proportion score multiplied by the intensity score, range from 0 to 12. IRS results 0-3 are classified into low expression (underexpression) and ≥4 is classified as high expression (overexpression) [9].

To examine differences of PD-L1 expression between early stage and advanced stage at NPC, the chi-squared test was performed. SPSS version 26.0. is used for statistical analyses.

### 3. RESULTS

There were thirty patients were included in the study. Table 1 shows the general characteristics of the patient. Mostly are stage IV. Most of patients were male (60%). Most range age was 40–59 years. The majority of patients were diagnosed with non-keratinizing undifferentiated carcinoma according to the histological classification of WHO. Based on T classification of primary tumors according to the AJCC, in early-stage NPC, T1 was the most common as much as 53.3%. Whereas in advanced NPC, T4 was the most commonly found as much as 46.7%,

It was found that the percentage of samples with high expression of PD-L1 with TPS was more in cases of advanced-stage NPC, which was 26.7% compared to cases of early-stage NPC, which was 20%. However, there was no statistically significant difference in PD-L1 expression between the early and advanced stages of NPC ( $p>0.05$ ) (Table 2). While, based on IRS was more in cases of advanced-stage NPC, which was 26.7% compared to cases of early-stage NPC, which was 0.7%. However, there was no statistically significant difference in PD-L1 expression between the early and advanced stages of NPC ( $p>0.05$ ) (Table 3).

**Table 1.** Patient characteristic

Characteristic	Early stages f (%)	Advanced stages f (%)
<b>Sex</b>		
Male	9 (60)	9 (60)
Female	6 (40)	6 (40)
<b>Age, SD</b>	<b>52,1±13,9</b>	<b>47,3±12,9</b>
20-39	2 (13,3)	4 (26,7)
40-59	7 (46,7)	7 (46,6)
≥60	6 (40)	4 (26,7)
<b>Histopathology</b>		
WHO type I	0 (0)	1 (6,7)
WHO type II	6 (40)	6 (40)
WHO type III	9 (60)	8 (53,3)
Basaloid	0 (0)	0 (0)
<b>T classification</b>		
T1	8 (53,3)	0 (0)
T2	7 (46,7)	5 (33,3)
T3	0 (0)	3 (20)
T4	0(0)	7(46,7)
<b>Stage</b>		
I	6 (20)	
II	9 (30)	
III		4 (13,3)
IV		11 (36,7)

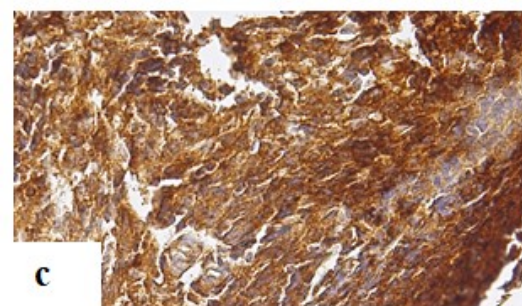
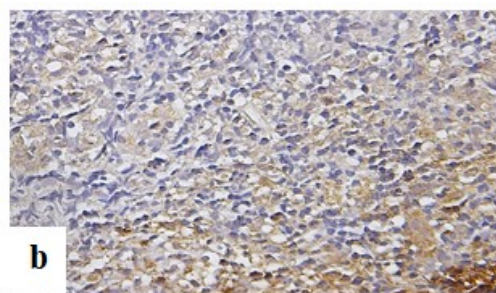
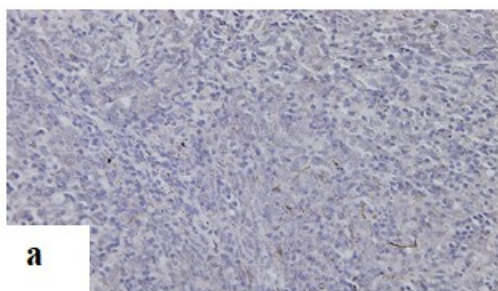
In recent years, a new method of cancer treatment is immunotherapy. Currently, one of the most widely used tumor immunotherapy methods is immune checkpoint blockade therapy. The pathway involving PD-1 and PD-L1 has been applied in the clinical treatment of various cancers that have been approved for various types of cancer, including melanoma, Hodgkin's lymphoma, non-small cell lung cancer (NSCLC), bladder cancer, breast cancer, renal cell carcinoma

(RCC), head and neck squamous cell carcinoma (HN-SCC), Merkel cell, carcinoma, hepatocellular carcinoma (HCC) and gastric cancer (GC). Several studies have reported that there are differences in PD-L1 expression based on NPC stage. Deng et al. [10] in Guangdong China found a significant difference in PD-L1 expression in early and advanced stage patients ( $p = 0.002$ ). PD-L1 expression was found to be increased in the advanced stage by 67.3% and in the early stage by 43.4%. In this study, no significant difference in PD-L1 expression based on sex, age and T classification. Li et al. [11] found a higher PD-L1 expression in stage IV significantly compared to stage I-III patients. Zhou [12] reported a significant increase in PD-L1 expression found as much as 74.3% in stage III-IV higher than stage I-II. In addition, Zhou also found an increase in expression that significantly related to the T classification of NPC, at T1-2 it was obtained as much as 41.9% and T3-4 as much as 74.3%. In the study of Yajuan Zhou et al. [13], a high PD-L1 expression was found as much as 67.6% in stage IV NPC and no significant difference was found based on the T classification.

In this study, based on TPS assessment, high expression of PD-L1 was found in 3 samples of early-stage NPC (20%) and 4 samples of advanced stage NPC (26.7%). Based on data analysis, the value of  $p=1.00$  ( $p>0.05$ ) means that there is no significant difference in PD-L1 expression between the early and advanced stages of NPC. In the IRS assessment, a high expression of PD-L1 was found in 1 sample of early-stage NPC (0.7%) and 4 samples of advanced stage NPC (26.7%). Based on data analysis, the value of  $p = 0.33$  ( $p>0.05$ ) which means that there is no significant difference in PD-L1 expression between the early and advanced stages of NPC. In this study, the assessment of PD-L1 expression on IHC examination used two scoring systems with Tumor Proportion Score (TPS) and Immuno Reactive Score (IRS). Minichsdorfer [8] said that so far there is no scoring system that has been validated for PD-L1 because that there are differences in the scoring system used in several studies. The TPS

assessment is a single qualitative score that assesses expression only by looking at the proportion of stained cells, while according to Fedchenko [9] the IRS assessment is a combination score that combines qualitative and semi-quantity consisting of two components, namely calculating the proportion score of positively stained cells and scoring the intensity of staining. Combination scores are considered more sensitive than single scores.

A meta-analysis study of the prognostic and clinicopathological value of PD-L1 expression in 1,315 NPC patients that included 11 studies from 2016-2019 conducted in China, Hong Kong, Philippines, Thailand, Taiwan and Japan found that increased PD-L1 expression was not associated with tumor stage. ( $p=0.1$ ), T classification of primary tumors, age and sex. In this study, it was found that increased PD-L1 expression was associated with poor overall survival (OS) in stage III-IV patients ( $p=0.049$ ), so it was concluded that PD-L1 could be used as a prognostic factor for NPC [14] Zhang [15] report that the high expression of PD-L1 in the advanced stage was 62.9% from 114 patients more than in the early stage was 59.6% of 57 patients but there was no significant difference in PD-L1 expression based on the NPC stage ( $p=0.647$ ). Meanwhile, based on the T classification, there was a statistically significant difference ( $p = 0.002$ ), the high PD-L1 expression was found in the T4 group of 69.3% of 75 patients, in the T3 group 65.4% of 78 patients, in the T2 group as many as 63.1% of 103 patients and in the T1 group as many as 28% of 25 patients. In this study, there was also no significant difference in PD-L1 expression based on age, sex and histopathological type. In this study, it was found that increased expression of PD-L1 was associated with increased expression of PD-1 and p-S6. S6 is a downstream target of the PI3K/Akt/mTOR oncogenic pathway. Overexpression of p-S6 can lead to dysregulation of mTor. The PI3K pathway is involved in the regulation of PD-L1 expression. Increased expression of PD-L1 is also associated with increased expression of PD-1 by CD8<sup>+</sup> T and secretion of IFN $\gamma$ .



**Figure 1.** Results of Immunohistochemical examination of PD-L1 on representative tissue of the study sample showed tissue with low expression (a) moderate expression (b) high expression (c), PD-L1 was expressed on the intracytoplasmic membrane in the cytoplasm of tumor cells. The stroma contains fibrocollagenous tissue with scattered inflammatory cells and blood vessels. Immunoperoxidase, obj 40x 200 $\mu$ m

Zhao [16] in his research in Fujian China also found no statistically significant difference in the increase in PD-L1 expression based on tumor stage ( $p = 0.118$ ), 46.3% were found in stages I-III and 53.7% in stage IV. Meanwhile, based on the T classification of primary tumors there was a significant difference ( $p= 0.033$ ), strong expression was found in 72.2% in the T3-T4 group and 27.8% in the T1-T2 group. This study concluded that activation of oncogenic signaling pathways and other related signaling pathways independent of inflammatory signals in the tumor microenvironment may regulate PD-L1 expression in tumor cells called the tumor-intrinsic mechanism.

Zhu [17] reported that 2 mechanisms of PD-L1 expression was regulated by tumor cells, namely: *innate immune resistance and adaptive immune resistance*. Oncogenic signaling pathways can induce increased PD-L1 expression as innate immune resistance. IFN- $\gamma$  secreted by active T cells can induce PD-L1 expression as adaptive immune resistance [18].

In addition, Ju, et al. [19] stated that PD-L1 expression is also regulated by genomic amplification, transcriptional regulation, epigenetic regulation and translational regulation. It is this mechanism that regulates tumor PD-L1 expression at different levels. PD-L1 is located on chromosome 9p24.1. Amplification of the 9p24.1 region is closely associated with increased levels of PD-L1 in various cancers. It has been found that copy number alteration (CNAs) of PD-L1 occurs in various tumor types which directly leads to upregulation of different PD-L1 expression. Several studies cited by Ju et al [19] reported that the highest CNA frequency of PD-L1 has been found in classic Hodgkin lymphoma (cHL), primary mediastinal B-cell lymphoma (PMBCL) and breast cancer, in 63%, 40% and 29%, respectively. However, in GC, NSCLC and diffuse large B-cell lymphoma (DLBCL), CNA was lower, with frequencies of 15%, 1.9%, 5.3% and 3%, respectively. Zerdes [20] also concluded that the increase in CNA was positively correlated with the level of the PD-L1 protein expressed by tumor cells.

In epigenetic regulation, epigenetic modifications such as microRNA (miRNA), promoter DNA methylation and histone modification can regulate the recognition and binding of transcription factors to DNA elements without affecting the DNA sequence, thereby altering chromatin structure and regulating PD-L1 expression levels. miRNAs are a class of non-coding single-stranded RNAs containing 22-24 nucleotides. miRNA inhibits translation or degradation of target mRNA by binding to the untranslated region 3 (3'UTR) of the target mRNA. A number of miRNAs have been found to regulate PD-L1 expression levels in various cancers, including miR513 [19,20]. Fang [21] who investigated the relationship between LMP1 and IFN in regulating PD-L1 expression in NPC and also reported that miR513 plays a role in the post-transcriptional mechanism that regulates expression. PD-L1. Tulalamba [1] also reported that several different cellular miRNAs overexpressed by NPC cause altered cellular gene expression that affects various signaling pathways in cell proliferation and apoptosis.

The transcription factors have been found to regulate PD-L1 transcriptional activation. These are MYC, STAT3, NF- $\kappa$ B, AP1, and hypoxia-inducible factor 1 (HIF-1). The MYC oncogene is a transcription factor that is overexpressed and activated in various tumors and involved in tumorigenesis. However, there is controversy regarding the regulation of PD-L1 expression by MYC. Casey et al [22] found that MYC inhibition in tumor cells resulted in decreased PD-L1

mRNA and protein expression. MYC can bind directly to the PD-L1 promoter and enhance the anti-tumor immune response. In contrast, Hogg et al [23] reported that MYC transcriptional levels inhibited PD-L1 mRNA expression. Fang [21] reported that STAT3 is another transcription factor involved to regulate of PD-L1 expression, STAT3 increases PD-L1 expression by binding to the PD-L1 promoter. It was also reported that LMP1 from the Epstein-Barr virus can induce PD-L1 expression through the induction of STAT3 phosphorylation. Noman [24] reported Hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) is another important carcinogenic factor and has clinical significance in regulating PD-L1 expression in tumor cells. HIF-1 $\alpha$  overexpression induces increased levels of PD-L1. It was also found that ubiquitination, deubiquitination, glycosylation and phosphorylation can affect the stability of PD-L1 protein in cancer cells, thereby regulating PD-L1 protein expression. Several proteins have been reported to regulate the stability of the PD-L1 protein through ubiquitination. CSN5 is the fifth component of the Component of Signalosome (CSN) complex, which contains the Jab1/MPN domain metalloenzyme (JAMM). CSN5 has deubiquitination activity through the JAMM motif and plays an important role during tumorigenesis. Lim et al [25] found that macrophages secrete TNF- to activate NF- $\kappa$ B and then induce CSN5 transactivation. Activation of CSN5 results in deubiquitination of PD-L1 in cancer cells and increases the stability of PD-L1 [19].

Minichsdorfer [8] in Austria only found a strong expression of PD-L1 as much as 20% in 55 Caucasian race NPC samples examined. The study found that the high expression of PD-L1 was associated with a decrease in the patient's OS. The study found that only 63% of patients showed positive EBV and concluded that negative EBV has a different microtumor immunological environment than EBV positive and this is related to differences in PD-L1 expression in NPC. Minichsdorfer [8] also used the antibody clone 22C3 Dako as in this study which is included in the diagnostic tool for staining PD-L1 in various types of solid malignancies that FDA-approved. The study also provides additional conclusions regarding the differences in PD-L1 expression reported in some literature that may be due to the absence of a comfortable antibody used for IHC staining for PD-L1. Buttner [26] also reported that IHC of PD-L1 staining results may differ depending on the antibody used. In addition, no validated PD-L1 scoring algorithm for NPC

has been developed so far and there is no consensus on widely accepted PD-L1 scoring.

Based on explanation of several studies above, it is possible that several factors that influence why PD-L1 is not significant in both groups in our study are there were differences of tumor-intrinsic mechanisms such as activation of oncogenic signaling pathways, there was differences expression of PD-1 by TILs, EBV might have an effect to expression PD-L1 in NPC, and genomic amplification, transcriptional regulation, epigenetic regulation and translational regulation mechanism that regulates tumor PD-L1 expression at different levels.

#### 4. CONCLUSION

Our result suggest that higher expression of PD-L1 was found to be more in advanced stages of NPC than in early stages of NPC and there was no significantly differences of PD-L1 expression between the early and advanced stages of NPC. Our recommendation are confirmation of quantitative value of PD-L1 expression data is required using RT-PCR and further investigation is needed for EBV and TILs that affect to PD-L1 expression in NPC.

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