Expression of PD-L1 as Prognostic Sign Factor in Carcinoma Nasopharyngeal Patient

Mila Habibasari1*, Abdul Kadir2, Nani Iriani Djufri3, Upik A. Miskad4, Abdul Qadar Punagi5

Abstract
It is well known that elevated levels of PD-1/PD-L1 are associated with poor prognosis in several tumor types, such as PD-1 in breast cancer and soft tissue sarcomas, and PD-L1 in melanoma and renal carcinoma. This study aims to measure PD-L1 levels in NPC patients at various stages and determine the prognosis of the disease. The method used was analytic observational research with a cross sectional research design. The total sample was 25 patients with NPC aged >18 years using consecutive sampling technique. Patients who have been diagnosed with NPC are then staged and examined for PD-L1 levels using immunohistochemical examination techniques. Test the analysis using Chi Square. The results showed that based on the comparison of PD-L1 based on cancer stage in NPC patients in this study, there were 42.9% PD-L1 at an early stage with negative values, 57.1% low, and 0% high. As for the advanced stage, there are 0% PD-L1 with negative values, 44.4% low, and 55.6% high, the Kruskall-Wallis test was carried out with p value = 0.002 (p<0.05), this indicates that the difference is significant. PD-L1 has sufficient sensitivity, specificity and accuracy in predicting advanced stage patients in NPC patients, it is useful in the prognosis of NPC. So it can be concluded that PD-L1 levels increase in advanced nasopharyngeal carcinoma so that it is suspected that there is a relationship with disease prognosis. So, the higher the PD-L1, the worse the prognosis for nasopharyngeal carcinoma.

Key Words: Nasopharyngeal Carcinoma, PD-L1, Immunohistochemistry, Prognosis.

Introduction
Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from mucosal epithelium or nasopharyngeal lymphoepithelial tissue, especially in the Rosenmuller fossa, NPC can spread to other lateral, posterosuperior, skull base, palate, nose, and oropharynx1. The incidence of NPC varies widely throughout the world. There are 15 to 30 per 100,000 new cases annually in Hong Kong, whereas in the United States it is only 1 per 100,000 population2.
Nasopharyngeal carcinoma based on its anatomical location is relatively inaccessible to curative surgical resection, generally managed with non-surgical treatment. In addition, these tumors are highly responsive to radiotherapy and chemotherapy. Thus the combination of chemotherapy and radiotherapy remains the initial definitive treatment option1. However, the problem that has arisen so far is that the recurrence rate of NPC after radiotherapy is still quite high, around 18%-45%, it is obtained data that between 80%-90% of early-stage NPC undergoing radiotherapy is in complete remission, but the survival rate during radiotherapy is complete. 5 years in advanced NPC only reaches 10% - 40%, and if distant metastases are obtained, 85% will die in the first year3. Immunotherapy has now been studied more extensively in enhancing the immune response against cancer cells.

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PD-1 binding and PD-L1 binding lead to inhibition of T cell activation and response. Blocking the PD-1/PD-L1 axis with monoclonal antibodies represents a target for cancer immunotherapy. PD-1 has two bonds, namely PD-L1 (CD274, B7-H1) and PD-L2 (CD273, B7-DC) which are members of the B7-CD28 protein family. PD-L1 is expressed on tumor cells but is also expressed on the surface of other cell types including T cells, B cells, dendritic cells, macrophages, mesenchymal stem cells, epithelial, endothelial, and adipose tissue. PD-L2 is typically expressed after stimulation of the cytokine interferon-γ (IFNγ), which is secreted by activating T cells. In the tumor microenvironment, PD-L1 is expressed but not only on immune cells associated with the microenvironment but also on the tumor cytomembrane and cytoplasm. Lyford-Pike et al compared noncancerous HPV+ tonsillar tissue with HNSCC cancer tissue and concluded that at the cellular level, local expression of PD-L1 is present in the inner tonsillar crypts, the site of initial HPV infection and the origin of HPV-HNSCC. PD-L1 in tumor cells is expressed mainly on the membrane (cell surface and partly in the cytoplasm).

It is well known that elevated levels of PD-1/PD-L1 are associated with poor prognosis in several tumor types, such as PD-1 in breast cancer and soft tissue sarcomas, and PD-L1 in melanoma and renal carcinoma. However, to date very few studies have been conducted on the role of PD-1 and PD-L1 as prognostic markers in NPC and have found inconsistent results. Here, we aim to measure PD-L1 levels in NPC patients at various stages and determine the prognosis of NPC.

Methods
This research is an analytic study using a cross sectional study method. This research was carried out at the T.H.T.K.L Polyclinic of Wahidin Sudirohusodo Hospital and Hasanuddin University Hospital during the period July 2021 – September 2021 until the number of samples was met. This study was conducted on 25 samples of NPC patients who met the inclusion criteria. Inclusion criteria were patients with NPC (all stages) who had a diagnosis based on histopathological examination, >18 years old, no tumors found in other organs, and no history of hemostatic disorders. The exclusion criteria were a history of other chronic infectious diseases.

This research has met the ethical requirements to be carried out from the Research Ethics Commission of the Hasanuddin University Teaching Hospital (Unhas Hospital) or the Dr. Central General Hospital. Wahidin Sudirohusodo (RSWS) with a letter of recommendation for ethical approval from the Health Research Ethics Commission (KEPK) RSPTN UN4.6.4.5.31/PP36/2021.

Immunohistochemical Stain
Samples were taken in the form of biopsy tissue from the nasopharyngeal mass after which it was mixed with paraformaldehyde (PFA) containing 2% sucrose in PBS at 4°C overnight and attached to paraffin using a tissue processor (EG1150, Leica, Germany). FFPE sections (3µm thickness) were cut with microton rotation (RM2255, Leica, Germany). The paraffin wax was rehydrated with alcohol, given 3% H2O2 for 10 minutes at room temperature and evaporated for 2.5 minutes to collect antigen using ethylene diamine tetra acetic acid buffer (pH=8.0). Subsequent staining of immunostaining was carried out for a 50-minute incubation period at 37°C with monoclonal antibodies for PD-1 and PD-L1. HIC staining of PD-1 and PD-L1 proteins was performed on two different slides. Tonsil tissue was taken as a positive control. Immune reactions were observed using the peroxidase/DAB kit. The images were taken using a phase contrast microscope.

Details of all reagents with reference to immunohistochemical staining procedures. Staining intensity was scored as follows:

- Percentage of tumor cells with positive cytomembranes (0: <5%, 1: 6-25%, 2: 26-50%, 3: 51-75%, 4: >75%) was added with intensity staining; (0: negative, 1: weak, 2: moderate, 3: strong (high). Patients with a score of 1 or 2 are considered low expression (low) 6.

Data analysis used IBM SPSS statistic version 22 and Chi Square statistical test procedure was used with a significance level of 0.05 (aLPHA = 5%).

Results
Data analysis was carried out on 25 patients aged >18 years with the distribution of sample characteristics shown in table 1.

Table 1. Characteristics of sample age in years based on cancer stage

<table>
<thead>
<tr>
<th>Variable</th>
<th>Early stage/I-II (n=7)</th>
<th>Advanced Stage/III-IV (n=18)</th>
<th>Nilai p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>SD</td>
<td>Min</td>
<td>Max</td>
</tr>
<tr>
<td>Age (Year)</td>
<td>43.14</td>
<td>11.71</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 1 shows that the average age of the study subjects was 43.14 (±11.71) years in early-stage...
patients with an age range of 26 to 58 years and the average age of the study subjects was 44.61 (±9.00) years in advanced-stage patients with an age range from 24 to 61 years. There is no statistically significant difference (p > 0.05) in the characteristics of the age data so that the data is said to be homogeneous.

Table 2. Characteristics of sex by cancer stage

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Early stage /I-II n (7)</th>
<th>% (100)</th>
<th>Advanced Stage /III-IV n (18)</th>
<th>% (100)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Man</td>
<td>7</td>
<td>100.0</td>
<td>10</td>
<td>55.6</td>
</tr>
<tr>
<td></td>
<td>Women</td>
<td>0</td>
<td>0.0</td>
<td>8</td>
<td>44.4</td>
</tr>
<tr>
<td>Age</td>
<td>21-30 Years</td>
<td>2</td>
<td>11.1</td>
<td>1</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>31-40 Years</td>
<td>3</td>
<td>16.7</td>
<td>2</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>41-50 Years</td>
<td>4</td>
<td>44.4</td>
<td>2</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>51-60 Years</td>
<td>4</td>
<td>22.2</td>
<td>2</td>
<td>28.6</td>
</tr>
<tr>
<td></td>
<td>≥ 61 Years</td>
<td>1</td>
<td>5.6</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 2 shows that showing the characteristics of the study sample based on gender, all patients with NPC in the early stages were male, namely 7 patients (100%), and at an advanced stage the sex was mostly male, namely 10 patients (55.6%), and for female as many as 8 patients (44.4%). After the Fisher's exact test was carried out, the value of p = 0.057 (p > 0.05) showed that patients in the early stages and patients in advanced stages were homogeneous in terms of gender. In the age distribution between early-stage patients and advanced-stage patients, there was no significant difference (p > 0.05) so that the data could be declared statistically homogeneous. Age frequency was analyzed using the Kruskall Wallis test where (p > 0.05) was not significant.

Table 3. Differences in PD-L1 by Cancer Stage

<table>
<thead>
<tr>
<th>PD-L1</th>
<th>Early stage /I-II n (7)</th>
<th>% (100)</th>
<th>Advanced Stage /III-IV n (18)</th>
<th>% (100)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>3</td>
<td>42.9</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>4</td>
<td>57.1</td>
<td>8</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>0</td>
<td>0.0</td>
<td>10</td>
<td>55.6</td>
</tr>
</tbody>
</table>

Based on the Kruskall-Wallis test, the value of p = 0.002 (p <0.05), this indicates that the difference is significant. There is a significant difference in PD-L1 levels between patients with early stage and advanced stage patients (Table 3).

Figure 1. PD-L1 levels

Figure 1 shows that PD-L1 levels at an advanced stage were found to be increased when compared to the early stage.
From the results of the ROC curve analysis in Figure 2, it was found that the area under the curve (AUC) for examining PD-L1 levels was 0.873 with an interval of 0.730 – 1000 and a significance of 95%. Diagnostic examination with an AUC value of 0.873 or 87.4%, meaning that if there are 100 patients studied, then as many as 87 patients will give the correct conclusion in determining the presence or absence of disease in that population. In this case there are 87 out of 100 patients who can be concluded correctly into the advanced stage criteria based on the PD-L1 examination.

Based on the Low/High and Negative categories in Table 4.4, 18 patients (81.8%) were found to be true positive, 4 patients (18.2%) were falsely positive, 0 patients (0.0%) were falsely negative, and 7 patients (28.0%) were true negatives. Based on the High and Low/Negative categories, 10 patients (100.0%) were found to be true positive, 0 patients (0.0%) were falsely positive, 0 patients (0.0%) were falsely negative, and 7 patients (100%) were true negatives.

Table 5 based on the PD-L1 cut-off point, the best in the low category with 100% sensitivity, 42.8% specificity, 81.8% positive predictive value, 100% negative predictive value, and 84.0% accuracy. This value is the most optimal value to distinguish patients who are categorized as advanced stage (III/IV) in patients with early-stage nasopharyngeal carcinoma (I/II) based on PD-L1 in this study.
Discussion

This study was conducted on 25 patients suffering from NPC, where the group of patients with early-stage NPC consisted of 7 patients and the group of patients with advanced NPC consisted of 18 people who had met the research criteria at Dr. RSUP Wahidin Sudirohusodo and RS. Hasanuddin University conducted in the period July 2021-September 2021.

In table 4.1, it can be seen that research subjects at an early stage had an average age of 43.14 (± 11.71) years with an age range of 26 to 58 years, not much different from those at an advanced stage which had an average age of 44.61 (± 9.00) years, with an age range of 24 to 61 years. After the unpaired t-test was performed, the p value = 0.739 (p>0.05), this indicates that the two groups have no difference in terms of average age. This finding is almost similar to a study conducted by Zhou et al in 2016 at the Hubei Cancer Hospital, Wuhan in Southern China. They reported that the mean age of patients with nasopharyngeal carcinoma was 47.05 years with an age range of 20 to 78 years7. This is also supported by research conducted by Sekar.M (2018) at Abdul Moeloek Hospital Bandar Lampung in 2018, 60% of NPC patients aged 45 years8. This is in line with research conducted by Melani (2013) which states that the average NPC patient is in the early adulthood to late adulthood age range9.

Table 4.2 shows the characteristics of the study sample based on gender, all patients with nasopharyngeal carcinoma in the early stages were male, namely 7 patients (100%), and at an advanced stage the most were male, namely 10 patients (55.6%).). The incidence of NPC is more experienced by men than women. This is because men are more often exposed to carcinogenic substances, including alcohol and cigarette smoke. This is in accordance with what was reported by Kristianti et al (2010) conducted at Hasan Sadikin Hospital, Bandung of 30 NPC patients studied, 83.3% of the patients were male and 70% were over 30 years old. Gender is also a confounding variable in this study and after the Fisher’s exact test was carried out with the result that there was no significant relationship between the two variables, it meant that the subject was homogeneous10.

Comparison of PD-L1 by Cancer Stage in Nasopharyngeal Carcinoma Patients

Based on the results of the analysis in table 4.3, it can be seen that in the early stages there were 42.9% PD-L1 with negative values, 57.1% low, and 0% high. As for the advanced stage, there are 0% PD-L1 with negative values, 44.4% low, and 55.6% high, the Kruskall-Wallis test was carried out with p value = 0.002 (p<0.05), this indicates that the difference is significant. There was a significant difference in PD-L1 levels between patients with early and advanced stages, this gave the result that there was a statistical relationship between PD-L1 expression and clinical stage (p<0.05). To date, various studies have confirmed that PD-L1 expression is associated with the stage and prognosis of cancer patients. Shi et al (2013) reported that high expression of PD-L1 in colorectal carcinoma is associated with tumor stage and prognosis11. Frigola et al (2011) showed that PD-L1 expression was associated with tumor stage and prognosis in patients with renal cell carcinoma12. Zhu Q (2017) at Sun Yat-Sen University (Guangdong, China) who measured PD-L1 expression as measured by immunohistochemistry in 2019 patients with nasopharyngeal carcinoma found that the level of PD-L1 expression was associated with the clinical stage of nasopharyngeal tumors13. The same thing was obtained by Wang et al (2018), they got results where there was a relationship between PD-L1 expression and survival in renal cell carcinoma patients. In addition, PD-L1 expression was found to be significantly associated with tumor stage14.

The opposite result was reported by Liu X (2019) at Nanfang Hospital (Guangzhou, China) who evaluated PD-L1 expression associated with TAM (Tumour Associated Machropagh) in 212 patients with nasopharyngeal carcinoma in which PD-L1 expression was found to be independent of nasopharyngeal tumor stage15, as well as a study conducted by Chan OS (2017) found that there was no correlation between nasopharyngeal tumor stage and PD-L1 expression16.

Relationship of PD-L1 to Predict Cancer Stage in Nasopharyngeal Carcinoma Patients

In our study, we showed that the PD-L1 cut off point in the Low category could predict advanced stage patients in nasopharyngeal carcinoma patients based on PD-L1 (Area Under Curve / AUC: 0.873). The PD-L1 Low value has a sensitivity of 100%, a specificity of 42.8%, a positive predictive value of 81.8%, a negative predictive value of 100%, and an accuracy of 84.0%.

Thus, it appears that PD-L1 has sufficient sensitivity, specificity and accuracy in predicting...
advanced stage patients in nasopharyngeal carcinoma patients and in this case can provide valuable prognostic information in nasopharyngeal carcinoma patients. The same thing was found in a study conducted by Zhou Y (2017) at the Wuhan Hospital, China where the PD-L1 value was suggested as an independent prognostic factor for nasopharyngeal carcinoma patients. Patients with high PD-L1 values have a greater risk of death, as well as a study conducted by Lee HV (2016) where they revealed that PD-L1 expression was associated with survival of nasopharyngeal carcinoma patients in this case can be used as information about prognosis of nasopharyngeal carcinoma patients. Similar results were obtained by Liu X (2019) at Nanfang Hospital (Guangzhou, China) who evaluated PD-L1 expression associated with TAM (Tumour Associated Macrophage) in 212 patients with nasopharyngeal carcinoma. PD-L1 levels in nasopharyngeal carcinoma are associated with significantly poor patient survival and may be useful as a prognostic factor for nasopharyngeal carcinoma patients. Zheng L (2015) using immunohistochemical staining by measuring the value of PD-L1 they found that high expression of PD-L1 in the cytomembrane correlates with poor prognosis in nasopharyngeal carcinoma patients. Similarly, Zhang et al (2015) in 139 patients diagnosed with nasopharyngeal carcinoma at Sun Yat Sen University Cancer Center (Guangzhou, China), they found that high PD-L1 expression in tumor cells predicts poor clinical outcome in NPC patients in this case PD-L1 expression can be an independent prognostic factor in NPC. Zhou Y (2017) at Wuhan University Hospital, China in their research results found that high PD-L1 expression correlates with shorter patient survival, in this case PD-L1 levels can be used as a prognostic factor in nasopharyngeal carcinoma. The opposite result was reported by Wotman. B (2020) found that there was no correlation between PD-L1 expression and patient survival, indicating that PD-L1 is not a suitable prognostic biomarker for nasopharyngeal carcinoma patients. Similarly, a study conducted by Chan OS (2017) at Pamela Youde Nethersole Eastern Hospital in Hong Kong showed no prognostic role for PD-L1 expression, in their study showing that PD-L1 expression is commonly found in nasopharyngeal carcinoma patients with a prevalence of 75% and 24% on immune cells and tumor cells when a 1% cut-off was used, respectively. This prevalence decreased when the 5% cut off was used. There was no correlation with respect to this prevalence with disease stage in this study and provides evidence that PD-L1 is commonly expressed in nasopharyngeal carcinoma but has no prognostic implications. Further studies are needed to confirm the prognostic value of PD-L1 expression in nasopharyngeal carcinoma tissue. There is variability in PD-L1 expression in some studies for various reasons, including tumor heterogeneity, number of samples studied, treatment options, antibody used, assay method, standard of PD-L1 assessment and PD-L1 positive threshold value used. From this study, it was found that in nasopharyngeal carcinoma patients, the expression of PD-L1 levels correlated with the stage of nasopharyngeal carcinoma patients and showed a poor prognosis. Many studies have shown that PD-L1 plays an important role in tumor immune evasion. Upregulation of PD-L1 will inhibit T cell function and cause T cell exhaustion and trigger immune evasion in cancer, indicating the importance of the PD-1/PD-L1 pathway in regulating tumor cell immunity. In the tumor microenvironment, PD-L1 is linked in the enhancement of T cell apoptosis and leads to tumor growth. PD-L1 is mainly expressed on the surface of tumor cells and tumor-associated APC (Agent Preventing Cell) in several types of cancer including pancreatic cancer, ovarian cancer, thymoma, and colorectal cancer. PD-L1 and its receptor PD-1 form the PD-1/PD-L1 signaling pathway that will mediate negative regulatory signals to produce immunosuppression which inhibits T cell function in anti-tumor immune responses and therefore blocks the PD-1/PD-L1 pathway. Can be effective as immunotherapy for NPC. This group of patients in our study may be ideal candidates for further clinical trials of anti-PD-1 or anti-PD-L1 therapy.

**Conclusion**

NPC patients with high PD-L1 expression show a poor prognosis where the PD-1/PD-L1 axis regulates anti-tumor immunity leading to T-cell exhaustion. Further studies with larger samples may be needed to confirm the prognostic value of PD-L1 expression in nasopharyngeal tissue. The combination of several factors, including the tumor microenvironment and several other molecular parameters, will better predict the prognosis and guide us in providing a theoretical basis for immunotherapy via the PD-1/PD-L1 pathway.
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