Profile of PD-1 and PD-L1 mRNA Expression in Peripheral Blood of Nasopharyngeal Carcinoma

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Background: Programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) expression is associated with prognostic and respond to immunotherapy with immune checkpoint inhibitor in several solid malignancies. However, the prognostic roles of PD-1 and PD-L1 expression in nasopharyngeal carcinoma (NPC) are less clear. This study aims to investigate PD-1 and PD-L1 mRNA expression levels in peripheral blood of Indonesian NPC patients and its association with clinicopathological features.

Materials and Methods: This study used blood samples of 21 NPC patients and 10 healthy volunteers as controls. Real-time polymerase chain reaction (PCR) was used to measure mRNA expression of PD-1 and PD-L1.

Results: PD-1 mRNA expression levels were significantly lower in NPC patients (ΔCT mean: 9.65±2.04) compared to healthy individuals (ΔCT mean: 8.04±1.51) (p=0.031). In contrast, PD-L1 mRNA expression levels were higher in NPC patients (ΔCT mean: 6.96±1.32) compared to healthy individuals (ΔCT mean: 7.11±0.55), but the difference was not statistically significant (p=0.554). The expression of PD-1 was associated with tumour-node-metastasis (TNM) stage (p=0.030) but not associated with age (p=1.000), sex (p=1.000), body mass index (p=0.350), tumor stage (p=0.338), nodal stage (p=0.579), metastasis stage (p=0.371), and Eastern Cooperative Oncology Group (ECOG) status (p=0.228). Meanwhile PD-L1 expression was not associated with all clinicopathological features.

Conclusion: The PD-1 mRNA expression levels were significantly lower, while PD-L1 expression levels were higher in NPC patients compared to healthy controls. PD-1 expression was correlated with TNM stage.

Keywords: nasopharyngeal carcinoma, immune checkpoint inhibitors, PD-1, PD-L1

Introduction

Nasopharyngeal carcinoma (NPC) is an endemic disease in Southern China and Southeast Asia, including Indonesia. Approximately 80,000 new cases worldwide are reported and estimated 50,000 deaths are found annually.¹ ² Advances in radiotherapy and tumor imaging have improved NPC patient’s outcome and increased 5 year disease free survival.

Date of submission: October 23, 2019
Last Revised: December 18, 2019
Accepted for publication: December 19, 2019

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to 77%. Despite the improvement in NPC treatment, distant metastasis and local recurrence are still become major problems in treating NPC. Approximately 5-15% NPC patients develop local and regional recurrences and 15-30% patients develop distant metastasis. Thus, effective treatment and reliable prognostic marker are needed to improve the survival.

Immunotherapy has emerged as an effective treatment for many types of cancer. One of the most promising approaches in immunotherapy is inhibiting programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) molecules using immune checkpoint inhibitor (anti PD-1 and anti PD-L1). Many clinical trials in immunotherapy especially therapy targeting PD-1 and PD-L1 have been done and showed good anti-tumor effect against several cancer types including NPC.

PD-1 is an inhibitory receptor that commonly expressed on T cell to regulate immune responses. PD-L1 is a ligand molecule for PD-1 and commonly expressed by immune cells such as B cell, resting T cell, and macrophages. PD-L1 also has been reported to be over expressed in many cancer types. Binding between PD-1 and PD-L1 causes T cell to inactive so that promotes tumor growth.

The expression of PD-L1 is related to response of therapy with immune checkpoint inhibitor where patient with positive PD-L1 expression tends to show good response. Immunohistochemistry (IHC) is commonly used to measure PD-L1 expression using patient’s tumor tissue. However, the use of immunohistochemistry has several drawbacks including differing IHC cut offs, variable detection antibodies, processing variability, and tissue preparation. Therefore, alternative method to check PD-L1 expression in blood might become an alternative method to replace IHC. Recent studies have shown that PD-1 and PD-L1 expression in peripheral blood was associated with cancer progression and potentially may assist in identifying type of patients who respond immune checkpoint inhibitor. The use of blood is easier, less invasive and could be used for monitoring sequential tumor response to immunotherapy.

However, until recently, not many studies have been done analyzing PD-1 and PD-L1 messenger RNA (mRNA) expression in peripheral blood of NPC patients. Prognostic role of PD-1 and PD-L1 in NPC patients is also not clear and inconsistent. Besides, research of PD-1 and PD-L1 in Indonesian cancer patients is not common. Therefore, we aim to analyze PD-1 and PD-L1 mRNA expression in peripheral blood of NPC patients in Dharmais Hospital-National Cancer Center Indonesia and investigate the association with clinicopathological features.

Materials and methods

Samples Collection
This cross-sectional study used peripheral blood of 21 NPC patients and 10 peripheral bloods of healthy individuals as healthy controls. NPC patients have been confirmed through anatomic pathology examination and haven’t received any therapy. All samples were collected with total sampling method in Dharmais Cancer Hospital from 2017 to 2019. Blood samples of patients were placed in ethylenediaminetetraacetic acid (EDTA) tube then directly proceed for RNA isolation. Clinicopathological data of patients were obtained from medical record of Dharmais Cancer Hospital. This study has been approved by Dharmais Hospital Ethics Committee with ethical approval number 086/KEPK/X/2017 and 075/KEPK/V/2018 for NPC patients and healthy individuals respectively. Patients were also asked to read and fill out written informed consent before the sample was taken.

RNA Extraction and Reverse Transcription
Total RNA was extracted using Total RNA Mini Kit (Blood/Culture Cell) (GeneAid Biotech, Taipei, Taiwan). RNA was treated with Dnase using Rnase-Free Dnase set (GeneAid Biotech) to remove any DNA contamination. Nanodrop spectrophotometer (Thermo Scientific, Massachusetts, USA) was used to measure RNA concentration and purity.

Reverse transcription was done using SensiFAST complementary DNA (cDNA) Synthesis Kit (GeneAid Biotech). A total of 50 ng of RNA was used in reverse transcription reaction to generate cDNA. Procedures for RNA extraction and reverse transcription followed the manual instructions provided by the kits.

Primers and Probes
This study used newly designed primers and probe. To avoid genomic DNA amplification, all primers used were designed to span exon-exon junction. Primers and probes sequences of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), PD-L1, and PD-1 were shown in Table 1. Each primer pair and probe were mixed and formulated into Custom TaqMan Gene Expression Assay (Applied Biosystem, California, USA).
Primer Sequence

| Primer          | Sequence                |
|-----------------|-------------------------|
| GAPDH for       | 5’-agcctcaagatcatcagca-3’ |
| GAPDH rev       | 5’-actgtggctagatccctc-3’ |
| GAPDH probe     | 5’-ctgcacaccaactgcttag-3’ |
| PD-L1 for       | 5’-tgtaggcaatggccccat-3’ |
| PD-L1 rev       | 5’-tgctagttcatgttcagaggt-3’ |
| PD-L1 probe     | 5’-attttgtgattttgacgtc-3’ |
| PD-1 for        | 5’-ccaagggcagatcaagaga-3’ |
| PD-1 rev        | 5’-tgggctgtgggcactt-3’ |
| PD-1 probe      | 5’-tgcctgctgctgataact-3’ |

Table 1. Primers and probes used in real-time PCR.

Real-time Polymerase Chain Reaction (PCR)/Quantitative PCR (qPCR) Analysis
Real-time PCR reaction was done using custom TaqMan gene expression assay and TaqMan gene expression master mix from Applied Biosystem. Real-time PCR instrument 7500 FAST from Applied Biosystem was used to perform qPCR reaction. The temperature for real-time PCR was started from hold stage at 50°C (2 minutes) and 95°C (10 minutes), followed by 40 cycles of denaturation at 95°C (15 seconds), and annealing and extension at 62°C (1 minute). Real-time PCR data was analyzed using difference of cycle threshold (∆CT) method with GAPDH as internal control.

To classify gene expression as low or high, average ∆CT value of 10 healthy controls was used as cut-off. Samples that have ∆CT values below healthy controls’ average ∆CT were classified as high expression, while ∆CT values of samples that above healthy controls’ average ∆CT were classified as low expression.

Statistical Analysis
SPSS Statistics 22 statistical software (IBM Corporation, New York, USA) was used to statistically analyze the data. Fisher-exact test or chi-square test was used to analyze the association between gene expression and clinicopathological features. Due to the abnormal data distribution, nonparametric test (Man-Whitney) was used to compare mRNA expression between NPC patients and healthy individuals. Differences with p<0.05 were considered statistically significant.

Results
PD-1 and PD-L1 mRNA Expression in NPC Patients and Healthy Individuals
A number of 21 NPC patients and 10 healthy individuals as control were used in this study. NPC patients have age range from 17 to 66 years (mean ages: 43.4±13.8), and healthy individuals’ age range from 23 to 49 years (mean ages: 39±9.4). As many as 3 NPC patients were female and 18 were male, while 5 healthy individuals were female and 5 were male.

PD-1 and PD-L1 expressions are represented as ∆CT values. Higher ∆CT values indicate lower PD-1 and PD-L1 mRNA expression. The result showed that PD-1 mRNA expression is significantly lower in NPC patients when compared to healthy controls (Figure 1). ∆CT mean of PD-1 is 9.65±2.04 in NPC and 8.04±1.51 in healthy individuals (p=0.031). Otherwise, PD-L1 mRNA expression tends to be higher in NPC patients, but the difference is not statistically significant. ∆CT mean of PD-L1 is 6.96±1.32 in NPC and 7.11±0.55 in healthy individuals (p=0.554) (Figure 1).

Association of PD-1 and PD-L1 mRNA Expression and Clinicopathological Parameters of NPC Patients
A total of 21 NPC patients were divided into low expression and high expression based on real time PCR result. As many as 5 patients (23.8%) were categorized into high PD-1 expression and 16 patients (76.2%) were categorized into low PD-1 expression. PD-1 expression was significantly associated with tumour-node-metastasis (TNM) stage (p=0.030), but not associated with the other clinical parameters examined (Table 2). A number of 13 patients (61.9%) were categorized into high PD-L1 expression and 8 patients (38.1%) were categorized into low PD-L1 expression. PD-L1 expression was not associated with all clinical parameters (Table 3).

Discussion
The result of this study found that PD-1 expression is significantly lower in NPC patient compared with healthy individuals. Previous study suggested that the increased of PD-1+ CD4+ T cells apoptosis might contribute to the decline of PD-1 expression.18 Low PD-1 expression may indicate the status of decreased number of T cells. Another study showed that low mRNA expression of PD-1 in blood might show the status of reduced T-cell immunosuppression
that may cause abnormal T cell activation.\textsuperscript{19} It has been explained that peripheral blood of NPC patients displayed abnormality in lymphocyte subsets.\textsuperscript{20,21} This aberration in immune status might be caused by abnormal cellular immune response.\textsuperscript{20} However, further studies need to be done to confirm this result.

Table 2. PD-1 expression and clinicopathological parameters of NPC patients.

| Parameter         | Low Expression n (%) | High Expression n (%) | p -value |
|-------------------|-----------------------|------------------------|----------|
| Age               | ≤50       | 10 (76.9) | 3 (23.1) | 1.000    |
|                   | >50       | 6 (75.0)  | 2 (25.0) |           |
| Sex               | Male      | 14 (77.8) | 4 (22.2) | 1.000    |
|                   | Female    | 2 (66.7)  | 1 (33.3) |           |
| Body Mass Index   | Normal    | 5 (100.0) | 0 (0)    | 0.350    |
|                   | Underweight| 6 (66.7)  | 3 (33.3) |           |
|                   | Overweight | 5 (71.4)  | 2 (28.6) |           |
| TNM Stage         | II-III    | 4 (100.0) | 0 (0)    | 0.030*   |
|                   | IVa       | 0 (0)     | 2 (100.0)|           |
|                   | IVb       | 8 (72.7)  | 3 (27.3) |           |
|                   | IVc       | 4 (100.0) | 0 (0)    |           |
| Tumor Stage       | T1-2      | 8 (88.9)  | 1 (11.1) | 0.338    |
|                   | T3-4      | 8 (66.7)  | 4 (33.3) |           |
| Nodal Stage       | N0-1      | 2 (66.7)  | 1 (33.3) | 0.579    |
|                   | N2-3      | 14 (77.8) | 4 (22.2) |           |
| Metastasis Stage  | X         | 3 (60.0)  | 2 (40.0) | 0.371    |
|                   | 0         | 9 (75.0)  | 3 (25.0) |           |
|                   | 1         | 4 (100.0) | 0 (0)    |           |
| ECOG Status       | 0-1       | 14 (82.3) | 3 (17.7) | 0.228    |
|                   | ≥2        | 2 (50.0)  | 2 (50.0) |           |

TNM: tumour-node-metastasis, ECOG:Eastern Cooperative Oncology Group.
In contrast with the PD-1 expression, PD-L1 expressions tend to be higher in NPC compared to healthy individuals. Several studies have been reported that PD-L1 expression was over expressed in various types of cancer including NPC.\textsuperscript{22,23} Study analyzing PD-L1 mRNA expression in NPC tissue showed that PD-L1 mRNA expression was detected in 66.6% NPC tissue samples, while there were no PD-L1 expressions in normal nasopharyngeal tissue.\textsuperscript{23} Another study in different cancer type found that PD-L1 mRNA expressions were significantly higher in oral squamous cell carcinoma (OSCC) tissue samples.\textsuperscript{24} The mRNA expression tended to be higher in blood of OSCC patients but the difference was not statistically significant.\textsuperscript{24}

Mechanism of PD-L1 up-regulation in NPC was not fully understood.\textsuperscript{15} In general, PD-L1 over expression may be regulated by innate immune resistance mechanism through constitutive oncogenic signaling such as signal transducer and activator of transcription 3 (STAT3) pathway or adaptive immune resistance mechanism through induction by inflammatory signal such as induction by interferon (IFN)-γ.\textsuperscript{8} In NPC, high expression of PD-L1 might be controlled by latent membrane protein 1 (LMP1) through nuclear factor kappa-B (NF-κB), activator protein 1 (AP-1), and STAT3 oncogenic pathways and immune modulation via excretion of IFN-γ. IFN-γ was known could increase the level of PD-L1 mRNA expression in Epstein–Barr virus (EBV)-infected NPC.\textsuperscript{25} Previous study also showed that PD-L1 mRNA and protein expression level were higher in EBV infected NPC than that in NPC with EBV negative.\textsuperscript{25} The study suggested that blockade of PD-1/PD-L1 as well
as LMP1 pathway may give clinical benefit in the treatment of NPC patients associated with EBV.

The result of this study also showed significant correlation between PD-1 expression and TNM stage. However, no significant correlation was found between PD-1 expression and age, sex, body mass index, TNM stage, tumor stage, nodal stage, metastasis stage, and ECOG status of NPC patients (Table 2). Meanwhile PD-L1 expression showed no significant correlation with all clinicopathological parameters (Table 3).

Previous study revealed significant correlation between PD-L1 expression with tumor stage and distant metastasis in Northern China NPC patients. Another study found significant correlation between PD-L1 expression and tumor stage, clinical stage, and hemoglobin levels of recurrent NPC patients in China. Meta-analysis study involving 1836 NPC patients from 15 different studies in Asia regions found no significant correlation between PD-1 and PD-L1 expression and clinicopathological characteristics of NPC.

It has been suggested that different region may vary in therapeutics and clinicopathological characteristics of NPC patients. Another similar study also suggested that there may be greater variability of PD-L1 expression in different area especially in endemic region. Indonesian NPC patients might have different PD-1 and PD-L1 expression pattern and clinicopathological features. In addition, the significant correlation between PD-1 and PD-L1 expression with clinicopathological features may be affected by the sample size. Therefore, to find more significant correlation, number of samples in our study need to be added.

To our knowledge, our study is the first to analyze PD-1 and PD-L1 mRNA expression in Indonesian NPC patients. We found that most NPC patients have higher PD-L1 expression. This finding suggests that checkpoint inhibitor therapy might give benefit to the subset of NPC patients in Dharmais Cancer Hospital. However, further research with larger samples is needed to confirm the result. Nevertheless, this study provides a preliminary data of PD-1 and PD-L1 mRNA expressions profile in Indonesian NPC patients.

Acknowledgements

The authors wish to thank to Dr. dr. Sri Hartini, SpPK(K), MARS for advices and discussions related to this study and Department of Research and Development, Dharmais Cancer Hospital-National Cancer Center for providing the funding and instrument used in this study.

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