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The correlation between interleukin-4 (IL-4) and programmed cell death-ligand 2 (PD-L2) expression with clinicopathological characteristics on prostate cancer

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ABSTRACT

Prostate cancer (PCa) is the most frequent cancer diagnosed worldwide and the second most common malignancy in men. IL-4 is one of cytokines related to the inflammation process. An increase level of IL-4 in patients with PCa might be related to progression to castrate-resistance prostate cancer. Programmed cell death-ligand 2 (PD-L2) plays an important role in the anti-tumor immune system, however the exact mechanism is not fully understood. This study aimed to investigate the correlation between IL-4 and PD-L2 expression with the clinicopathological characteristic of PCa. The IL-4 and PD-L2 examinations were performed using quantitative real-time polymerase chain reaction (qRT-PCR) while clinicopathological characteristics were described by the Gleason score and International Society of Urological Pathology (ISUP) grade. Data collected were then analyzed using Pearson and Spearman test. In total, 20 patients with PCa tissue were collected between 2015 and 2020. The mean level of IL-4 and PD-L2 were higher in metastatic PCa/M-PCa (105.64 and 665.42 ng/mL) compared to non-metastatic PCa/NM-PCa (41.62 and 215.06 ng/mL). A significant difference with medium correlation between IL-4 and PD-L2 with Gleason score and ISUP grade was observed on all samples (p = 0.035 and 0.045; r = 0.454 and 0.473). However, no significant difference with weak correlation was observed on each group (p = 0.136 and 0.858; r = 0.065 and 0.506). Interestingly, there was a significant difference with very strong correlation observed between IL-4 and PD-L2, both on all samples (p = 0.001; r = 0.955) and on each group (p = 0.001 and 0.001; r = 0.917 and 0.955). In conclusion, there is a correlation between IL-4 and PD-L2 with the clinicopathological characteristics of PCa.

Keywords:
clinicopathological;
IL-4;
mRNA;
PD-L2;
prostate cancer

ABSTRAK

Kanker prostat (PCa) adalah kanker yang paling sering terdiagnosis di seluruh dunia dan merupakan keganasan kedua paling umum pada pria. IL-4 merupakan salah satu sitokin yang berhubungan dengan proses inflamasi. Peningkatan kadar IL-4 pada pasien PCa berhubungan dengan perkembangan kanker prostat kebal kastrasi. *Programmed cell death-ligand* 2 (PD-L2) mempunyai peran penting dalam sistem kekebalan anti tumor, namun mekanisme pastinya belum sepenuhnya dipahami. Penelitian ini bertujuan untuk mengkaji hubungan ekspresi IL-4 dan PD-L2 dengan karakteristik patologi klinik PCa. Pemeriksaan IL-4 dan PD-L2 dilakukan dengan *quantitative real-time polymerase chain reaction* (qRT-PCR) dan karakteristik patologi klinik digambarkan dengan *Gleason score* dan *International Society of Urological Pathology* (ISUP) *Grade*. Data yang terkumpul kemudian dianalisis menggunakan uji Pearson dan Spearman. Total 20 pasien dengan jaringan PCa dikumpulkan antara tahun 2015 dan 2020 dan dianalisis. Rerata kadar IL-4 dan PD-L2 lebih tinggi pada PCa metastasis /M-Pca (105,64 dan 665,42 ng/mL) dibandingkan dengan PCa non-metastasis/NM-PCa (41,62 dan 215,06 ng/mL). Terdapat perbedaan signifikan dengan korelasi sedang antara IL-4 dan PD-L2 dengan *Gleason score* dan ISUP *grade* pada semua sampel (p = 0,035 dan 0,045; r = 0,454 dan 0,473). Namun, tidak ada perbedaan nyata dengan korelasi lemah pada masing-masing kelompok (p = 0,136 dan 0,858; r = 0,065 dan 0,506). Menariknya, terdapat perbedaan nyata dengan korelasi sangat kuat antara IL-4 dan PD-L2 yang diamati pada semua sampel (p = 0,001; r = 0,955) dan setiap kelompok (p = 0,001 dan 0,001; r = 0,917 dan 0,955). Kesimpulannya, terdapat hubungan antara IL-4 dan PD-L2 dengan karakteristik patologi klnik PCa.

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INTRODUCTION

Nowadays, prostate cancer (PCa) is the most frequent cancer diagnosed worldwide. It is the fourth most non-skin cancer in human and the second most diagnosed cancer in men in the world. In 2018, approximately 1.3 million new cases of PCa were diagnosed and 359.000 deaths occurred. Prostate cancer is positioned as the fifth cause of death in men worldwide.[1] Early diagnosis and management of PCa contribute to the decrease of mortality rate in many countries such as in the United States, North America, Oceania, North and West Europe, and several developing countries in Asia.[1-4]

Chronic inflammation has emerged as an important factor in the development and progression of PCa through the release of proinflammatory cytokines. It was reported that interleukin-4 (IL-4), an inflammatory mediator, plays a dual role in the development and progression of PCa.[5,6] On one hand, IL-4 was shown to have tumor-promoting effects in prostate cancer. Interleukin-4 induces T-cell anergy and loss of T-cell-mediated cytotoxicity leading to the promotion of tumor development and cancer progression.[7] Moreover, IL-4 serves a direct role in the progression of PCa from androgen-responsive to advanced castrate-resistance PCa. The IL-4 also can activate the androgen receptor (AR) which plays in the transition from androgen-dependent to androgen-independent PCa after androgen therapy.[8] On the other hand, IL-4 also has tumor-suppressive properties in prostate cancer, especially in benign PCa.[5] At high concentrations, IL-4 inhibits the proliferation of breast and colorectal cancer cells line.[9,10] In addition, IL-4 induces natural killer (NK) cell cytotoxicity and increases NKG2D receptor expression.[11]

Programmed cell death-ligand 2 (PD-L2) is one of two ligands of the programmed cell death-1 (PD-1) receptor, a protein that plays an important role in immune cell activation. It has been widely used as targeted therapy in some solid tumor and hematology cancers with promising results.[12] Programmed cell death-ligand 2 plays a role in the regulation of antitumor immune response, however, the exact mechanism can not be fully explained.[13] The high expression of PD-L2 in prostatectomy samples also shows a prognostic value with the findings of worse biochemical recurrence, metastatic status, and specific survival in prostate cancer.[14] This study aimed to investigate the correlation between IL-4 and PD-L2 with clinicopathological characteristics on PCa patients.

MATERIAL AND METHODS

Study design and subject

This was an observational cross-sectional study involving twenty paraffin embedded tissue samples of PCa patients in the Dr. Sardjito General Hospital, Yogyakarta, Indonesia, from 2015 to 2020 who meet the inclusion and exclusion criteria. The PCa patients were diagnosed based on the histopathological examination either from prostate biopsy or transurethral resection of the prostate. The inclusion criteria of samples were PCa patients with available paraffin embedded tissue samples and complete pathological anatomy grading. The exclusion criterias were tissue samples aged more than 3 years and had invalid DNA integrity.

This study has been approved by the Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta with number KE/FK/0109/EC/2022.

Examination of IL-4 and PD-L2 expression

RNA extraction

The RNA genome was extracted from formalin-fixed paraffin-embedded (FFPE) prostate tissue using the GeneAll®

Exgene™ Hybrid™ miRNA Kit (Cat. No. 104-150). In FFPE tissue specimens, a deparaffinization procedure was carried out using xylol and absolute ethanol. Cell lysis was carried out using FARB buffer and 3.5 μL β-mercaptoethanol. For RNA binding and elution, ethanol 70% (RNase-free) was used, and then RNA was extracted.

RT-qPCR

The RNA extraction product was then examined using the Bioneer AccuPower® GreenStar™ RT-qPCR PreMix (Cat. No. K-6400). The PCR was performed using veriti thermal cycler under the following conditions: reverse transcription at 50-70°C for 15 min followed by 1 cycles of pre-denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, 40 cycles annealing/extension/detection at 55-60°C for 30 sec, and 1 cycle of melting. The primer used was Bioneer Oligonucleotide - AccuOligo® which has free bio-RP purification (Cat. No. SR-1002)

Prostate cancer classification

The PCa classification was created to group patients with similar clinical findings. Generally, it was used to determine the stage of the disease and to decide the most appropriate management.[15]

TABLE 1. TNM classification of PCa

Class	Characteristics
T- Tumor (primary)	
• Tx	Primary tumor can't be assessed
• T0	No evidence of tumor
• T1	Tumor can't be palpated clinically
• T1a	• Incidental tumor finding ≤ 5 % of the resected tissue
• T1b	• Incidental tumor finding > 5 % of the resected tissue
• T1c	• Tumor identified using needle biopsy
• T2	Tumor can be palpated and confined in prostate
• T2a	• Tumor palpated in less than half of the lobe, in one lobe
• T2b	• Tumor palpated in more than half of the lobe, in one lobe
• T2c	• Tumor palpated in both lobes
• T3	Tumor infiltrate the prostate capsule
• T3a	• Extracapsular extension (unilateral or bilateral)
• T3b	• Tumor infiltrate seminal vesicle
• T4	Tumor is fixated or infiltrate other organ beside seminal vesicle: external sphincter muscle, rectum, levator muscle and or abdominal wall
N-Node	
• Nx	Regional lymphnode can't be assessed
• N0	Regional lymphnode metastatic is absent
• N1	Regional lymphnode metastatic is present
M-Metastatic	
• M0	Distant metastatic is absent
• M1a	Metastatic in non-regional lymphnode
• M1b	Metastatic in bone
• M1c	Metastatic in visceral organ

Note: TNM classification of PCa. T is assessed using digital rectal examination (DRE). N and M is assessed using radiologic imaging, the most common used is multi-slice computed tomography (MSCT) with contrast.

In addition, grading in PCa was also needed for the treatment decision. It was also needed to group the disease into risk groups. The Gleason score was measured by finding the most common histopathological pattern type and second most common pattern type, while ISUP grade was grouped according to its Gleason score.[15]

Statistical analysis

The SPSS version 25.0 was used for statistical analysis. The data were previously tested for the normality using Saphiro Wilk test. The correlation between variables were analyzed using Pearson analysis for normally distributed data and using Spearman analysis for data that not normally distributed. A p value < 0.05 was considered significant.

RESULTS

Twenty PCa patients were involved in this study. The patients were divided into two groups with 10 patients in each group i.e. metastasis prostate cancer (M-PCa) and non-metastasis prostate cancer (NM-PCa). The mean age for M-PCa group was 69.6 ± 9.51 yr, while in NM-PCa group was 75.7 ± 5.52 yr. The IL-4 and PD-L2 expression as well as Gleason score and ISUP grade on PCa patients are presented in TABLE 3.

Significant moderate positive correlation ($r > 0.400$; $p < 0.05$) between IL-4 and PD-L2 with clinicopathological characteristics (Gleason score and ISUP grade) on group of PCa was observed (TABLE 4). However, no significant correlation ($p > 0.05$) on subgroup of PCa was observed (TABLE 5).

Interestingly, strong correlation between IL-4 and PD-L2 both in group or subgroup of PCa was observed ($r>0.900$; $p < 0.05$) as presented in TABLE 6 and FIGURE 1A and 1B.

TABLE 2. Prostate cancer grading according to the International Society of Urological Pathology

Gleason score	ISUP grade
2-6	1
7 (3+4)	2
7 (4+3)	3
8	4
9-10	5

Note: Gleason score and ISUP grade as the part of prostate cancer grading to know disease severity and guide for management decision.

TABLE 3. IL-4 and PD-L2 expression (ng/mL) and Gleason score and ISUP grade on PCa patients

Variable	n	Mean ± SD	Med. (Min. – Max.)
IL-4			
• NM-PCa	10	41.628 ± 19.85	41.62 (17.15 – 68.59)
• M-PCa	10	105.64 ± 26.81	105.64 (73.52 – 168.9)
PD-L2			
• NM-PCa	10	215.061 ± 117.41	215.06 (64.0 – 415.87)
• M-PCa	10	665.424 ± 204.16	665.42 (445.72 – 1024.0)
Gleason score			
• NM-PCa	10	7.6 ± 1.58	7.6 (6 – 10)
• M-PCa	10	9 ± 1.25	9 (6 – 10)
ISUP grade			
• NM-PCa	10	2.9 ± 1.73	2.9 (1 – 5)
• M-PCa	10	4.5 ± 1.27	4.5 (1 – 5)

Note: SD= standard deviation; Med. = median; Min. = lowest value; Max. = highest value; PCa: prostate cancer; NM-PCa = non-metastasis prostate cancer; M-PCa = metastasis prostate cancer (NM-PCa); PD-L2 = programmed cell death-ligand 2; ISUP grade = The International Society of Urological Pathology grade.

TABLE 4. Correlation between IL-4 and PD-L2 with Gleason score and ISUP on group of PCa

Variable	r	p
IL-4 – Gleason score	0.470	0.036
IL-4 – ISUP grade	0.454	0.045
PD-L2 – Gleason score	0.473	0.035
PD-L2 – ISUP grade	0.454	0.044

Note: PCa: prostate cancer; PD-L2 = programmed cell death-ligand 2; ISUP grade = The International Society of Urological Pathology grade.

TABLE 5. Correlation between IL-4 and PD-L2 expression with Gleason score and ISUP grade on subgroup of PCa

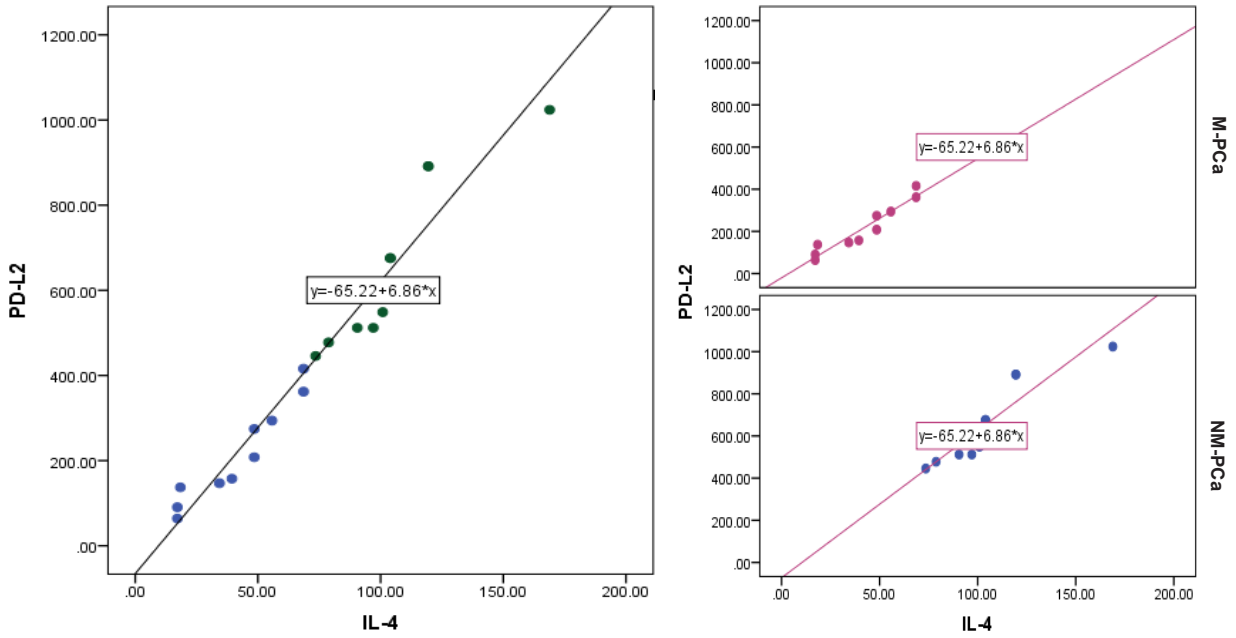
Variable	r	p
IL-4 – Gleason score		
• NM-PCa	0.145	0.690
• M-PCa	0.504	0.137
IL-4 – ISUP grade		
• NM-PCa	0.176	0.626
• M-PCa	0.065	0.858
PD-L2 – Gleason score		
• NM-PCa	0.162	0.655
• M-PCa	0.506	0.136
PD-L2 – ISUP grade		
• NM-PCa	0.181	0.617
• M-PCa	0.065	0.857

Note: PCa: prostate cancer; NM-PCa = non-metastasis prostate cancer; M-PCa = metastasis prostate cancer (NM-PCa); PD-L2 = programmed cell death-ligand 2; ISUP grade = The International Society of Urological Pathology grade.

TABLE 6. Correlation analysis between IL-4 and PD-L2 expression in all group and subgroup

Variable	r	p
All samples	0.950	0.001
NM-PCa	0.955	0.001
M-PCa	0.917	0.001

Note: NM-PCa = non-metastasis prostate cancer; M-PCa = metastasis prostate cancer (NM-PCa); PD-L2 = programmed cell death-ligand 2.



A B

FIGURE 1. Scatterplot for correlation of IL-4 and PD-L2 on A) group and B) subgroup of PCa.

DISCUSSION

Correlation between IL-4 and PD-L2 with Gleason score and ISUP grade on all group

Significant moderate positive correlation between IL-4 and PD-L2 with clinicopathological characteristics on group of PCa were observed (TABLE 4). In this study the clinicopathological of PCa was expressed by Gleason score and ISUP grade. The increase of Gleason score was followed by the increase of ISUP grade as the Gleason score is the component in ISUP grade measurement. The increase of both of this clinicopathological aspect is related to advance prostate cancer. This is similar to the results reported by Erb *et al.*[16] stating that IL-4 as one of cytokines which affects the immune response, cell proliferation, differentiation, and related to prostate cancer and tumor microenvironment (TME). IL-4 was found a lot in cell in TME, such as tumor-associated stromal cells and tumor-infiltrating immune cells,

and this is known to be increasing significantly in patients with advance prostate cancer.

Significant moderate positive correlation between PD-L2 with clinicopathological characteristics on group of PCa observed in this study was also reported by Zhao *et al.*[17] The authors demonstrated that PD-L2 affects the immune system response in the development of prostate cancer. Not like PD-L1, studies concerning PD-L2, one of ligands of PD-1, were limited. Even though PD-L2 has two until six time higher affinity compared to that PD-L1.[12] The role of PD-L1 and PD-L2 in the progressivity of prostate has not been clearly explained. Both PD-L1 and PD-L2 may bond to PD-1 receptor in T cell lead to increase prostate cancer cell proliferation.

Correlation of IL-4 and PD-L2 with Gleason score and ISUP grade on each group

Although significant moderate positive correlation between IL-4 and PD-L2 with clinicopathological characteristics (Gleason score and ISUP grade) on all group of PCa were observed, however there was no significant correlation ($p > 0.05$) on subgroup of PCa (TABLE 5). This result is not concordance with other previous studies. Relatively little sample involving in each group (10 samples) in this study might cause the correlation was not significant. Very low correlation in NM-PCa group and low correlation in M-PCa were observed. This result was also supported by the higher of the mean of IL-4 and PD-L2 in M-PCa. Goldstein *et al.*[5] reported that IL-4 proportionally increase with the PCa development, both in castrate-resistance and M-PCa. IL-4 activates androgen receptor in PCa cell, even in the condition of low circulating level of androgen. Moreover, Zhang *et al.*[14] reported that the increase of PD-L2 level is associated with worst biochemical recurrence, metastatic status, and cancer specific survival in PCa.

Correlation of IL-4 with PD-L2

Solinas *et al.*[12] reported that PD-L2 expression was identified in a variety of tumor cells including tumor-associated macrophages (TAMs), and tumor-infiltrating lymphocytes (TILs), dendritic cells tumor, and stromal cells tumor, which able to induce the release some cytokines such as IL-4, granulocyte-monocyte colony stimulating factor (GM-CSF), IF-γ and IF-β. The PDL-2 expression in esophagus cancer cells is associated to Th2 response mediated by IL-4 and IL-13. Whereas in colorectal cancer cells, it is mediated by IFN-γ and in melanoma, it is mediated by IFN-β and IFN-γ.

A significant strong correlation between IL-4 and PDL-2 both in M-PCa and NM-PCa was observed in this study (TABLE 6 and FIGURE 1). However, the reason underlying the correlation between IL-4 and PDL-2 in PCa can not be explained in this study. Further study is needed to explain the correlation.

This study used limited samples, 10 samples for M-PCa and 10 samples for NM-PCa, which might not represent the general population. Previous studies concerning this topic are also limited. Further studies are needed to prove the findings of this study.

CONCLUSION

In conclusion, there is correlation between IL-4 and PD-L2 with the clinicopathological characteristics of patients with PCa. In addition, a strong correlation between IL-4 and PD-L2 is reported in patients

with PCa. Further study with larger samples is needed to be conducted to confirm this finding.

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