A Preliminary Study of Tumor Microenvironment Interleukin-4 as Promoter in Immune Escape Event in Prostate Cancer

Raden Danarto (✉ dr.danarto@yahoo.co.id)

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Ery Kus Dwianingsih

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Yurisal Akhmad Dany

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Lucky Frannata

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Ibnu Widya Argo

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Indrawarman Soerohardjo

Universitas Gadjah Mada, Dr. Sardjito General Hospital

Research Article

Keywords: Prostate Cancer, IL-4, PD-1, CTLA-4, PD-L1 and PD-L2

Posted Date: December 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1077393/v1>

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Abstract

Introduction : This study aims to investigate the relationship between IL-4 expression with Apoptosis-associated gene receptors (PD-1, CTLA-4) and Programmed Death-1 Ligands (PD-L1, PD-L2) in the microenvironment of prostate cancer tissue.

Methods : The samples were collected from single-center hospital in a period from 2014 to 2020. Deparaffinize formalin-fixed paraffin-embedded and RNAs extraction by manufacturer's protocol with slight modification was performed. The RNAs expressions were investigated by using quantitative real-time polymerase chain reaction. Then we categorize them into 4 groups. The ANOVA test is used to compare mean expression between groups and followed by a correlation test using Pearson test.

Result : In the BPH group sample, CTLA-4 had the highest expression level, followed by the expression of IL-4, PD-L2, then PD-1 and PD-L1. The concentration of IL-4 in prostate cancer, both metastatic and non-metastatic, is higher than in BPH, with a p-value of 0.006. the correlation between IL-4 and PD-L1 is the strongest ($r=0.919$), between IL-4 and PD-L2 comes the second (0.832) and between PD-1 is comes the third ($r=0.626$).

Conclusion : In this study, we find that the expression of IL-4 and Apoptosis-Associated Gene Receptors (PD-1, CTLA-4) and Programmed Death-1 Ligands (PD-L1, PD-L2) in the prostate cancer tissue microenvironment have a significant relationship. In conclusion, it is possible that IL-4 is a promoter of the Immune Escape mechanism in prostate cancer.

Introduction

Prostate cancer is currently the most commonly diagnosed cancer in 105 countries and the 2nd most common type of malignancy in male malignancies worldwide.¹ In 2018, there were 1.3 million new cases of prostate cancer and 359,000 related deaths worldwide.

Tumor cells express various cytokines, cytokine receptors, and proteins which then in situ helps tumor cells in tumor survival, tumor progression, tumor invasion, tumor metastasis, and apoptosis resistance to immune cell responses, this mechanism is called Immune Escape.^{1,2}

Programmed Death-1 (PD-1) or CD279 is an apoptosis-associated gene receptor for T cell activity that plays a role in inhibiting the immune response.^{3,4} PD-1 has protein binding with Programmed Death-1-Ligand 1 (PD-L1) and Programmed Death-1-Ligand 2 (PD-L2).⁵ PD-L1 and PD-L2 are expressed on immune cells, stromal cells, tumor cells, and antigen-presenting cells (APCs) from the Tumor Micro Environment (TME).⁶⁻⁹

IL-4 is known to be expressed in malignancies such as melanoma, colorectal, non-Hodgkin's lymphoma, gastric, breast, skin, and bladder. The expression of IL-4 was increased under conditions of low androgen levels and increased the expression of Mir-21.^{10,11} Mir-21 increases Androgen Receptor expression in the

Androgen Receptor negative group and has a feedback loop mechanism.¹² Interleukin-4 is able to activate the Androgen Receptor (AR) by increasing the expression of CBP/p300 and the interaction of the AKT signal which results in the growth of tumors without androgen (androgen-independent).^{13–15}

The purpose of this study was to determine the relationship between IL-4 expression with Apoptosis-associated gene receptors (PD-1, CTLA-4) and Programmed Death-1 Ligands (PD-L1, PD-L2) in the microenvironment of prostate cancer tissue.

Method

Cluster Random Sampling was used as the sampling method. The sample is the FFPE of a patient's tissue who was diagnosed with prostate cancer histopathologically. FFPE samples were obtained from prostate tissue after prostate biopsy or transurethral prostatectomy. Exclusion criteria includes: no baseline Total PSA data, no baseline Total Testosterone data, FFPE age greater than 3 years, and invalid DNA integrity.

The Prostate biopsy and transurethral prostatectomy were carried out according to the guidelines and standard operating procedures in our center hospital. All experimental protocols were approved by ethics committee of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, with the ethical approval number 759/UN1/FK-KMK.2/IB/PT/2021. Informed consent was obtained from all subjects and/or their legal guardian(s).

RNA Extraction

The RNA genome was extracted from formalin-fixed paraffin-embedded (FFPE) prostate tissue. In FFPE tissue specimens, a deparaffinization procedure was carried out using deparaffin liquid, RNA was extracted using the Hybrid-R miRNA kit.

RT-qPCR

The RNA extraction product was examined by RT-qPCR using the Bioner Accupower Greenstar RT-qPCR Master Mix. The PCR was performed using Veriti Thermal Cycler under the following conditions: Reverse Transcription at 50-70°C for 15 minutes followed by 1 cycle of pre-Denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, 40 cycles Annealing/Extension/Detection at 55-60°C for 30 seconds and 1 cycle of melting. The primer sequence for IL-4 is Forward CCGTAACAGACATCTTTGCTGCC and Reverse GAGTGTCTTCTCATGGTGGCT. And The primer sequence for PD-1 Forward GACTATGGGGAGCTGGATTT, PD-1 Reverse AGAGCAGTGTCCATCCTCAG, CLTA4 Forward GCTCTACCTCTTGAAGACCT, CLTA4 Reverse AGTCTCACTCACCTTTGCAG, PD-L1 Forward TGATACACATTTGGAGGAGACG, PD-L1 Reverse CCCTCAGGCATTTGAAAGTATC, and PD-L2 Forward GCTTCACCAGATAGCAGCTTT ATTC and for PD-L1 Reverse CTCCAAGGTTACATGACTTCCA.

Statistical Analysis

The descriptive and analytic data analysis methods were used. An analytical test was performed to ensure that the data was normal. If the sample size is greater than 50 using the Kolmogorov Smirnov Test and greater than 50 using the Shapiro Wilk Test, and the data distribution is normal, the statistical test employs a comparative parametric test Independent T-Test for two groups of categories and ANOVA Test for more than two groups of categories. If the data distribution is not normal, the statistical test employs the comparative non-parametric test, Mann Whitney U test for two groups of categories, and Kruskal Wallis Test for more than two groups of categories, followed by the Pos Hoc Test if the test is significant. The comparative test is preceded by the correlation test. The Pearson Test is used for parametric tests, while the Spearman Test is used for non-parametric tests.

Result

The samples taken were 40 FFPE preparations, including 12 samples diagnosed with Benign Prostate Hyperplasia (BPH), 8 samples of non-metastatic prostate cancer, and 20 samples of metastatic prostate cancer. The mean age of the patients is 68.5 years.

Table 1. Sample Characteristics

Variabel	Total	BPH	Prostate Cancer			P-value (normality test)
			Non-MPCa	M1b PCa	M1c PCa	
Number of patients, n (%)	40	12 (29.3)	8 (19.5)	8 (19.5)	12 (29.3)	
Mean Age, years ± SD	68.5 ± 8.3					>0.05*
Mean Expression ± SD						
IL-4 (Mean ± SD)		10.7 ± 7.7	69.7 ± 35.9	50.4 ± 28.5	63.3 ± 61.1	>0.05*
CTLA-4 (Mean ± SD)		82.5 ± 52.3	64 ± 30.8	38.5 ± 20.3	71.8 ± 30.5	>0.05*
PD-1 (Mean ± SD)		2.6 ± 0.5	4.2 ± 1.1	4.8 ± 1.1	4.3 ± 1.2	<0.05
PD-L1 (Mean ± SD)		1.5 ± 0.4	92.5 ± 56	70.9 ± 48.1	76.1 ± 87.1	>0.05*
PD-L2 (Mean ± SD)		6.9 ± 2.6	480 ± 310.9	241.7 ± 160.1	462.3 ± 588.5	>0.05*

*Normal distribution

The distribution of samples for each gene was tested using the Shapiro-Wilk test. The data distribution of the IL-4, CTLA-4, PD-L1, and PD-L2 genes cytokine were normal, but the distribution of PD-1 was not normal. In table 1, the midpoint concentration of IL-4 appears to be directly proportional to the expression

of PD-L1 and PD-L2 in both BPH, Non-Metastatic Prostate Cancer (Non-MPCa), and in Metastatic Prostate cancer (M1b PCa and M1c PCa) with p-value 0.006.

In the BPH group sample, CTLA-4 had the highest expression level, followed by the expression of IL-4, PD-L2, then PD-1 and PD-L1. However, in the third column of prostate cancer, in non-metastatic prostate cancer and metastatic prostate cancer, PD-L2 had the highest expression level, followed by PD-L1, CTLA-4, IL-4, and PD-1 with a p-value 0.012.

Table 2. Univariat Comparative Analysis

Variabel	BPH	Prostate Cancer			P-value
		Non-MPCa	M1b PCa	M1c PCa	
IL-4 (Mean ± SD)	10.7 ± 7.7	69.7 ± 35.9	50.4 ± 28.5	63.3 ± 61.1	0.006*
CTLA-4 (Mean ± SD)	82.5 ± 52.3	64 ± 30.8	38.5 ± 20.3	71.8 ± 30.5	0.089*
PD-1 (Mean ± SD)	2.6 ± 0.5	4.2 ± 1.1	4.8 ± 1.1	4.3 ± 1.2	0.390**
PD-L1 (Mean ± SD)	1.5 ± 0.4	92.5 ± 56	70.9 ± 48.1	76.1 ± 87.1	0.004*
PD-L2 (Mean ± SD)	6.9 ± 2.6	480 ± 310.9	241.7 ± 160.1	462.3 ± 588.5	0.012*

*ANOVA test

** Kruskal-Walis Test

From Table 2 we can observe that the concentration of IL-4 in prostate cancer, both metastatic and non-metastatic, is higher than in BPH, with a p-value of 0.006. There is also a significant difference in the average concentration of PD-L1 and PD-L1 between BPH and prostate cancer, with p-values of 0.004 and 0.012 respectively. In the case of PD-1 and CLTA-4, there was no significant difference in average concentration.

Table 3. Correlation Analysis IL-4

Variable	IL-4		
	r	p-value	
PD-1	0.626	0.000*	From table 3, we can observe that between IL-4 and the three variables, the correlation between IL-4 and PD-L1 is the strongest (r=0.919), between IL-4 and PD-L2 comes the second (0.832), and between PD-1 comes the third (r=0.626). The relation between those 3 variables shows a strong correlation (r-value between 0.5-1). We can also see from table 3, IL-4 correlated significantly with PD-L1, PD-L2, and PD-1. Shown by the p-value of all three variables 0.000.
PD-L1	0.919	0.000	
PD-L2	0.832	0.000	
CLTA-4	0.265	0.099	

*Spearman correlation test

Discussion

From this study, significant results were found between the expression of IL-4 with Apoptosis-associated gene receptors (PD-1, CTLA-4) and Programmed Death-1 Ligands (PD-L1, PD-L2) in the microenvironment of prostate cancer tissue. Interleukin-4 is the main signal expressed in the tissue microenvironment of Chronic Lymphocytic Leukemia (CLL).¹⁶ This is similar to what was found in this study where IL-4 expression was increased in prostate cancer. In this study, there was a significant comparison between the expression of IL-4 and PD-1 in prostate cancer compared to Benign Prostate Hyperplasia (BPH) tissue. IL-4 is known to be expressed in other malignancies such as melanoma, colorectal, non-Hodgkin's lymphoma, gastric, breast, skin, and bladder.¹⁷

PD-L1 is upregulated and expressed in several solid tumors and in hematological malignancies. The expression of PD-1 and PD-L1 on the cell surface can be detected using Immunohistochemistry (IHC). This study also found the results of an increase in PD-1 and PD-L1 in prostate cancer.¹⁸ The increase in PD-L2 expression also showed significant results. PD-L2 expression in tumor cells is associated with Th2 cell responses through an intermediary mechanism and induces the highest levels of PD-L2 expression sequentially from IL-4, IL-13, and IFN- cytokines, through IL-4R α /IL-4, IL binding. -4R α /IL-13, and Stat6 on M2 macrophage cells.¹⁹

Immunotherapy in cancer treatment has long been proposed and shows promising results in recent studies. By blocking inhibitory immune pathways such as PD-1/PD-L1, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), demonstrated clinical improvement in advanced melanoma, lung cancer, and kidney cancer.²⁰

Serum IL-4 increases as prostate cancer progresses. Data from a study conducted by Goldstein et al showed that IL-4 increased as it developed into Castrate Resistant. This is also consistent with the evidence that after radical prostatectomy, the prognosis worsens when a significant increase in IL-4 is found.²¹

As research conducted by Ueda et al, showed that serum IL-4 in hormone-refractory prostate cancer patients compared with pre-treatment prostate cancer patients was significantly significant.²² However, serum IL-4 between healthy patients, patients with benign prostate enlargement, and patients with pre-treatment prostate cancer was not significant. Furthermore, IL-4 levels were also not significantly different in pre-treatment patients in terms of clinical stage, histologic grade, and tumor Gleason score.

Furthermore, increased levels of IL-4 receptors have been reported in a variety of human cancers and IL-4 may actually promote tumorigenesis by a direct effect on the malignant cells. Aberrantly increased cell proliferation is a requisite of successful tumor progression and the ability to metastasize at distant sites. Although studies have found examples of IL-4 having both negative and positive effects on cell proliferation in general, studies with cancer cells have suggested that IL-4 promotes malignant cell proliferation, though the mechanism is still unclear. The results presented here demonstrate that IL-4 is a potent inducer of prostate cancer PC3 cell proliferation when the cells are subjected to nutrient-depletion stress.²³

A study on prostate cancer showed elevated IL-4 levels in patients with hormone-refractory cancer. Moreover, recombinant IL-4 application also upregulated the expression of the two proteins - annexin A5 and syncytin that play important roles in several cell-cell fusion processes. IL-4 inhibition, indeed, lowers their expression and suppresses cell proliferation and fusion. In pancreatic tumors, IL-4 autocrine origin is essential in the control of normal macrophages transition into tumor-promoting macrophages. IL-4 expression was low in normal islets and increased by 4.5-fold at the hyperplastic stage.²⁴

Conclusion

In this study, we find that the expression of IL-4 and Apoptosis-Associated Gene Receptors (PD-1, CTLA-4) and Programmed Death-1 Ligands (PD-L1, PD-L2) in the prostate cancer tissue microenvironment have a significant relationship. As a result, it is possible that IL-4 is a promoter of the Immune Escape mechanism in prostate cancer. This study is the first study in Indonesia to look into the relationship between IL-4 expression in the tumor microenvironment of prostate cancer tissue. However, because it is still a local study, it is recommended that a larger sample size be used for future research in Indonesia using a multicenter approach.

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