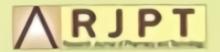
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Enhancement of Physicochemical and Pharmacokinetic Characteristics of Ranolazine drug substance using Cocrystalization Technique

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Simultaneous determination of Cefepime and Tazobactam by using Hyphenated Liquid Chromatography (UPLC-MS/MS)

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A Green UPLC Method for the Simultaneous determination of Tenofovir and Amoxicillin in Biological Fluids and Dosage Forms

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In Vitro Evaluation of Alpha Amylase and Alpha Glucosidase Inhibitory Activity of Kamadhenu Ark extracts of Climate Smart Crop Millets

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Evaluation of the Bioactive compounds and its Functional role in the Aquatic weed Pistia stratiotes

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Author(s): Galanki Vasantha, Ch Dayakar, D Vasudha, Iragavarapu Tejolahari, S Bala Chandrika

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RESEARCH ARTICLE

Correlation between PD-L1 and Ki-67 Expression at various T-stage Clear Cell Renal Cell Carcinomas

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ABSTRACT:

Renal cell carcinoma (RCC) is a malignant neoplasm originating from renal epithelium, with the clear cell renal cell carcinoma (ccRCC)being the most common type (80%) and the most common cause of death among other types of kidney cancer. Pathological stage is an important parameter that affects ccRCC survival, followed by nuclear grade. Pathological staging of RCC according to the AJCC (American Joint Committee on Cancer) TNM system 8th edition is based on local extension of the main tumor (T), involvement of lymph node (N), and metastasis (M). Ki-67 is a marker of proliferation used to assess tumor grade. High Ki-67 correlates with poor prognosis, advanced clinical and pathological features, thus Ki-67 can be used as a biomarker in the management of RCC.Ki-67 is routinely used to see the proliferation index in various cases of malignancy, but not in kidney malignancy. Programmed death ligand 1 (PD-L1) acts as a negative regulator of T cell-mediated anti-tumor immune responses. PD-L1 is expressed on T cells, B cells, macrophages, dendritic cells, endothelial cells and in various tumor cells including ccRCC. This study aims to determine the correlation between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas. Material and Method: This was an observational analytical study with cross-sectional approach toward 52 cases of ccRCC whose diagnosis was made histopathologically at the Anatomical Pathology Installation of Dr. Soetomo General Academic Hospital Surabaya from January 2014 to December 2020. Immunohistochemical stainingwas carried out using Ki-67 and PD-L1 antibodies, followed by an assessment using a scoring system. T-stage data were obtained from the patients' medical records which were then analyzed statistically with the Spearman test. Result: The study included 52 cases of ccRCC obtained from nephrectomy specimens at RSUD dr. Soetomo between 2014–2020. The age distribution of the subjects was 29-69 years and the mean and median age was 53 years. The ratio of male patients compared to female patients was 2.5:1. The majority was stage T2 (50%). Statistical test results showed no correlation between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas (p=0.965 and p=0.680). Conclusion: This study showed no correlation between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas. Nonetheless PD-L1 can be considered as an important biomarker with a poorer prognosis and aggressive clinicopathological findings in patients with RCC.

KEYWORDS: Clear cell renal cell carcinoma, T-stage, Ki-67, PD-L1, Kidney Cancer. INTRODUCTION: Renal cell carcinoma (RCC) is a malignant neoplasm originating from renal epithelium and the clear cell (ccRCC) is the most common type (80%) and the most common cause of death 1-3. Pathological stage is an important parameter that affects ccRCC survival, followed by nuclear grade 4.5. Pathological staging of RCC according to the AJCC

(American Joint Committee on Cancer) TNM system 8th edition is based on local extension of the main tumor (T), involvement of lymph node (N), and metastasis (M)⁶. RCC therapy is surgery, palliative therapy, followed by chemotherapy. Many cases of RCC are resistant to conventional therapy (hormonal therapy, radiotherapy, and chemotherapy)⁷. Targeted therapy and immunotherapy were developed especially in cases of tumors with metastases or advanced stages^{8,9}. Single targeted therapy in the form of anti-angiogenesis alone does not show any lasting response and may cause toxic side effects^{10,11}. PD-1/PD-L1-based immune-checkpoint inhibitor (ICI) regimen is a new hope as the first line of RCC therapy in metastatic cases¹⁰.

Programmed death ligand 1 (PD-L1) acts as a negative regulator of T cell-mediated anti-tumor immune responses. PD-L1 is expressed on T cells, B cells, macrophages, dendritic cells, endothelial cells and in various tumor cells including ccRCC^{12,13}. PD-L1 expression in tumor cells is associated with high nuclear grade, tumor size, clinical stage, poor prognosis, and overall survival in ccRCC patients¹⁴. Previous studies suggested that anti-PD-L1, as an ICI, holds great therapeutic potential in ccRCC, especially in advanced cases which are often resistant to conventional therapy¹⁰.

Ki-67 is a marker of proliferation used to assess tumor grade^{15,16}. High Ki-67 correlates with poor prognosis, advanced clinical and pathological features, thus Ki-67 can be used as a biomarker in the management of RCC¹⁷⁻¹⁹. Ki-67 is routinely used to see the proliferation index in various cases of malignancy, but not in kidney malignancy. This study analyzed the correlation between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas.

MATERIAL AND METHODS:

This was an observational analytical study with cross-sectional approach carried out on 52 paraffin blocks of ccRCC whose diagnosis was made histopathologically from nephrectomy specimens at the Anatomical Pathology Installation of Dr. Soetomo General Academic Hospital Surabaya from January 2014 to December 2020who fulfilled the inclusion criteria using the consecutive sampling technique. Data on age, sex, location and number of tumors, as well as the TNM stage data were obtained from the patients' medical records and the Anatomical Pathology report. Tumor grade data were obtained from re-reading HE slides.

This study has received ethical approval from the Health Research Ethics Commission, RSUD dr. Soetomo Surabaya. The subjects were obtained from paraffin blocks contained in the medical records of the Anatomical Pathology Installation of RSUD dr. Soetomo Surabaya.

Immunohistochemical staining was carried out using Ki-67 and PD-L1 antibodies. The paraffin blockswere cut 4 µm, deparaffinized, and rehydrated with alcohol periodically. It was then heated with citrate buffer pH 7–7.3 for 20 minutes in the microwave. The primary antibodies, namely PD-L1 (28-8) Rabbit Monoclonal Primary Antibody (438R-14 Cell Marque/RabMab) and Ki-67 (SP6) Rabbit Monoclonal Primary Antibody (901-325-09282 Biocare Medical), were dripped with a dilution of 1:100 at 40°C overnight. The secondary antibody was then dripped and incubated for 20 minutes. As the final step, diaminobenzidine (DAB) was dripped and the counterstain was carried out with Meyer Hematoxylin.

The stained slides were then graded and scored to obtain the data. The results of the assessment of Ki-67 and PD-L1 expression in patients with ccRCC will be compared with the T-stage data of patients described as stage T1, T2, T3 and T4. All data obtained were analyzed by Spearman test.

RESULTS AND DISCUSSION:

The subjects of this study were 52 cases of ccRCC obtained from nephrectomy specimens at RSUD dr. Soetomo Surabaya between 2014 – 2020. Data on the age distribution were: 21 - 30 years (3.8%), 31 - 40 years (1.92%), 41 - 50 years (34.61%), 51 - 60 years (34.61%), and 61 - 70 years (25%), with a median and median age of 53 years. The age range was 29 - 69 years which is slightly lower than the average age in the literature 20,21 .

Some literature states that the majority of ccRCC are sporadic (95%), while the remaining cases (5%) are associated with genetic disorders such as Von Hippel Lindau disease and tuberous sclerosis²⁰. Sporadic ccrCC occurs mainly in the sixth to eighth decades, with a median age at diagnosis of 64 years (19 – 80 years)^{21,22}. In the Asian group, this figure is lower with an average age of 57.6 years^{20,23}. Sporadic renal carcinoma is rarely found in young age, but in recent years the incidence of renal carcinoma in this group tends to increase and may be associated with certain genetic disorders^{21,22}.

The number of male ccRCC patients is higher than female patients with a ratio of 2.5:1. This is in accordance with Globocan data 2020, which state that the incidence rate in male patients is higher than female patients with a ratio of 1.5:1^{24,25}.

The majority of patients presented to RSUD dr. Soetomo had malignancy in one of their kidneys (71%). As many as 54% of them had tumor on both kidneys, 24% on the

superior pole, 18.8% on the medial pole, and 2.7% on the inferior pole. There were 29% cases of multifocal and 80-85% cases of primary RCC originate in the renal cortex. Based on the literature, ccRCCtumors are usually solitary in the renal cortex and can occur in both kidneys in equal proportions and there are renal carcinomas associated with genetic disorders such as VHL (<5% of cases), multifocal and/or bilateral tumors, and found in young age²⁶.

In this study, the majority of patients had high grades, namely 27 patients of grade 4(51.9%) and 14 patients of grade 3(27%). A high grade indicates a poor prognosis. Grade in RCC is a prognostic factor which has been used since a hundred years ago. Determination of grade in RCC mainly uses nuclear morphology, which is recommended to assess visible or prominent nuclei and eosinophilic color in nuclei, as well as the presence of large tumor cells, sarcomatoid and rhabdoid components²⁷. The RCC grading system is determined by the International Society of Urological Pathology (ISUP) and the World Health Organization (WHO), which is then included in the WHO/ISUP grading classification in the fourth edition of the WHO classification of tumors of the urinary system and male genital organs (2016). This grading system applies to clear cell and papillary RCC types²⁷. The consensus used is to determine the nuclei that are visible or prominent as grade 1 to 3 and the presence of large tumor cells, sarcomatoid and rhabdoid components as grade 4^{28,29}.

This study on Renal Cell Carcinomagave different results from other studies. It could be due to the process of staining techniques in which the results do not accurately reflect the actual PD-L1 expression status. Besides that, it could also be because tumor cells are strongly influenced by the time and location of the tumor so that it can affect the interpretation of the results. The clonal differences and tumor microenvironment can also influence PD-L1 expression in. All of these factors can lead to differences in PD-L1 expression. However, the use of PD-L1 as an exclusive biomarker for cancer immunotherapy should also be considered as an alternative for patients to choose targeted therapy regimens to benefit from immunotherapy³

Table 1: Characteristic of clinicopathological profile.

Characteristics		n	(%)
Year	2014	8	15.4
	2015	6	11.5
	2016	11	21.1
	2017	7	13.5
	2018	10	19.2
	2019	3	5.8
	2020	7	13.5
Age	21–30 years	2	3.9

31–40 year	·c	1	
		-	1.9
41–50 years		18	34.6
51–60 years		18	34.6
61–70 years		13	25
Female Male		15	28.8
		37	71.2
Unifocal	Superior pole	9	17.3
	Medial pole	7	13.5
	Inferior pole	1	1.9
	Entire kidney	20	38.4
Multifocal		15	28.8
pT1 pT2 pT3 pT4		6	11.6
		26	50
		15	28.8
		5	9.6
G1 G2 G3		3	5.8
		8	15.4
		14	26.9
G4		27	51.9
Negatif 1-10 >10		7	13.5
		15	28.8
		30	57.6
Negatif 1-10 >10		0	0
		49	94.2
		3	5.8
	61-70 year Female Male Unifocal Unifocal PT1 PT2 PT3 PT4 G1 G2 G3 G4 Negatif 1-10 Negatif 1-10	61–70 years Female Male Unifocal Unifocal Medial pole Inferior pole Entire kidney Multifocal pT1 pT2 pT3 pT4 G1 G2 G3 G4 Negatif 1-10 Negatif 1-10	61-70 years 13 Female 15 Male 37 Unifocal Superior pole 9 Medial pole 7 Inferior pole 1 Entire kidney 20 Multifocal 15 pT1 6 pT2 26 pT3 15 pT4 5 G1 3 G2 8 G3 14 G4 27 Negatif 7 1-10 15 >10 30 Negatif 0 1-10 49

Table 2: The difference between Ki67 and PDL-1 with stage T

T	Ki67	PDL1
Kruskal-Wallis H	0.274	1.509
df	3	3
Asymp. Sig.	0.965	0.680

The majority of patients in this study had stage T2, namely: T2a, the tumor size $> 7 \, \mathrm{cm}$ and $\le 10 \, \mathrm{cm}$ which is only in the kidney and pT2b, the tumor size $> 10 \, \mathrm{cm}$ which is only in the kidney, 26% or 50% respectively. The results of this study are in accordance with previous studies, which obtained the most suffering from stage T2³¹. Another study obtained different results, stating that most patients were at stage T3 and T4 because most were asymptomatic, making it difficult to detect. These results will affect the determination of patient management and patient survival³².

Other factor that greatly influences the patient's prognosis is the histological type. Different histological types in RCC can result in different survival. Different histological subtypes have cytogenetic and molecular differences and they also determine differences in stage and grade³³. Stage and grade, together with age and year at diagnosis, are the most important prognostic factors for survival in RCC patients. In ccRCC, there is a strong relationship between grade, tumor size or stage, but stage is by far the strongest prognostic factor^{33,34}.

As previously noted, ccRCC is the most lethal and highly aggressive malignancy of the urinary tract and is often resistant to chemotherapy and radiotherapy³⁵. In the last few decades, the management of ccRCC has experienced tremendous development. The existence of various new therapeutic methods will give hope to

ccRCC patients. One of the therapies that has been developed is targeted therapy, for example inhibition of the vascular endothelial growth factor (VEGF) pathway and mammalian target of rapamycin (mTOR) inhibitors. Several other new therapies are immunotherapy/immune checkpoint inhibitors (ICI), which consist of antiprogrammed death 1 (PD-1), anti-programmed death ligand 1(PD-L1), and anti-cytotoxic T lymphocytes antigen 4 (CTLA-4)¹¹.

One of the body's ways to fight tumor cells is by activating the immune system, both innate and adaptive immunity. Innate immunity is in the form of dendritic cells which will capture antigens released by tumor cells and then presented to MHC I and II contained in T cells³⁶⁻³⁸. This process will activate effector T cells which are components of adaptive immunity. These activated T cells will enter the circulation through the blood vessels and will then arrive at the site of the tumor cells. T cells will recognize and bind to these tumor cells and kill them. This process cannot take place effectively in cancer patients so they cannot detect tumor cell antigens. The antigen will become self-antigen so that it will activate the response from regulatory T cells. Activation of regulatory T cells and other factors in the tumor microenvironment will prevent T cells from eliminating tumor cells 11,39-43.

The inhibition of checkpoint molecule programmed death-1 (PD-1) plays an important role in maintaining homeostasis of the body's immune response to tumor cells by binding to its ligands, namely PD-L1 and PD-L2. PD-1 is expressed on the surface of T cells, including regulatory T cells (Treg), B cells, monocytes, dendritic cells and natural killer cells (NK cells). The bond between PD-1 and PD-L1 expressed by tumor cells and inflammatory cells will act as a negative regulator of the elimination process of tumor cells⁴⁴. This process will eventually lead to exhaustion of T cells¹⁰. Blockade of this process will restore the function of T cells in eliminating tumor cells⁴⁴.

The expression of PD-1 and PD-L1 is associated with clinicopathological features in ccRCC, namely large tumor size, high nuclear grade, presence of tumor necrosis and presence of sarcomatoid differentiation. PD-1 expression can be used as a biomarker in detecting poor outcomes in metastatic ccRCC patients receiving targeted therapy^{35,45}.

High PD-L1 expression has been confirmed as a poor prognostic factor but currently its use as a predictive marker is still controversial for various reasons, including: (1) there are several types of antibodies against PD-L1 available, for example 22C3, 28-8, 73-10, and SP142, (2) studies that have compared PD-L1 22C3

and 28-8 found a fairly high match between the two antibodies^{46,47}, and (3)PDL-1 SP142 antibody has a lower sensitivity when compared to the two^{11,46}.

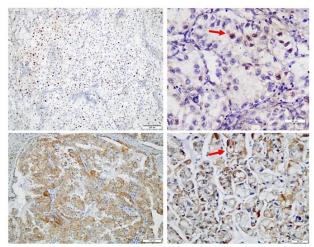


Figure 1: Staining of Ki-67 and PD-L1 on CCRCC. Ki-67 is positive in the nuclei, while PD-L1 is positive in the membrane cell and cytoplasm of tumor cells.

This study showed no correlation between PD-L1 and stage indicating that in ccRCC, tumor cells are strongly influenced by time and location so that it can affect the interpretation of the results. Clonal differences and tumor microenvironment can also influence PD-L1 expression in tumors. Non-uniform PD-L1 expression, a better understanding of the tumor microenvironment and the use of other biomarkers such as gene markers and composite indices are needed to better identify patients who would benefit from PD-1/PD-L1 checkpoint blockade therapy³⁰.

Statistical test results in Table 2 showed that there was no relationship between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas (p=0.965 and p=0.680), indicating that in patients with clear cell renal cell carcinoma, the expression PD-L1 in tumor cells is not related to tumor size or clinical stage, and Ki-67 can only be used to determine nuclear grade, not stage.

This is different from a study which states that RCC patients with high Ki-67 expression tend to have higher TNM stages, pathological T-stages, positive metastases, and higher nuclear grades. Mechanism of Ki-67 which is a well-known biomarker of cell proliferation in many tumors plays an important role in mitosis by regulating chromatin recombination, so that Ki-67 is considered as a molecular marker of aggressive behavior and response to therapy for the assessment of survival outcomes in several cancers including RCC, as well as explaining as a prognostic factor that influences the clinicopathology of RCC patients⁴⁸.

Meta-analysis studies proved that PD-L1 expression did not represent an accurate biomarker for selecting treatment for ccRCC patients because the PD-L1 negative group and the PD-L1 positive group both benefited from immunotherapy⁴⁹.

Another factor to consider is that PDL1 expression on TIL, but not on tumor cells, was shown to be significantly associated in more aggressive and higher stage RCC patients. These data indicate that TIL PD-L1 expression in RCC patients contributes to cancer aggressiveness⁵⁰.

PD-L1 can be considered as an important biomarker with a poorer prognosis and aggressive clinicopathological findings in patients with RCC. PD-L1 expression has a significant relationship with nuclear level and tumor necrosis (p = 0.025 and 0.010, respectively). However, PD-L1 expression does not correlate with tumor size, lymphovascular invasion, and sarcomatoid differentiation⁵¹.

CONCLUSION:

This study showed no correlation between the expression of PD-L1 and Ki-67 in various T-stage clear cell renal cell carcinomas. Nonetheless PD-L1 can be considered as an important biomarker with a poorer prognosis and aggressive clinicopathological findings in patients with RCC.

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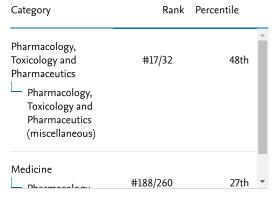
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: Korelasi Antara Ekspresi PD-L1 dan Ki-67 Pada Berbagai Stadium T Karsinoma

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Komite Etik Penelitian Kesehatan RSUD Dr Soetomo menyatakan bahwa dokumen diatas sesuai dengan The Office for Human Research Protections (OHRP) dibawah persyaratan the U.S. Department of Health and Human Services (HHS) Regulasi 45 CFR bagian 46 untuk exempt review.

Prof. Dr. Hendy Hendarto dr., SpOG(K)

Ketua Panel 1

Dra. Siti Farida SpFRS, Apt

Sekretaris Panel 1