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Cytotoxic T lymphocyte associated antigen-4 (CTLA4) expression with renal cell carcinoma subtype and staging

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ABSTRACT

In Indonesia, approximately 45% of renal cell carcinoma (RCC) patients are at an advanced stage that requires checkpoint inhibition combination immunotherapy. Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is associated with poor prognosis of RCC and it is the first checkpoint developed in cancer immunotherapy. This study aimed to investigate CTLA-4 expression among RCC subtypes and staging. Formalin fixed paraffin embedded (FFPE) tissue of RCC patients from 2018-2020 were obtained from the Department of Anatomical Pathology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital, Yogyakarta. Expression of CTLA-4 among RCC subtypes and stage was measured using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) and compared. Among the 40 patients involved in this study, the CTLA-4 expression was higher in papillary RCC/pRCC (95.88 ± 31.58) compared to clear cell RCC/ccRCC (90.94 ± 43.05). However, no significantly different in CTL-4 expression based on histologic subtypes and tumor stage (p>0.05). In conclusion, neither the histologic subtype nor the tumor stage of RCC can be predicted by CTLA-4 expression.

ABSTRAK

Sekitar 45% pasien karsinoma sel ginjal (RCC) di Indonesia pada stadium lanjut yang memerlukan kombinasi imunoterapi dengan penghambat *check point*. Antigen *cytotoxic T lymphocyte associated antigen-4* (CTLA-4) berhubungan dengan prognosis buruk dari RCC dan penghambat *check point* pertama yang dikembangkan dalam imunoterapi kanker. Penelitian ini bertujuan untuk mengetahui ekspresi CTLA-4 pada subtipe dan stadium RCC. Jaringan formalin fixed paraffin embedded (FFPE) pasien RCC tahun 2018-2020 diperoleh dari Departemen Patologi Anatomi Fakultas Kedokteran Kesehatan Masyarakat dan Keperawatan Universitas Gadjah Mada/RSUP Dr. Sardjito Yogyakarta. Ekspresi CTLA-4 di antara subtipe dan stadium RCC diukur menggunakan *quantitative reverse transcription-polymerase chain reaction* (qRT-PCR) dan dibandingkan. Di antara 40 pasien yang terlibat dalam penelitian ini, ekspresi CTLA-4 lebih tinggi pada RCC/pRCC papiler (95,88 ± 31,58) dibandingkan dengan RCC/ccRCC *clear cell* (90,94 ± 43,05). Namun, ekspresi CTL-4 berdasarkan subtipe histologis dan stadium tumor tidak berbeda nyata (p>0,05). Kesimpulannya, subtipe histologis maupun stadium tumor RCC tidak dapat diprediksi dengan ekspresi CTLA-4.

Keywords:
renal cell carcinoma;
CLTA-4;
stage;
subtype;
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INTRODUCTION

Renal cell carcinoma (RCC) originates from the renal epithelium and accounts for more than 90% of renal cancers. There are wide variety of histopathological appearances and molecular subtypes with clear cell RCC (ccRCC) as the most common type of renal cancer (80%) and also the most common renal cancer-associated death.[1,2] In Indonesia, the incidence of renal cancer is 2.4-3 cases/100000 people with 45% of patients first discovered already at an advanced stage. Besides ccRCC, there are other RCC subtypes like papillary RCC (10-15%), and chromophobe RCC (5%).[3]

Diagnosis of RCC can be challenging because most RCC cases (85%) typically have unclear clinical presentations.[4] This causes a delay in diagnosis which explains why most RCC cases are diagnosed in advanced stages.[5] A checkpoint inhibitor (CI) combination immunotherapy showed better overall survival (OS) than tyrosine kinase inhibitor (TKI) monotherapy.[6] Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is a checkpoint that has been first studied and used in the management of melanoma and non-small cell lung cancer.[7] Renal cell carcinoma guidelines in Europe and Indonesia now include the combination of CIs in the management of advanced RCC.[8,2]

Identification the RCC subtype and stage is very important because it is associated with the treatment and prognosis of the tumor.[9] Five-year cancer-specific survival rates for clear cell RCC (ccRCC), papillary RCC (pRCC), and chromophobe RCC (chRCC) were 68.9%, 87.4%, and 86.7%, respectively. Clear cell RCC had shown a worse prognosis than another RCC subtype.[10] Stage T1 RCC has a survival rate of 83%, while the survival rate for stage T2 is 57%. For stage T3, the survival rate is 42%, and for stage T4, it drops further to 28%.[9]

The function of CTLA-4, which is a homologous protein of CD28, is to inhibit the costimulatory signals initiated by CD28-B7 through a competitive binding mechanism. This causes a decrease in the ability of T cells to interact with antigen presenting cell (APC).[10] CTLA-4 expression has a strong correlation with local recurrence, pathological stage, the degree of immune infiltration, lower overall survival, and cancer-specific survival rate.[11,12] While CTLA-4 expression increases with tumor severity, CTLA-4 should be high in ccRCC tumors as one of the RCC subtypes with a worse prognosis and late stage of RCC.[12] In this study, we compared the expression of CTLA-4 in RCC patients to determine the patterns and explored relationships between CTLA-4 and RCC subtype and stage.

There are several methods to detect gene or protein expression like polymerase chain reaction (PCR), Western blot or immunohistochemistry (IHC). PCR has become a crucial method in biochemistry and molecular biology, allowing for the quick detection of genetic material present in small quantities and the identification of individual copies of genomes. On the other hand, IHC uses monoclonal and polyclonal antibodies to identify specific tumor antigens expressed in tissue sections, serving as a valuable diagnostic tool for various medical conditions. However, not all proteins are uniformly well-preserved and detectable using this technique.[13] Furthermore, IHC demonstrates lower sensitivity and specificity compared to PCR-based molecular diagnostic methods. There is an alternative method called fluorescence in situ hybridization (FISH) which is cytogenetic technique that uses fluorescent probes that bind to only particular parts of a nucleic acid sequence with a high degree of sequence complementarity.[14]

CTLA-4 was detected using various methods at the mRNA level or the protein level. Examinations at the

mRNA level include Real-time PCR and quantitative reverse-transcription PCR (qRT-PCR), whereas at the protein level include Western blotting, immunohistochemistry, flow cytometry, ELISA, and fluorescence microscopy.[15] CTLA-4 protein has small structure which is approximately 24 kDa. This causes challenges associated with detecting the CTLA-4 protein. There is also possibility that potential protein damage can occur while preserving the tissue.[16]

It is believed that qRT-PCR is the best option for detecting CTLA-4 expression in RCC. This study aimed investigate the CTLA-4 expression in RCC patients by using qRT-PCR. Furthermore, the correlation between this expression with RCC was analyzed to assess the possibility of CTLA-4 in predicting subtype and stage.

MATERIAL AND METHODS

Subjects and data collection

Patients of RCC with formalin fixed paraffin embedded (FFPE) tissue from 2018-2020 in Pathology Anatomy Installation at Dr. Sardjito General Hospital, Yogyakarta were included in this study. Exclusion criteria for this study were the tissue findings outside ccRCC, pRCC and chRCC diagnosis. A total of 40 samples meeting the inclusion and exclusion criteria were selected, and patient data were subsequently gathered from the medical records. This study has been approved by the Medical and Health Research Ethics Committee, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/Dr. Sardjito General Hospital numbered KE/FK/0017/EC/2021.

While qRT-PCR amplification results can be compared with IHC, this study focused solely on the qRT-PCR procedure. The qRT-PCR procedure was conducted in the Department of Anatomical Pathology, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada, Yogyakarta.

qRT-PCR analysis

The harvested tissue was extracted and then isolated in Mini Kit FavorPrep™. Total RNA followed by cDNA synthesis using AccuPower®Greenstar™ RT-qPCR Master Mix. Reverse transcription PCR was conducted using DT-lite real time PCR system with the following conditions: 95 °C for 1-3 sec (denaturation), 95 °C for 3 min (annealing), and 60 °C for ≥ 20 sec (extension). Qualitative PCR was done for CTLA-4 and GAPDH (as the internal control). The primer genes were summarized in TABLE 1. Details of qRT-PCR condition were denaturation, followed by up to 40 cycles: 95 °C for 1-3 sec, annealing, and 95 °C for 3 min, followed by elongation at 60 °C for more than 20 sec. Products of PCR were visualized in 2% agarose gel along with a 100-bp DNA ladder (Bioron, Germany, Cat. No. 306009) for RNA amplification process. Relative quantification by Livak method to determine CTLA-4 expression was used. Each of PCR signal from CTLA-4 and GADPH was measured. Delta cycle threshold (ΔCT value) was determined by subtracting the CTLA-4 signal and the GADPH. Tonsil as calibrator for this study was used. Delta ΔCT value (ΔΔCT value) was calculated by subtracting ΔCT value CTLA-4 with ΔCT value from tonsil. CTLA-4 expression value was obtained by using 2ΔΔCT value.

TABLE 1. Primer sequence of CTLA-4 and GADPH

Gene	Forward primer	Reverse primer
CTLA-4	GCTCTACCTCTTGAAGACCT	AGTCTCACTCACCTTTGCAG
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA

Statistical analysis

The statistical significance in CTLA-4 difference for tumor subtype was determined using independent t-test for normally distributed data, and Mann-whitney if only the data was not normally distributed. To determine CTLA-4 expression difference in tumor stadium, we used one-way Anova for the normally distributed data or Kruskal-Wallis test for normally undistributed data. The difference was significant if the p value < 0.05. Expression of CTLA-4 then was divided by median as cut-off with upper than median considered as high expression and lower than median considered as low expression. Level of CTLA-4 then was compared among RCC subtypes.

RESULTS

The RCC samples from 40 patients consisting of 31 ccRCC and 9 pRCC patients were included in this study. The patients consisted of 27 men and 13 women with mean age was 56.3 ± 11.7 y.o. The mean ages of the patients with ccRCC subtypes were 56.68 ± 12.3 y.o. and those pRCC subtypes were 55.33 ± 10.1 y.o. No significant difference in patients' age between tumor subtype was observed ($p>0.05$). The surgical approaches in this study included 18 open surgeries and 22 laparoscopic approaches. In total of 40 RCC, tumor ISUP grading for grade I, II, III and IV were 2, 13, 14 and 7, respectively were observed. Only 4 RCC cases with undefined grading were observed. No significant difference between ISUP grading and tumor subtypes was observed. In this study, 11 cases with vascular invasion and 29 cases without vascular invasion were found. Significant difference in vascular invasion between ccRCC and pRCC was observed ($p<0.05$). Thirty-nine patients with tissue invasion and only a patient without tissue invasion were found. In addition, 19 patients with tumor infiltrating lymphocyte (TIL) and 21 patients without TIL were found (TABLE 2).

TABLE 2. Characteristics of patients

Variable	Mean ± SD/ [n(%)]	p
Age (mean ± SD yr)	56.30 ± 11.7	
• Clear cell type	56.68 ± 12.3	0.767
• Papillary type	55.33 ± 10.1	
Gender [n (%)]		
• Male	27 (67.5)	0.624
• Female	13 (32.5)	
Histologic type [n (%)]		
• Clear cell type	31 (77.5)	N/A
• Papillary type	9 (22.5)	
TNM staging [n (%)]		0.307
• cRCC		
✓ I	4 (10.0)	
✓ II	11 (27.5)	
✓ III	3 (7.5)	
✓ IV	13 (32.5)	
• pRCC		
✓ I	3 (7.5)	
✓ II	2 (5.0)	
✓ III	2 (5.0)	
✓ IV	2 (5.0)	
Operation technique		
• Open Surgery	18 (45.0)	0.341
• Laparoscopic	22 (55.0)	
Vascular invasion		
• No	29 (72.5)	0.037
• Yes	11 (27.5)	
Tissue invasion		
• No	1(2.5)	0.225
• Yes	39(97.5)	
TIL		
• No	19 (47.5)	0.457
• Yes	21 (52.5)	
ISUP grading		
• I	2 (5.0)	
• II	13 (32.5)	0.821
• III	14 (35.0)	
• IV	7 (17.5)	
CTLA-4 (with median cut-off)		0.705
• ccRCC		
✓ >87.48	15 (37.5)	
✓ ≤87.48	16 (40.0)	
• pRCC		
✓ >87.48	5 (12.5)	
✓ ≤87.48	4 (10.0)	

The data showed the mean expression of CTLA-4 in all of our patients were 92.05 ±_40.43. Data of CTLA-4 expression were normally distributed proven by normality test (p=0.11). Subtype of pRCC (95.88 ± 31.58) had higher CTLA-4 expression compared to ccRCC (90.94 ± 43.05). However, it was not significantly different in CTLA-4 gene expression among RCC histologic subtype (TABLE 3).

Based on tumor stage, the mean of CTLA-4 expressions in ccRCC for stadium I, II, III, and IV were 85.99 ± 31.09, 88.31 ± 47.89, 80.59 ± 54.43, and 100.71 ± 41.14, respectively and in pRCC were 87.01 ± 12.13, 126.67 ± 59.71, 76.36 ± 29.20, and 98.89 ± 31.58 (TABLE 3 and FIGURE 1). No significantly different in CTLA-4 expression between each tumor stage was observed (p>0.05). Further classified of the tumor was performed to be early stage (stages I and II) and advanced stage (stages III and IV) and dichotomous data from median CTLA-4 expression was analyzed (TABLE 4). However, no significantly different in CTLA-4 expression between early stage and advanced stage was also observed (p>0.05).

TABLE 3. Means comparation between CTLA-4 expression on tumor subtype and stage

Variable	CTLA-4	> median	< median
ccRCC	90.94 ± 43.05	15	16
• I	85.99 ± 31.09	2	2
• II	88.31 ± 47.89	3	8
• III	80.59 ± 54.43	2	1
• IV	100.71 ± 41.14	8	5
pRCC	95.88 ± 31.58	5	4
• I	87.01 ± 12.13	2	1
• II	126.67 ± 59.71	1	1
• III	76.36 ± 29.20	1	1
• IV	98.89 ± 31.58	1	1

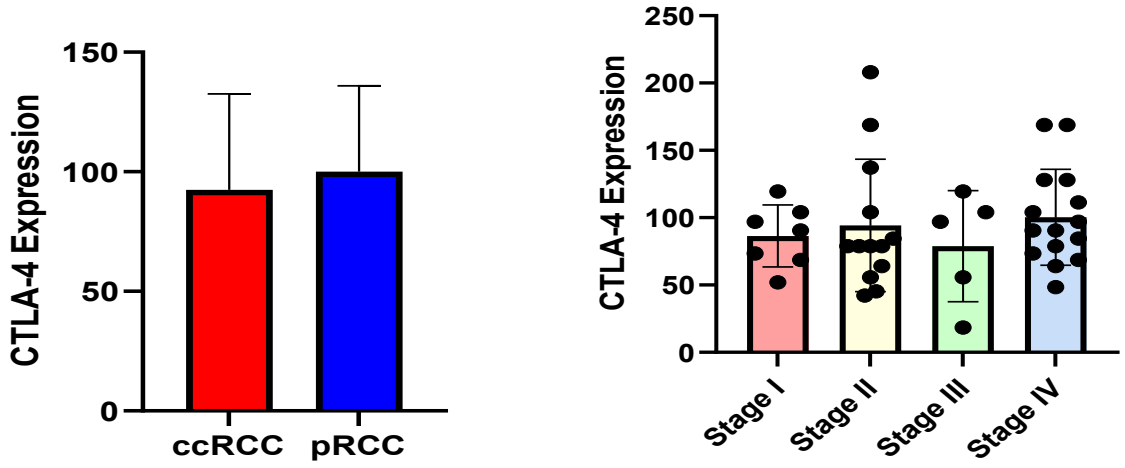

FIGURE 1. Expression of CTLA-4 in tumor subtype and stage1, stage2, stage3, stage4

TABLE 4. Comparing dichotomous data from median CTLA-4 expression and early and advanced or tumor stage

Variable	> median	< median
Advance stage	12	8
Early stage	8	12

DISCUSSION

In this study, CTLA-4 expression in pRCC was higher than ccRCC. However, it was not significantly different (p>0.05). It was in contrast to previous studies that CTLA-4 expression increases with tumor severity and it has a strong correlation with local recurrence, pathological stage, the degree of immune infiltration, lower overall survival (OS) and cancer-specific survival (CSS) rate.[11,12] It was also reported that CTLA-4 is more upregulated in ccRCC tissue and its expression was related to poorer prognosis.[17,18] Another study reported that polymorphisms of CTLA-4 gene are associated in higher risk for high-stage ccRCC.[19]

Although the availability is limited in Indonesia, CTLA-4 inhibitor (ipilimumab) in combination with nivolumab can be used for ccRCC as recommended by Indonesian Urological Association (IAUI) guideline for advance RCC.[2] Grimm *et al.*[20] reported that combination ipilimumab and nivolumab only increased 10% of objective response rate (ORR). Quite contrary from IAUI guideline and previous study, this study showed that CTLA-4 expression in pRCC might be as high as in ccRCC, this indirectly indicated that anti-CTLA-4 might have potential in management of pRCC. Park *et al.*[21] reported that renal metastatic adenocarcinoma cell line with systemic injection of JX-594 with ipilimumab and nivolumab reduced primary tumor burden and had stronger therapeutic effects both in early and advance model compared to JX-594 monotherapy alone. This also showed that recent discovery of anti-CTLA-4 efficacy in non-ccRCC.[21]

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) was known to attenuate T cell activation through cell intrinsic and extrinsic mechanisms.[22,23] Activation of CTLA-4 can inhibit IL-2 production, T cell proliferation, and arresting cell cycle. Regulatory T cell can inhibit activation of cytotoxic T cell through CTLA-4 which might cause T cell dysfunction.[17,24] Dysfunction of T cell characterized by reduced proliferative capacity, decreased effector function and overexpression of multiple inhibitor receptor especially CTLA-4.[24] Yang *et al.*[25] reported anti CTLA-4 could induce RCC regression by prevention of tumor immunosuppression mechanism. This explained CTLA-4 expression should be high in late-stage RCC.[25] In this study, there was no significant difference between CTLA-4 expression in tumor subtype and stage.

The association of PD-1/PD-L1 and CTLA-4 with RCC patient overall survival and cancer-specific survival was discovered and first reported by Kahlmeyer *et al.*[12] It was reported that the CTLA-4 expression is more marked in poorer prognosis. Furthermore, ccRCC had poorer prognosis among pRCC and ccRCC which indirectly implied CTLA-4 expression more likely to be high.[18,26,27] It is inconsistent with the results of this study, which found pRCC, a subtype with better prognosis, had higher CTLA-4 expression compared to ccRCC. This result suggest that CTLA-4 expression might be high in pRCC because most of

the pRCC cases included in this study were above stage II and had high ISUP grading. Antibody of CTLA-4 could also increase IFNγ-producing CD4+ cells in metastatic bladder cancer but not early stage of tumor.[28] This showed CTLA-4 role in the pathogenesis of advance staged tumor.

By using qRT-PCR, Klümper *et al.*[29] determined methylation of CTLA-4 and reported hypomethylation of CTLA-4 as a strong biomarker for poor prognosis in ccRCC. A hypomethylated DNA was associated with specific protein upregulation.[30] By using immunohistochemistry staining, Liu *et al.*[17] also reported CTLA-4 upregulation was associated with worse prognosis in ccRCC, whereas by using PCR, Tupikowski *et al.*[19] reported polymorphism of CTLA-4 genes was associated with its aggressive course in ccRCC. Conversely with the previous studies, this study showed CTLA-4 expression in less aggressive subtype (pRCC) might be as high as RCC.

Ipilimumab, a CTLA-4 checkpoint inhibitor, can be combined with nivolumab for treatment-naïve patients with advance ccRCC.[31] This indirectly indicated that CTLA-4 expression should be higher in late stage of tumor. Although not statistically significant, this was consistent with this study which stage IV ccRCC had highest CTLA-4 expression. There was toxicities regarding of nivolumab/ipilimumab usage but quality of life and overall survival (OS) for intermediate and poor-risk patients showed better result than sunitinib.[31]

Polymorphism of CTLA-4 +49 A/G decreased risk of cancer incidence in Asian population but not in Caucasian.[32] Different in genetic characteristics may contribute to divergent result because the distribution of the CTLA-4 allele frequency varies among Asians and Caucasians. The findings of this study might be applicable on Asian population because only Indonesian population were included. Only 40 samples had been used in this study which indirectly implied internal and external validity of this study may not be very good. It was the first study exploring CTLA-4 expression with tumor subtype and staging using Indonesian population. Less risk bias with large sample and heterogeneous ethnicity may be required to have definitive conclusion regarding CTLA-4 expression association with RCC tumor subtype.

CONCLUSION

In conclusion, the CTLA-4 expression in pRCC and ccRCC are not different. Late stadium of RCC does not always have high CTLA-4 expression. Neither the histologic subtype nor the stage of RCC can be predicted by CTLA-4 expression.

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