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Original Article

Positive Correlation Found Between CXCL12/PLK1 Expression and T Stage of Clear Cell Renal Cell Carcinoma

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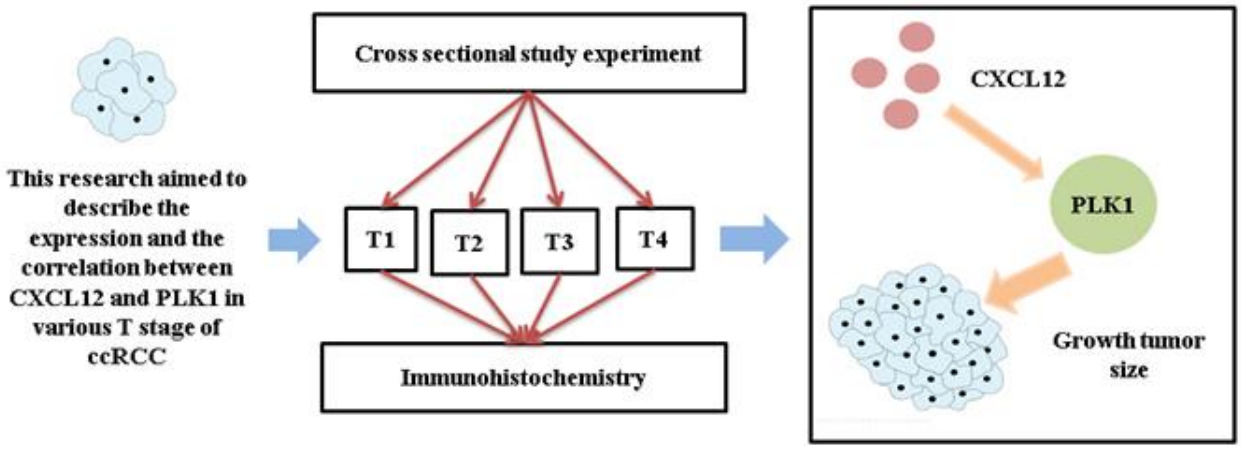
ABSTRACT

Clear cell renal cell carcinoma (ccRCC) is the most common type of kidney cancer and the incidence is steadily increasing. T Stage is one of the most important information to assess the disease magnitude. Both CXCL12 and PLK1 play important role in several pathways that lead to proliferation of tumor cells. The disruption of this pathway is seen in many cancers including clear cell renal cell carcinoma. This research aimed to describe the expression and the correlation between CXCL12 and PLK1 in various T stage of ccRCC. This was an analytic observational study with cross-sectional approach. The study included 50 samples throughout January 2014 until June 2022. The samples were divided into T1, T2, T3, and T4 based on histopathology examination. Immunohistochemistry examination was performed using CXCL12 and PLK1 antibodies. The correlation was analyzed using statistical tests. There was positive correlation between CXCL12 and PLK1 expression in various T stage of ccRCC (p=0.005), but when correlated separately, there was no significant result in both CXCL12 and PLK1 (p=0.443 and p=0.292, respectively). CXCL12 and PLK1 expression varied throughout different T stages in this study, but in tandem, the work of CXCL12 positively affect PLK1, resulting greater tumor size.

GRAPHICAL ABSTRACT



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Introduction

Renal cell carcinoma (RCC) is malignant tumor originating from epithelial cells of renal tubules, and is the third most common type of malignancy of the urinary tract [1, 2]. It is the 13th leading cause of malignancy related death in the world and the incidence increases every year [3-6]. Clear cell renal cell carcinoma (ccRCC) is the most prevalent subtype of kidney cancer (75-85%) [7-9]. This subtype's prognosis is also worse with an 8% 5-year survival rate which is linked with tumor aggressiveness and a higher rate of proliferation [10]. By increasing the expression of CXCL12/CXCR4 and accumulating HIF, pVHL inactivation in ccRCC can cause an imbalance in cell proliferation and vascular development (angiogenesis) [11]. According to studies, increased CXCL12 expression and interaction with its receptor are linked to larger tumors, metastasis, and tumor resistance to chemotherapy [12, 13]. In addition, increased transcription of several target genes that influence tumor growth, including PLK1, will occur when the CXCL12/CXCR4/FOXM1 pathway is activated. PLK1 plays various activities during the cell cycle, including regulating the start of mitosis, DNA replication, and the cell's reaction to injury. PLK1 has been linked to a poor prognosis and is widely expressed in many cancer cells, including ccRCC. This is brought about by a rise in the capacity of cancer cells to multiply, spread, and resist chemotherapy [14, 15]. According to certain studies, inhibiting PLK1 expression can both cause tumor cell death and effectively slow tumor growth [16, 17]. This study had hypotheses, that there is correlation between CXCL12/PLK1 expression and T stage of clear cell renal cell carcinoma. Knowing the correlation between CXCL12/PLK1 and ccRCC T stage can be a basis for further research on ccRCC prognostic factors. The authors declare that this study is original and that no similar studies have been done before.

Materials and Methods

Study designs

Samples from 50 patients who underwent radical nephrectomy and were identified as having ccRCC at Dr. Soetomo General Academic Hospital between January 2014 and June 2022 were used in the study. Tissue was routinely processed for histopathological examination. Using the World Health Organization (WHO) categorization of tumors of the urinary system and male genital organs, the samples were categorized into T1 (7 cases), T2 (21 cases), T3 (16 cases), and T4 (6 cases). All tissue samples were re-examined before this study. Slides with marginally healthy tissue and tumors were chosen for manual immunohistochemical staining.

Immunohistochemistry

Paraffin blocks from HE preparations were cut 3-5 micron thick, and then deparaffinised using xylol. Rehydration was done with 96%, 90%, and 80% alcohol, respectively, for 2 minutes, followed by 5 minutes of washing in both running and distilled water. Slides were then placed in 3% H_2O_2 in methanol for 15 minutes at room temperature, washed with distilled water for 5 minutes, and warmed in a deep decloaking chamber for 20 minutes at 95 °C using Target Retrieval Solution (TRS)/Buffer citrate at pH 9 for CXCL12 and pH 6 for PLK1. PBS was used to wash the preparation for five minutes. The background snipper was then dripped for 15 minutes, followed by the primary antibody, which was rabbit monoclonal antibody IgG against CXCL12 (GeneTex A18225) at a dilution of 1:100 and a dilution of 1:1000 for the polyclonal antibody against PLK1 (ABclonal N2C2). After 60 minutes of room temperature incubation, the slides underwent a 5-minute PBS wash. Trekkie Link, a secondary antibody, was dripped and incubated for 20 minutes [31]. Secondary antibody (HRP label) was then dripped for 10 minutes. Diaminobenzidine (DAB) was dripped and incubated for five minutes at room temperature, and then mayer hematoxylin was added. The last steps were dehydration with 80%, 90%, and 96% alcohol. It is then placed in xylol, and a cover glass was used to close the slide. Two pathologists who were blinded to each patient's T stage independently assessed each immunostaining using binocular microscope Olympus CX31. The use of a double headed

microscope was used to settle disagreements between observers.

Scoring

The tumor cell's cytoplasm was examined for CXCL12 immunostaining localization. There were four levels of staining intensity: (0) negative, (1) weak, (2) moderate, and (3) strong. The percentage of tumor cells that were positive was rated as follows: (0) no positive cells, (1) positive in 10% tumor cells, (2) positive in 10-50% tumor cells, (3) positive in 51-80% tumor cells, and (4) positive in >80% tumor cells. The immunoreactive score (IRS), which was the result of multiplying the proportion of positive cells (0-4) by the staining intensity (0-3), had a range of 0-12 [18, 19]. PLK1 expression was observed in the cytoplasm of tumor cells. Cells were considered PLK1 positive only when strong expression were observed. In each case, PLK expression was assessed based on the percentage of positive tumor cells. The final value was the percentage of strongly positively stained cells [20, 21].

Statistical analysis

Statistical analysis was performed using a statistical package for social sciences (SPSS 25.0, Chicago, IL, USA). By using the Spearman's correlation test, we assessed the correlation of PLK1 and CXCL12 in the various T stage of ccRCC. Results of correlation are considered significant if the p value is less than 0.05.

Ethical approval

The authors did not use humans or animals as research subjects, but paraffin blocks were used from clear cell renal cell carcinoma. This study was approved by Research Ethical Committee of Dr. Soetomo General Academic Hospital in concordance with The Office for Human Research Protection (OHRP) dated January 24th, 2023, with Reference No. 1197/LOE/301.4.2/I/2023.

Results and Discussion

The study's findings included the expression of CXCL12 and PLK1, as well as information on age, sex, and the T stage, which is split into stages T1 through T4 (Table 1).

Patient characteristics

In this study, patients with ccRCC were an average age of 57.3 years. The oldest patient is 75, and the youngest patient is 32 years old. The patient's age distribution data was then divided into 6 groups with a 10-year time interval. The age group of 51-60 years had the highest prevalence of patients, with 19 (38%) cases.

Table 1: Characteristics of clear cell renal cell carcinoma patients

Characteristics	Total
Population	50
Sex	
Male	35 (70%)
Female	15 (30%)
Age	
31-40	2 (4%)
41-50	10 (20%)
51-60	19 (38%)
61-70	15 (30%)
71-80	4 (8%)
T stage	
T1	7 (14%)
T2	21 (42%)
T3	16 (32%)
T4	6 (12%)

These findings are consistent with research conducted by RSUP Dr. Hasan Sadikin Bandung in 2018, which found that the peak events occurred when people were between the ages of 51 and 65, with 58 being the average age [22, 23]. The average age at diagnosis for the 87.325 American patients with ccRCC studied by Feng *et al.* was 62 years. The ccRCC incidence rises with age and peaks between the ages of 60 and 79 before decreasing after that [24].

The mean age of sporadic clear cell renal cell carcinoma is 64 years. This is because ccRCC takes time to develop, which results in ccRCC diagnosis being made in later life [22, 25].

The highest smoking rates were found in people between the ages of 34 and 65, according to 2013 Basic Health Research Data. This could promote the development of ccRCC. The incidence rate is unusual for people under the age of 40; this investigation discovered 2 individuals who were 35 and 36 years old. This substance is linked to a genetic illness called VHL disease or Xp11.2 translocations [26].

50 cases of ccRCC made up the study's sample, with a male to female sex ratio of 2.3:1 and a combined total of 35 male patients (70%), and 15 female patients (30%). According to GLOBOCAN data for 2020, men experience ccRCC on average 1.5 times more frequently than women. This is linked to the habits that promote carcinogenesis, such as smoking [27, 28].

Men are also more likely than women to suffer from hypertension, according to basic health research data from 2013 to 2018. Each rise of 10 mm Hg increases the probability of developing CCRCC by 10-22%, and a history of hypertension is linked to a 67% higher risk of ccRCC events [22].

Categorization of the pathological stage T was classified according to the WHO Classification Tumors of the Urinary System and Male Genital Organ, which evaluates the primary tumor for both size and invasiveness, into 4 groups. The T2 group had 21 cases (42%), followed by the T3 group with 16 cases (32%), the T1 group with 7 cases (14%), and the T4 group with 6 cases (12%).

Stage T2, which had a maximum tumor diameter of over 7 cm and was restricted to the kidney, was the most prevalent stage T in this study, with an average diameter of 11.2 cm.This is in line with a study by Mutuiri and Kenya on the clinicopathology characteristics of ccRCC. According to data from SEER (Surveillance, Epidemiology, and End Results), stage T1-T2 renal cell carcinoma was the most common stage at which patients were diagnosed. This may be due to the fact that most patients complain of renal masses, whereas complaints are more likely to be associated with tumors larger than 4 cm [24, 29].

CXCL12 expression

In this study, CXCL12 expression was seen in all four T stage groups. By examining the immunoreactivity score (IRS), which is the result of multiplying the proportion of tumor cells stained with the positive staining intensity of ccRCC tumor cells, the expression of CXCL12 was evaluated (Figure 1).

Table 2: CXCL2 score distribution

CXCL12	T stage								Total	*P*-value
	T1		T2		T3		T4			
Score	n	%	n	%	n	%	n	%	(n)	
0	1	14.33	2	9.65	0	0	0	0	3	0.443
1	2	28.5	0	0	1	6.25	0	0	3	
2	2	28.5	11	52.3	8	50	6	100	27	
3	0	0	0	0	0	0	0	0	0	
4	1	14.33	5	23.8	3	18.75	0	0	9	
6	1	14.33	2	9.65	3	18.75	0	0	6	
9	0	0	1	4.7	1	6.25	0	0	2	
Total (n)	7	100	21	100	16	100	6	100	50	

Expression levels of CXCL12 varied across all samples. The most prevalent score, 2, was found in 27 out of a possible 50 samples (54%). The T1 group's highest score was 1 and 2 (2 cases each), while the T4 group's highest score was 2 (all cases). No cases scored > 9 (Table 2).

Spearman correlation test was used to analyze variations in CXCL12 expression. The analysis's findings did not demonstrate a significant difference between CXCL12 expression and the T stage of ccRCC, with a p-value of 0.443. The primary role of CXCL12, also known as stromal derived factor 1 (SDF1), is to promote the migration and adhesion of hematopoietic progenitors, stem cells, leukocytes, and endothelial cells. In several organs, CXCL12 is generated by fibroblasts, macrophages, and other stromal cells. Both tumors with hereditary VHL syndrome and those that develop sporadically exhibit high expression of CXCL12 [13, 23]. One example of this malignancy is ccRCC. This study demonstrated independent CXCL12 staining over different T stages. According to statistical analysis, there was no association between CXCL12 expression and T stages of ccRCC (p = 0.122). A meta study done by Samahendra *et al.* suggested that different tumor types express CXCL12 in different ways [30]. While expression is lower in breast cancers, CXCL12 is abundantly expressed in tumor cells of the gastro-esophageal, lung, and pancreas, which results in a high mortality rate from local invasion. Low levels of CXCL12 in tumor cells are linked to the spreading of malignancies to tissues with high CXCL12 level, such as liver, bone marrow, and lung. The majority of research found that tumor cells were the primary producers of CXCL12. However, it is possible that the CXCL12 produced by stromal cells and tumor cells plays various roles in the development of tumors. According to a different study, it is insufficient to analyze the CXCL12 performance by itself. The ratio of CXCL12 to its receptors, specifically CXCR4 and CXCR7, can serve as a better measure of CXCL12 activity [31]. The ratio of receptors to ligands varies depending on the type of tumor [30]. Study by Wang *et al.* showed CXCL12 expression in ccRCC tumor cells was lower than in stromal and healthy tissue surrounding the tumor [32]. This affects how a tumor spreads to neighboring and distant tissues and organs.

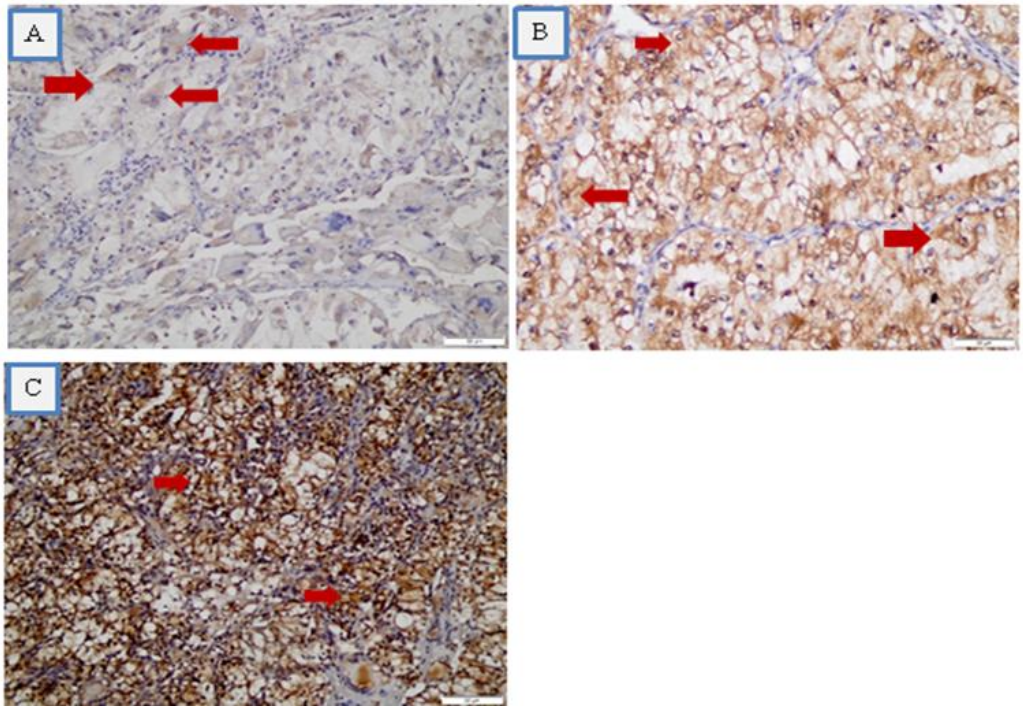

Figure 1: CXCL12 staining observed in tumor cell's cytoplasm (red arrow). (A) Weakly stained (400x), (B) moderately stained (400x), and (C) strongly stained (400x)

PLK1 expression

In this study, the percentage of PLK1 expression was observed in the cytoplasm of the strongly stained tumor cells. PLK1 expression varied at various T stages. The percentage that was most common in 8 cases (16%) was in the range of 1 to 10%. No cases had a percentage of greater than 90, while five cases received a score of zero (Figure 2). Spearman correlation test was used to analyze variations in PLK1 expression. The analysis's findings did not demonstrate a significant difference between PLK1 expression and the T stage of ccRCC, with a p-value of 0.292 (Table 3).

PLK1 performs a variety of functions during the cell cycle, including regulating the start of mitosis and the G2/M checkpoint, coordinating centrosomes with the cell cycle, regulating chromosomal segregation, facilitating DNA replication, and participating in cytokinesis and meiosis. PLK1 plays a role in the way the cell reacts to injury. Previous studies have shown that PLK1 is significantly expressed in a variety of cancer cells [33]. Studies about PLK1 expression in ccRCC gave variety of results. Dufies *et al.*'s study found that metastases and resistance to ccRCC therapy were associated with increased PLK1 expression. An analysis of PLK1 expression in 90 RCC cases revealed a relationship between elevated PLK1 expression and the clinicopathological characteristics of RCC patients (tumor size, clinical staging, and grading) [34]. Zhang *et al.*'s study on PLK1 expression shown that decreased PLK1 expression (PLK1 knockdown) led to a significant increase in the proportion of cells in the G2/M phase (cycle arrest) [14].

Table 3: PLK1 percentage score distribution

PLK1	T stage								Total	*P*-value
	T1		T2		T3		T4			
Percentage	n	%	n	%	n	%	n	%	(n)	
0	1	14.3	3	14.3	0	0	1	16.7	5	0.292
1-25	0	0	5	23.8	7	43.7	2	33.3	14	
26-50	3	42.9	6	28.6	3	18.7	3	50	15	
51-75	2	28.5	5	23.8	2	12.5	0	0	9	
>75	1	14.3	2	9.5	4	25.1	0	0	7	
Total (n)	7	100	21	100	16	100	6	100	50	

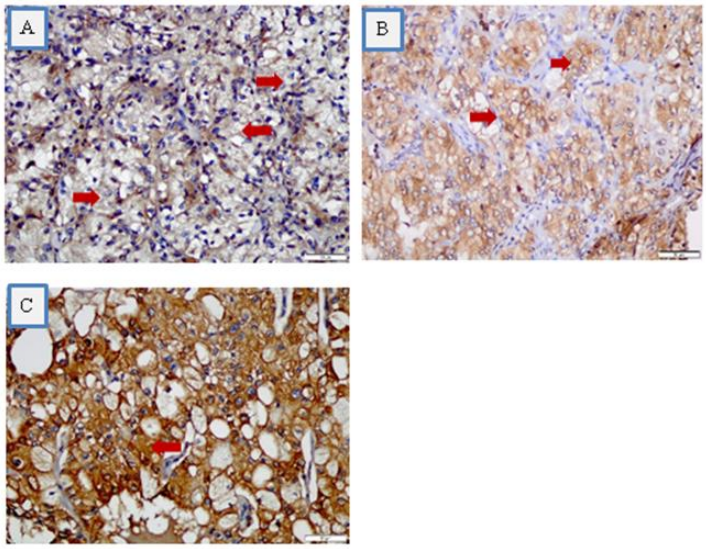

Figure 2: PLK1 staining observed in tumor cell's cytoplasm (red arrow). (A) Weakly stained (400x), (B) moderately stained (400x), and (C) strongly stained (400x).

Table 4: Parameters and *p*-value

Parameter	*P*-value
Correlation of CXCL12 expression with T stage	0.443
Correlation of PLK1 expression with T stage	0.292
Correlation of CXCL12 and PLK1 with T stage	0.005

PLK1 has been linked to tumor grade, metastasis, and aggressiveness, according to a study that performed genomic profiling on ccRCC. Injection of a PLK1 inhibitor decreased tumor growth/volume in ccRCC xenograft nude mice, while other research has indicated that anti-PLK1 siRNA injection did not have the same effect in experimental animals [35].

Correlation between CXCL12 and PLK1 expression

Correlation between CXCL12 expression and PLK1 expression with ccRCC's T stage was tested using Spearman's non-parametric correlation test. The findings of the statistical analysis revealed a significant correlation with a correlation coefficient of 39.4% and a p-value of 0.005 ($p< 0.05$) (Table 4).

Up to now, there's no other studies that assessed the correlation between CXCL12/PLK1 and T stage of ccRCC, and in this study, we found a significant correlation. Clear cell renal cell carcinoma is characterized by the inactivation of the tumor suppressor gene von Hippel Lindau, which is responsible for the degradation of HIF1 and HIF2. Hypoxic circumstances and pVHL inactivation cause a high concentration of HIF [36]. Increased HIF increases CXCL12 expression in tumor cells [37].

CXCL12 interacts to CXCR4 on the cell membrane, causing GTP to be converted into GDP. This, in turn, causes the subunit dissociation Gα of the Gβ/Gγ dimer. Phosphoinositide-3 kinase (PI3K) is activated by either the Gβ/Gγ dimer or the Gαi subunit, which phosphorylates many proteins involved in focal adhesion and aids in cell migration [38, 39].

PIP2 (phosphatidylinositol biphosphate) will be actively phosphorylated by the PI3K to produce PIP3 (phosphatidylinositol triphosphate activation). PTEN (phosphatase and tensin homologs) inhibits this activity. AKT, a protein serine-threonine kinase, and PDK1, a pyruvate dehydrogenase kinase, bind at PIP3. Activated AKT can activate a number of downstream targets including mTOR, NF-B, and MDM2 (mouse double minute 2 homolog), which play important roles in cell survival and proliferation. Through paracrine actions, the AKT pathway can also raise CXCL12 expression [40].

FOXO3 is a transcription factor that causes cell growth arrest and apoptosis. Increased AKT has the ability to phosphorylate FOXO and cause its destruction in the cytosol via the ubiquitin-proteasome pathway. The potent oncogene FOXM1, whose activity is negatively controlled by FOXO3, is increased when FOXO3 is inactivated [41]. In addition, FOXM1 is necessary for the production of the proteins Aurora B kinase and Polo-like kinase 1 (PLK1), which control cell cycle and death. At the T210 centrosome, Aurora A binds to the cofactor Bora and activates PLK1. To perform its functions, the phosphorylated PLK1 is then translocated to the nucleus cells [42].

High PLK1 expression causes the epithelial-mesenchymal transition that leads to stage T elevation as well as tumor cell proliferation [43]. Through these pathways, CXCL12 believed to be positively affects PLK1 and involved in ccRCC T stage development. Until now, there is no known studies that used the same antibodies (CXCL12 and PLK1) on ccRCC in Indonesia. Previous study with fewer sample about CXCL12's receptor, CXCR4 yield the same positive effect on ccRCC's T stage [31]. Other similar studies in Indonesia used CD113, EGFR, HIF, and VEGF on ccRCC with fewer sample [6].

Involvement of other pathways

When correlated separately, neither CXCL12 nor PLK1 had direct effect on the T stage. The involvement of other pathways, including p53 (a tumor suppressor gene), may have contributed to the varied results of CXCL12 and PLK1 expression in this study. P53 performs a variety of tasks, including senescence, cell cycle arrest, and apoptosis [44, 45]. The tumor suppressor gene mutation known as the p53 mutation is

most frequently observed in a variety of cancers [46]. In addition to lose its ability to control tumor growth, mutant p53 (Mutp53) frequently exhibits an oncogenic gain of function (OGF) that promotes carcinogenesis. Depending on the type of tumor, p53 gene mutations can happen either early or late in the tumorigenic process. According to studies by Gorova *et al.*, Zhang *et al.*, and Xie *et al.*, p53 mutation frequency in ccRCC was low [47-49]. AKT activity is crucial for maintaining a balance between survival and aptosis and contributes to the development of RCC tumors. PTEN is a tumor suppressor gene that blocks the lipid phosphatase activity of the PI3K/AKT pathway, hence inhibiting the AKT activity. AKT expression and PTEN have been linked in studies, while other research suggests that PTEN is activated by a different method [47]. Several cancers, including RCC, have been linked to PTEN deletions and mutations, but only 23 (5%) of 538 ccRCC cases had PTEN alterations, according to research by Fan *et al.* Patients with the PTEN mutation may have different signalling and metabolic changes from those with wild-type PTEN, which may affect the prognosis and course of their malignancy [47, 49]. These could be the factors that cause high levels of CXCL12 and PLK1 in tumors with low T stage, and vice versa.

Conclusion

This study analyzed correlation between CXCL12/PLK1 and T stage of ccRCC, which incident is increasing in Indonesia. This study used two proliferation markers: CXCL12 and PLK1. These two proteins were never analyzed together in ccRCC cases. Both CXCL12 and PLK1 expression varied throughout different T stages in this study, but in tandem, the work of CXCL12 positively affect PLK1, resulting greater tumor size. Further research to assess the expression of CXCL12 and PLK1 in clear cell renal cell carcinoma based on other parameters, such as metastatic status or therapy resistance with a more even distribution of patients can be done. It is necessary to identify the role of other pathways such as PTEN and p53 in the proliferation process of clear cell renal cell carcinoma in upcoming research.

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Disclosure Statement

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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