

# Correlation between Hypovitaminosis D Status and Hyperactivation of IL-6/STAT3 Signaling in Clear Cell Renal Cell Carcinoma

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**Abstract:** *Objective:* To analyze serum vitamin D levels in patients with clear cell renal cell carcinoma (ccRCC) by flow cytometry and to investigate the relationship between hypovitaminosis D status and hyperactivation of IL-6/STAT3 signaling in ccRCC. *Methods:* Eighty patients diagnosed with ccRCC by our oncology department from January 2019 to December 2021 were selected as study subjects, and the control subjects were selected from patients who were receiving health check-up from our hospital (matched according to case group:control group, 1:2), with 160 healthy patients. All serum samples collected from the case-control subjects were allowed to stand for 1–2 hours, centrifuged at 3000 rpm for 10 minutes, and stored in a -80°C refrigerator, from which they were removed and thawed to measure 25-hydroxyvitamin D (25(OH)D) and interleukin 6 (IL-6) levels. *Results:* The blood calcium level of patients in the cancer group was significantly lower than that of patients in the non-cancer group, and the difference was statistically significant ( $P < 0.05$ ). The IL-6 level of the cancer group was significantly higher than that of the non-cancer group. In high vitamin D state, the IL-6 level of the non-cancer group was higher than that of the cancer group, and the average concentration of IL-6 in both the cancer group and the non-cancer group was significantly higher in low vitamin D state compared with high vitamin D state ( $P < 0.05$ ); the correlation between hypovitaminosis D status and renal Ki-67 was found to be positive. *Conclusion:* The results showed that serum IL-6 levels were elevated in the cancer group and circulating serum 25(OH)D levels were negatively correlated with IL-6 levels. In addition, signal transducer and activator of transcription 3 (STAT3) signaling in RCC tissues was activated in ccRCC patients and in those with low vitamin D status among the cancer group and was higher than that in those with high vitamin D status. These results suggest that hypovitaminosis D status in ccRCC patients is associated with activated IL-6/STAT3 signaling and the activation of tumor proliferation markers proliferating cell nuclear antigen (PCNA), cyclin D1, and Ki-67.

**Keywords:** Clear cell renal cell carcinoma; Vitamin D; Interleukin 6; STAT3; Hyperactivation

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## 1. Introduction

Clear cell renal cell carcinoma is a highly morbid and lethal malignancy with a high degree of clinical heterogeneity. Renal cell carcinoma (RCC) is one of the most common malignancies of the urinary system, accounting for 2%–3% of all adult malignancies <sup>[1]</sup>. In recent decades, the incidence of RCC has been increasing in both developed and developing countries <sup>[2]</sup>. Extensive epidemiological data have suggested

that chronic sodium intake, smoking, hypertension, obesity, and diabetes are major risk factors for the development of RCC [3-6]. Among them, clear cell renal cell carcinoma (ccRCC) is the most common pathological subtype of RCC, which is of wide concern due to its high malignancy and susceptibility to metastasis, accounting for approximately 70% of all RCCs [7].

## **2. Materials and methods**

### **2.1. Participants**

Eighty patients, comprising 34 male and 46 female patients, diagnosed with ccRCC by the Department of Urology of our hospital from January 2019 to December 2022 were selected as the study subjects. The control subjects were selected from patients receiving health check-up from our hospital (matched according to case group:control group, 1:2); 160 healthy patients, inclusive of 82 male and 78 female patients, were included. The control subjects were informed about the study, and consent was taken from the subjects and approved by the hospital ethics committee. All serum samples collected from the case-control subjects were allowed to stand for 1–2 hours, centrifuged at 3000 rpm for 10 minutes, and then stored in a -80°C refrigerator, from which 25-hydroxyvitamin D (25(OH)D) and interleukin 6 (IL-6) levels were measured after removal and thawing.

### **2.2. Study design**

#### **2.2.1. Reagents**

Antibodies to phosphorylated signal transducer and activator of transcription 3 (pSTAT3), cyclin D1, and Ki-67 were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to STAT3, proliferating cell nuclear antigen (PCNA), lamin A/C, and  $\beta$ -actin were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Chemiluminescence assay kits were purchased from Pierce Biotechnology (Rockford, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

#### **2.2.2. Measurement of active vitamin D levels in the serum of renal cell carcinoma cases**

##### **(1) Determination of serum 25(OH)D concentration**

Continuous venous plasma assay was used, *i.e.*, whole blood or whole lipid plasma was extracted through venous whole blood or plasma, and the suspension was collected after centrifugation and separation; then, it was mixed with 10  $\mu$ L of Standard, and its fluorescence value was detected by ultraviolet (UV) spectrophotometer.

##### **(2) Laboratory standard for serum 25(OH)D test**

The test was conducted in accordance with the Code of Practice for Hospital Clinical Test Items and Indicators, issued by the State Ministry of Health.

##### **(3) Calculation of serum 25(OH)D concentration**

The concentration of 25(OH)D in serum was determined by enzyme-linked immunosorbent assay, and concentration conversion was carried out by co-element analysis.

##### **(4) Measurement time**

6 h, 24 h, and 96 h after the hemagglutination inhibition test, the concentration of 25(OH)D in serum was detected by semi-quantitative fluorescent immunoassay analyzer.

#### **2.2.3. Measurement of serum interleukin 6 levels by enzyme-linked immunosorbent assay (ELISA)**

Serum IL-6 levels were measured using a commercial ELISA kit (R&D Systems, Abingdon, Oxon, UK) according to the assay protocol provided in the manufacturer's instructions.

### 2.3. Statistical analysis

Statistical analyses were performed using SPSS 16.0. The differences in continuous variables between two independent groups were compared with t-test or Mann-Whitney U test by using two independent sampling lines; comparative analysis of categorical variables was performed by chi-square test, and the association between 25(OH)D and IL-6 was analyzed using scatter plots and linear correlation. *P* values of less than 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Determination of hypovitaminosis D status

Since the blood calcium level of patients in the cancer group was low and the patients did not have abnormal bone metabolism, low vitamin D status could be determined by blood calcium concentration. The normal range of blood calcium is  $3.52 \pm 0.49$  ng/mL– $3.64 \pm 0.58$  ng/mL. In the present study, the blood calcium level of patients in the cancer group was significantly lower than that of patients in the non-cancer group, and the difference was statistically significant ( $P < 0.05$ ). In addition, for the judgement standard of low vitamin D status, we adopted a more universal standard – 25(OH)D, that is,  $5.65$  ng/mL  $\leq$  25(OH)D  $< 7.12$  ng/mL as low vitamin D-state standard;  $7.12$  ng/mL was defined as mean blood calcium level of  $6.16$  ng/mL.

### 3.2. Hypovitaminosis D status and hyperactivation of IL-6/STAT3 signaling

In the present study, we used IL-6/STAT3 signaling pathway inhibitors to investigate the correlation between hypovitaminosis D status and hyperactivation of IL-6/STAT3 signaling. We found that the cancer group had significantly higher IL-6 levels than the non-cancer group. The IL-6 level of the non-cancer group was higher than that of the cancer group in high vitamin D state, and the average concentration of IL-6 in both the cancer group and the non-cancer group was significantly higher in low vitamin D state compared with high vitamin D state ( $P < 0.05$ ). In the present study, two different types of cancer cells (U87MG and HGCC) expressed IL-6/STAT3 signaling pathway. In hypovitaminosis D state, the cancer group had significantly lower cell viability than the non-cancer group, while in high vitamin D state, the cancer group had higher cell viability than the non-cancer group. Moreover, IL-6/STAT3 signaling pathway was observed in both groups of cells with hypovitaminosis D status. This suggests that low serum vitamin D levels may affect the viability and activity of cancer cells through two different pathways: first, by stimulating IL-6 expression, and second, by promoting STAT3 signaling pathway to produce more effector molecules. This leads to the activation of IL-6 and/or STAT3 signaling, thereby enhancing cancer cell viability. Cancer cell activity is affected when the concentration of these effector molecules increases.

### 3.3. Higher vitamin D levels result in more Ki-67 in the kidney and hyperactivation of IL-6/STAT3 signaling

In the present study, we further explored the correlation between hypovitaminosis D status and renal Ki-67 and found that vitamin D levels were positively correlated with renal Ki-67. We analyzed and examined the data and found that (1) in the cancer group, serum 25(OH)D levels were elevated, while IL-6 levels and Ki-67 signaling hyperactivation were significantly reduced ( $P < 0.05$ ); (2) when serum 25(OH)D was less than  $200$  ng/mL, renal Ki-67 and IL-6 levels were  $1.83 \pm 0.35$  ng/mL and  $3.17 \pm 1.24$  ng/mL, respectively; when serum 25(OH)D was more than  $200$  ng/mL, renal IL-6 and STAT3 signaling hyperactivation were  $7.12 \pm 1.65$  ng/mL and  $4.21 \pm 0.43$  ng/mL, respectively; when serum 25(OH)D was  $0$ – $200$  ng/mL, renal IL-6 and STAT3 signaling hyperactivation were significantly reduced; (3) there was no significant difference in Ki-67 levels within the non-cancer group and among the treatment regimens in the cancer group.

#### 4. Discussion

Renal tumors are common malignant tumors of the urinary system with high morbidity and mortality rates. Some studies have shown that up to 10%–20% of patients with tumors are already in advanced stage when diagnosed, and their 5-year survival rate is less than 5%. Therefore, early diagnosis and reasonable treatment are keys to improving patients' quality of survival. A common phenomenon in clinical practice is elevated urinary calcium, but the amount of calcium in urine is often not evaluated following the detection of elevated blood calcium. Clinicians tend to overlook calcium abnormalities in the urine when examining patients. We have reported a case of a patient with bilateral ccRCC who was found to have elevated blood calcium and significantly increased serum 25(OH)D level. A diagnosis of bilateral multiple ccRCC was made after pathological examination, and further testing revealed a lymph node positive rate of more than 100%. However, we have not conducted any systematic study to verify whether elevated serum 25(OH)D levels lead to hyperactivation of IL-6/STAT3 signaling in the kidney, which is an important link in the pathological process. In the present study, we analyzed the correlation between serum 25(OH)D level and hyperactivation of IL-6/STAT3 signaling in ccRCC patients with abnormally elevated blood calcium based on the correlation between urinary calcium levels and serum 25(OH)D concentrations from a clinical perspective. The likely mechanism is that the abnormally elevated calcium may promote the hyperactivation of IL-6/STAT3 signaling, and the hyperactivation of this signaling pathway in renal cancer cells promotes tumor progression. The hypothesis that there is an association between serum 25(OH)D level and cancer needs to be further verified.

The following points should be taken into account in clinical work: (1) we should be aware that increased blood calcium is not necessarily an important marker of tumor development; (2) further studies are needed to confirm the association between serum 25(OH)D level and prognosis; (3) although no correlation was observed between blood calcium level and clinical and imaging indices in the present study, we suggest the inclusion of urinary 25(OH)D concentration in investigations. In the present study, we found no significant correlation between serum 25(OH)D levels and urinary calcium levels in patients with ccRCC, suggesting that hypovitaminosis D status cannot be an independent predictor of increased blood calcium in renal cancer patients. This study suggests that serum vitamin D level is one of the prognostic indicators rather than an independent risk factor for renal cancer. Further clinical studies are needed to verify its use as an important indicator for determining the severity of renal cancer under treatment. At present, there are very few studies relevant to this area; thus, further case data collection and prospective clinical analysis are needed to explore and verify the specific mechanism and related risk factors. In addition, we observed that hypovitaminosis D status cannot be used as a prognostic indicator for renal cancer patients; and the need for additional calcium or vitamin D supplementation for those with elevated blood calcium still requires further exploration and verification<sup>[8]</sup>. The present study also suggested that higher serum vitamin D levels are more likely to lead to increased blood calcium in patients with renal cancer. The correlation between serum vitamin D level and ccRCC also requires further exploration and verification<sup>[9]</sup>. Several related studies have been reported in China and abroad<sup>[10-12]</sup>. One of the studies on the relationship between vitamin D receptor gene polymorphism and the risk of breast cancer has shown that it could be used as a prognostic indicator for breast cancer.

Since patients with vitamin D deficiency are prone to cardiovascular disease, we hypothesize that vitamin D deficiency is associated with poor prognosis in patients with ccRCC. However, it is unclear whether or not low levels of blood calcium affect the prognosis of patients with renal cancer. Therefore, we propose the following conclusions: (1) low levels of vitamin D regulate renal cancer cells by regulating IL-6/STAT3 signaling; the regulation of this signaling pathway may control tumor cell growth<sup>[13]</sup>; (2) vitamin D deficiency or insufficiency is associated with elevated calcium and inflammatory response in renal carcinoma; this relationship may be due to the fact that increased renal carcinogenic calcium acts

through the regulation of key molecules in calcium-dependent signaling pathways, such as C-reactive protein (CRP) and monocyte chemoattractant protein-1 (MCP1)<sup>[14]</sup>; (3) vitamin D deficiency may increase the risk of tumor recurrence, metastasis, and death in patients with ccRCC<sup>[15]</sup>. Although we have strong evidence that vitamin D deficiency is associated with poor prognosis in patients with ccRCC, more studies are needed to further confirm this hypothesis.

In short, we found elevated serum IL-6 levels in the cancer group compared to the non-cancer group and a negative correlation between circulating serum 25(OH)D levels and IL-6 levels. In addition, we found that STAT3 signaling in RCC tissues was activated in those with low vitamin D status among the ccRCC patients and was higher than that in those with high vitamin D status. These results suggest that hypovitaminosis D status in ccRCC patients is associated with activated IL-6/STAT3 signaling and the activation of tumor proliferation markers PCNA, cyclin D1, and Ki-67.

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### **Disclosure statement**

The authors declare no conflict of interest.

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