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Determination of serum vitamin D in patients with renal, bladder, and prostate cancer by ultra-performance liquid chromatography-tandem mass spectrometry

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The purpose of this study is to detect the vitamin D (VitD) levels in patients with renal cell carcinoma (RCC), bladder cancer (BC), and prostate cancer (PC) using ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technology to assess the VitD status in subjects using different methods, to understand the true level of VitD in RCC, BC, and PC patients. A total of 170 subjects were included in this study, and their serum VitD metabolite levels were measured, including 25-hydroxyvitamin D_2 [25(OH)D_2], 25-hydroxyvitamin D_3 [25(OH)D_3], 3-epi-25-hydroxyvitamin D_3 [C_3-epi-25(OH)D_3, C_3-epi], and calculations for 25(OH)D, 25(OH)D_2/25(OH)D_3, and C_3-epi/25(OH)D_3 were made. The variations in serum VitD, calcium (Ca), inorganic phosphorus (IP), vitamin D receptor (VDR), and renal function indicators were measured, and their correlations were analyzed. The levels of 25(OH)D, 25(OH)D_3, C_3-epi, C_3-epi /25(OH)D_3, and free 25(OH)D [F25(OH)D] in RCC, BC, and PC patients were significantly lower than that in the healthy control (HC) group (all $p<0.05$). The ratio of 25(OH)D_2/25(OH)D_3 was significantly higher in these groups compared to the HC group (all $p<0.05$). 25(OH)D_3 distinguished the HC group from common cancers of the urinary system (including RCC, BC, and PC) in male patients and showed good diagnostic performance. The level of 25(OH)D_3 in all three groups was positively correlated with F25(OH)D levels, and in the disease groups, C_3-epi levels were positively correlated with both 25(OH)D_3 and F25(OH)D levels. This study found that RCC, BC, and PC patients had lower serum levels of 25(OH)D_3, C_3-epi, and F25(OH)D compared to healthy individuals, with most RCC, BC, and PC patients displaying VitD deficiency.

Key words: vitamin D; renal cell carcinoma; bladder cancer; prostate cancer; ultra-high performance liquid chromatography-tandem mass spectrometry

Cancer is an important public health problem we face. According to the latest cancer data statistics, there are more than 19 million new cancer cases and nearly 10 million new cancer deaths in the world, of which there are more than 2.4 million new cases of kidney cancer (KC), bladder cancer (BC), and prostate cancer (PC), accounting for 12.5% of all cancers, and nearly 770,000 new deaths, accounting for 7.7% of all cancers [1]. Renal cell carcinoma (RCC), BC, and PC are the most common types of cancer in the urogenital system. Despite significant progress in diagnosis and treatment, the mortality rate remains high [2]. RCC is the most common solid lesion in the kidney, originating from renal tubular epithelial cells and accounting for approximately 90% of KC. Although RCC, BC, and PC are common malignant tumors in the urogenital system, they do not have specific clinical symptoms, so most patients are unexpectedly discovered during physical examinations. Advanced RCC is a fatal disease with a low 5-year survival rate, and conventional renal function testing indicators cannot effectively indicate the occurrence of common cancers in the urinary system. Therefore, early diagnosis and adjuvant treatment of common cancers in the urinary system are particularly important.

Vitamin D (VitD) is a lipid-soluble steroid derivative that contains various forms and has different chemical structures

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but similar biological effects. There are two main forms of VitD metabolites, namely ergocalciferol (VitD2) and cholecalciferol (VitD3), which are hydroxylated by the liver and kidney to form the most active 1,25-dihydroxy vitamin D [$1,25(OH)_2D_3$] and then combine with vitamin D receptor (VDR) to exert biological effects [3]. As is well known, VitD has a classic role in regulating calcium-phosphorus balance and promoting bone formation. In addition, research has reported that VitD can also widely participate in and regulate metabolic reactions in the body, which is closely related to the occurrence, development, and prognosis of various diseases [4]. Studies have shown that low levels of VitD are associated with the development and progression of common cancers of the urinary system [5], it can inhibit the migration and invasion of cancer cells through a variety of different signaling pathways. For cancer patients, VitD can have anti-inflammatory, antioxidant, and DNA damage repair effects in the initial stage, inhibit cancer cell proliferation, angiogenesis, clone formation, and metastasis in the development stage, and VitD can improve some possible risk factors such as hypertension, diabetes, and obesity [6]. This study used ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) technology [7] to detect serum 25-hydroxyvitamin D_2 [$25(OH)D_2$], 25-hydroxyvitamin D_3[$25(OH)D_3$], 3-epi-25-hydroxyvitamin D_3 [C_3-epi-$25(OH)D_3$, C_3-epi] levels in male RCC, BC, PC patients, and the healthy control (HC) group. The total 25(OH)D level, 25 $(OH)D_2/25(OH)D_3$, and C_3-epi/$25(OH)D_3$ ratio were calculated, and the subjects were evaluated using different methods. The VitD status in the body of the subjects more accurately reflects the storage status of VitD in the body. At the same time, we also tested the renal function indicators, calcium and phosphorus content, and VDR of each group of subjects to explore the changes and correlations of serum VitD, VDR, and renal function indicators in common urological cancer patients, providing new ideas for early diagnosis and new treatment strategies.

Patients and methods

Study subjects. A total of 170 subjects, including 44 in the RCC group, 39 in the BC group, 45 in the PC group and 42 in the HC group, were collected from Mianyang Central Hospital from May 2022 to December 2022. General clinical data such as age, gender, and medical history of all subjects were recorded in this study. The baseline characteristics of the participants are presented in Table 1.

Inclusion criteria. 1) Age ≥18 years old; 2) diagnosed by pathology as RCC, BC, or PC; 3) has not received chemotherapy, radiation therapy, or resection before sampling; 4) the HC group consisted of patients who had normal liver and kidney function indicators, blood analysis, and urine routine during the same period of physical examination in our hospital.

Exclusion criteria. 1) Pregnant and lactating women; 2) patients with other kidney diseases; 3) Secondary renal dysfunction; 4) pathologically confirmed as a non-tumor or benign tumor; 5) cancer patients undergoing radiotherapy, chemotherapy, and immunotherapy for surgical resection; 6) patients with liver cirrhosis and hematological diseases; 7) subjects using drugs or preparations containing hormones or VitD.

Sample collection. After fasting overnight, the subjects' venous blood was collected to the scale (about 5.0 ml) with a vacuum collection vessel (BD Vacutainer®, USA) containing separating gel and coagulant, centrifuged at 3000 rpm for 10 min, and the upper serum was divided into two parts, one of which completed the detection of renal function indicators and calcium and phosphorus within 2 h. The other was stored in the –80 °C refrigerator for VitD and VDR testing.

Table 1. Baseline characteristics table of all subjects in this study.

group	HC (n = 42)	RCC (n = 44)	BC (n = 39)	PC (n = 45)	F, p-value
Male/female (n)	42/0	44/0	39/0	45/0	/
Age (years)	63.4 ± 8.2	63.6 ± 6.5	65.0 ± 8.0	66.4 ± 5.6	1.685, 0.172
WHO/ISUP classification (n)					
G1	/	14	/	6	/
G2	/	23	/	2	/
G3		5	/	15	/
G4	/	2	/	9	/
G5	/	/	/	13	/
Grade				/	
Low	/	/	21	/	/
High	/	/	18	/	/
Gleason score (n)					
≤6	/		/	6	/
6–9	/		/	26	/
≥9			/	13	/

Abbreviations: HC-healthy control; RCC-renal cell carcinoma; BC-bladder cancer; PC-prostate cancer

Renal function, calcium, and phosphorus testing. Complement 1q (C1q) (20220821) was detected using immunoturbidimetry, with the reagent kit provided by Shanghai Beijia Biochemical Reagent Co., Ltd. Inorganic Phosphate (IP) (02021) and total calcium (Ca) (59094) were measured using transmission immunoturbidimetry, with reagent kits provided by Wako Pure Chemical Industries, Ltd., Japan. Uric Acid (UA) (CH0204054) was detected using the uricase method; neutrophil gelatinase-associated lipocalin (NGAL) (CH0204061) was measured using immunoturbidimetry; urea (CH0204051) was tested using urease-glutamate dehydrogenase method; creatinine (Cr) (CH0204053) was measured using creatinine oxidase method; cystatin C (CysC) (1022031) was detected using immunoturbidimetry, with reagent kits all provided by Sichuan Maike Biotech Co., Ltd. These tests were performed on the LST008 Automatic Biochemical Analyzer (Hitachi, Japan).

The estimated Glomerular Filtration Rate (eGFR) was calculated using the CKD-EPI Crea-CysC formula based on serum Cr and CysC recommended in the 2012 KDIGO guidelines [8]; $eGFR = 135 \times \min(Cr/\kappa, 1)^{\alpha} \times \max(Cr/\kappa, 1)^{-0.601} \times \min(CysC/0.8, 1)^{-0.375} \times \max(CysC/\ 0.8, 1)^{-0.711} \times 0.995^{age}$ ($\times$ 0.969 female).

In the above formula, Cr is serum creatinine (mg/dl) (1 mg/dl = 88.4 μmol/l); CysC is serum cystatin C (mg/l). κ represents the correction factor of Cr, which is 0.7 for women and 0.9 for men. α was 0.248 for females and 0.207 for males. min (Cr/κ, 1) represents the minimum value or 1 of Cr/κ, and max (Cr/κ, 1) represents the maximum value or 1 of Cr/κ. min (CysC/0.8, 1) indicates the minimum value or 1 of CysC/0.8, and max (CysC/0.8, 1) indicates the maximum value or 1 of CysC/0.8.

Detection of VitD metabolites using UPLC-MS/MS technique. The detection method for VitD metabolites followed the previously described procedure [9], briefly, 10 μl of internal standard was added to 200 μl of serum sample and vortex-mixed for 5 min. Then, 1.0 ml of releasing agent (tert-butyl methyl ether, CNW, Germany) was added and centrifuged at 13,000×g for 5 min to collect 800 μl of supernatant. The solution was then dried using an MD200-1A nitrogen blow-down evaporator (Ousheng, China). Subsequently, 125 μl of reconstitution solution was added and vortex-mixed for 5 min before centrifugation at 13,000×g for 5 min. Samples were analyzed using Jasper™ HPLC (Shimadzu, Japan) coupled with an AB SCIEX Triple QuadTM 4500MD (ABI, USA) UPLC-MS/MS. Chromatographic conditions were as follows: F5 column, column temperature 45 °C, flow rate 0.6 ml/min, injection volume 10 μl, mobile phase A consisting of deionized water with 0.1% formic acid, and mobile phase B consisting of methanol with 0.1% formic acid, eluted in a gradient mode. Mass spectrometry conditions included the use of atmospheric pressure chemical ionization (APCI) ion source for analysis. Throughout the MS/MS run, positive ion multiple reaction monitoring (MRM) mode with a 40 ms dwell time was used to continuously monitor all ions.

Free 25(OH)D and VDR testing. Both free 25(OH)D [Free 25(OH)D, F25(OH)D] and VDR were detected using the ELISA method. The F25(OH)D testing was strictly conducted as per the instructions of the kit (E-EL-H2043c, DIA Source Future Diagnostics, Belgium). VDR testing was performed strictly according to the kit instructions (KAPF1991, Eli Lilly, China) and analyzed on a Rayto enzyme-linked immunosorbent assay analyzer (Rayto, China).

Statistical analysis. The results of this experiment were statistically analyzed using SPSS 25.0 software package. The results of the non-normal distribution were expressed as the median (interquartile distance) [M (P25~P75)]. The Mann-Whitney U test was selected to compare the differences in the observational indicators between the two groups. The Kruskal-Wallis H rank sum test was selected to compare the differences between multiple groups, and the Bonferroni method (i.e., adjusted α level method) was used for pairwise comparison. Counting data is expressed as a percentage (%). A p-value <0.05 was considered to be statistically significant.

Ethics approval. This study protocol was reviewed and approved by the Medical Ethics Committee of Mianyang Central Hospital (P2020030), and all patients signed the informed consent.

Results

Renal function indicators, calcium, and phosphorus levels. In this experiment, renal function indicators varied significantly across the four groups, with notable differences between cancer patients and healthy controls. UA levels were significantly elevated in the RCC and BC groups compared to HC, while the PC group showed a significant decrease in UA levels compared to both the HC and other cancer groups ($p<0.001$). NGAL levels were markedly increased in all cancer groups (RCC, BC, and PC) compared to HC ($p<0.001$), suggesting renal stress or injury in these subjects. Similarly, Cr levels were significantly higher in the RCC group compared to both HC and PC ($p<0.001$), while CysC levels were elevated in all cancer groups compared to HC ($p<0.001$). The eGFR was notably reduced in all cancer groups compared to HC ($p<0.001$), reflecting impaired renal function. Regarding mineral metabolism, IP levels were significantly higher in the PC group compared to HC, but no significant differences were observed in Ca levels across the groups ($p=0.530$) (Table 2). These results indicate significant renal dysfunction and alterations in phosphorus metabolism, particularly in cancer patients, with more profound effects seen in those with RCC and PC.

Serum VitD metabolites and VDR. The levels of VitD metabolites and VDR showed significant differences among the four groups of male subjects. Both 25(OH)D and 25(OH)D_3 levels were significantly lower in the RCC, BC, and PC groups compared to HC, with the lowest levels observed in the BC and PC groups ($p<0.001$). F25(OH)D levels followed a

Table 2. Renal function indicators and total calcium and inorganic phosphorus levels of four groups of subjects in this experiment.

	HC (n=42)	RCC (n=44)	BC (n=39)	PC (n=45)	H/F, p-value
UA	**350.05 (317.75–385.13)**	**373.15 (334.93–425.73)^**	**370.40 (298.40–438.10)^**	**298.80 (248.40–359.15)***	**21.495, <0.001**
C1q	170.93±31.22	184.84±32.92	189.69±34.66*	193.44±36.73*	3.575, 0.015
NGAL	**72.50 (53.75–87.25)**	**136.50 (111.25–193.75)***	**180.00 (142.00–242.00)***	**138.00 (101.00–201.50)***	**73.271, <0.001**
Urea	5.74 (4.53–6.48)	6.11 (5.01–6.79)	6.25 (5.03–7.55)	6.13 (4.66–7.36)	2.446, 0.485
Cr	**75.60 (68.93–82.38)**	**101.55 (76.28–119.75)*^**	**81.80 (70.60–103.70)**	72.40 (64.05–85.45)	28.086, <0.001
CysC	0.93 (0.86–1.01)	1.17 (0.99–1.53)*	1.07 (1.01–1.29)*	1.11 (0.97–1.38)*	33.114, <0.001
eGFR	84.30 (77.73–91.18)	67.90 (52.28–79.33)*	73.70 (61.50–77.90)*	71.10 (57.90–81.00)*	33.114, <0.001
IP	1.04 (0.91–1.09)	0.98 (0.91–1.12)^	1.08 (0.98–1.14)	1.15 (0.94–1.27)*	15.503, 0.001
Ca	2.26 ± 0.20	2.24 ± 0.14	2.26 ± 0.14	2.29 ± 0.17	0.740, 0.530

Abbreviations/Notes: UA-Uric Acid (μmol/l); C1q-Complement 1q (mg/l); NGAL-Neutrophil Gelatinase-Associated Lipocalin (μg/l); Urea (mmol/l); Cr-Creatinine (μmol/l); CysC- Cystatin C (mg/l); eGFR-estimated Glomerular Filtration Rate (ml/min/1.73 m^2); IP-Inorganic Phosphate (mmol/l); Ca-Calcium (mmol/l); HC-healthy control; RCC-renal cell carcinoma; BC-bladder cancer; PC-prostate cancer; *p-value compared to HC; ^p-value compared to PC

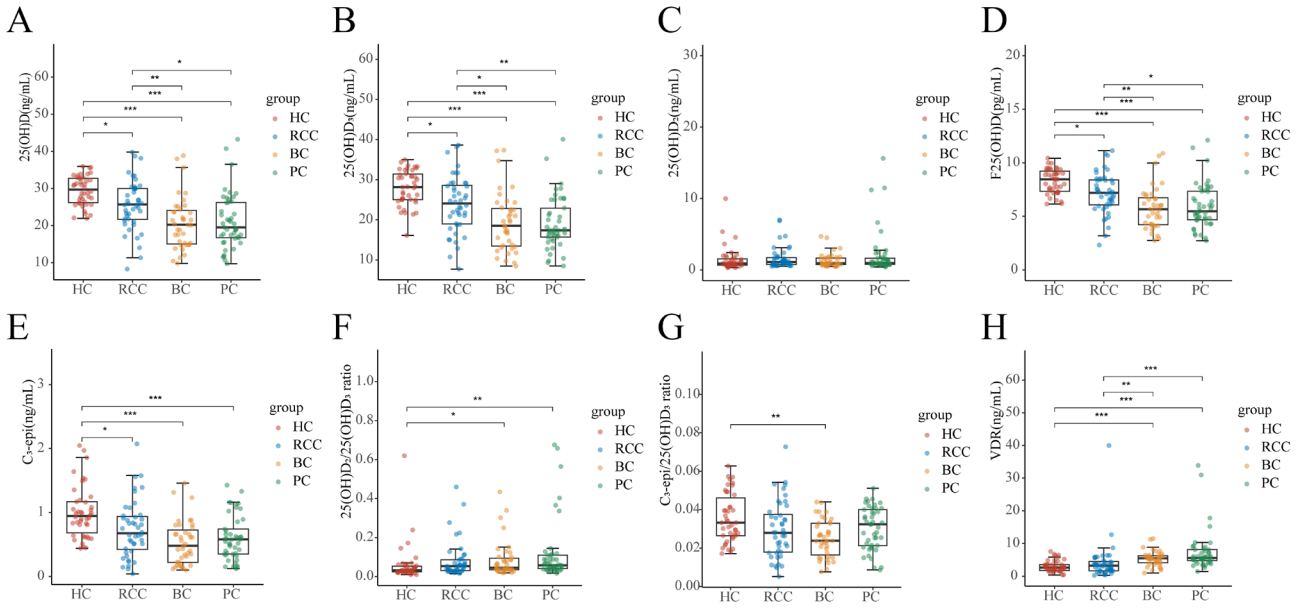

Figure 1. The levels of serum VitD metabolites and VDR in four groups of subjects. Levels of A) 25(OH)D, B) 25(OH)D_3, C) 25(OH)D_2, D) F25(OH)D, E) C_3-epi, F) 25(OH)D_2/25(OH)D_3 ratio, G) C_3-epi/25(OH)D_3 ratio, and H) VDR for four groups. 25(OH)D = 25(OH)D_2 + 25(OH)D_3; Abbreviations: HC-healthy control; BC-bladder cancer; PC-prostate cancer; RCC-renal cell carcinoma; *p<0.05, **p<0.01, ***p<0.001

similar trend, being markedly decreased in all cancer groups compared to HC (p<0.05). The C_3-epi was also significantly lower in the cancer groups, with the most pronounced reduction in the BC and PC groups (p<0.001). Furthermore, the ratio of 25(OH)D_2 to 25(OH)D_3 was significantly increased in the BC and PC groups compared to HC (p<0.05), suggesting altered VitD metabolism in cancer patients. Notably, VDR expression was significantly elevated in the BC and PC groups compared to both HC and RCC (p<0.01), indicating a potential upregulation of VDR in these cancers (Figure 1).

Assessment of VitD nutritional status in participants. According to the global standards for assessing VitD nutritional status recommended by the Institute of Medicine, serum 25(OH)D levels of <20 ng/ml are considered deficient, 20–30 ng/ml as insufficient, and ≥30 ng/ml as sufficient [10]. Moreover, the literature reports that the biological activity of 25 (OH) D_3 is 2–3 times that of 25(OH) D_2, and C_3-epi is a diastereomer of 25(OH)D_3 with little or no biological activity. Therefore, in order to more accurately evaluate the VitD of subjects, we used three different evaluation methods to accurately assess the nutritional status of VitD [9]. Method 1 = 25(OH)D_2 + 25(OH)D_3 + C_3-epi, that is the total 25(OH)D value obtained by our various immunological methods. Method 2 = 25(OH)D_2/3 + 25(OH)D_3 + C_3-epi, this is converting 25(OH)D_2 to 25(OH)D_3 activity, which is the actual 25(OH)D in the body. Method 3 = 25(OH)D_2/3+25(OH)D_3, that is after removing the possible diastereomer (C_3-epi) of 25(OH)D_3. Methods 2 and 3 yielded slightly lower VitD values compared to method 1 across all groups, but the overall trend remained consistent. In terms

of VitD nutritional status, the HC group had the highest percentage of subjects with sufficient VitD levels across all methods, with no subjects classified as deficient using method 1. In contrast, the cancer groups, especially BC and PC, exhibited a higher proportion of subjects with VitD deficiency and insufficiency.

In the PC and BC groups, nearly half of the subjects were classified as deficient across all methods, with method 1 identifying 46.2% and 48.9% of BC and PC subjects, respectively, as deficient. The differences in VitD status between the HC and cancer groups were significant, indicating that cancer patients tend to have poorer VitD storage and nutritional status (Table 3 and Table 4). From this, it can be seen that when we evaluate the nutritional status of subjects based on the different activities of VitD, regardless of whether they belong to the disease group or the healthy group, their deficiency rate, insufficiency rate, and adequacy rate will change. This indicates that detecting a total 25(OH)D in the body solely through immunological methods often overestimates VitD content. Therefore, it is particularly necessary to use UPLC-MS/MS to simultaneously detect different VitD metabolites.

ROC analysis. ROC analysis was also performed on subjects in the HC group, RCC group, BC group, and PC group, and the results were as follows (Figure 2). The 25(OH)D_3 distinguished the RCC group from the HC group in male patients (Figure 2A) with an AUC of 0.689 and a confidence interval of (0.576, 0.803). In male patients, the AUC of 25(OH)D_3 was 0.854 and the confidence interval was (0.761, 0.948) between the HC and BC groups (Figure 2B), and the AUC of 25(OH)D_3 was 0.866 between the HC and PC groups (Figure 2C). Confidence intervals of (0.786, 0.946) and 25(OH)D_3 distinguished the HC group from common cancers of the urinary system (including RCC, BC, and PC) in male patients (Figure 2D). The AUC of 0.802 and confidence intervals of (0.735, 0.868) showed good diagnostic performance.

Correlation analysis. The correlation analysis results are shown in the following Figure 3 and Figure 4. The correlation analysis between different VitD metabolites revealed a positive correlation between serum F25(OH)D and 25(OH)D_3 in the HC group, RCC group, BC group, and PC group, and a positive correlation between serum C_3-epi and 25(OH)D_3 and F25(OH)D in each group. We conducted a correlation analysis between the biochemical indicators detected in this experiment and the different VitD metabolites measured and found that 25(OH)D_3 and F25(OH)D were significantly negatively correlated with NGAL and CysC, while eGFR was positively correlated with them. These results suggest that a decrease in VitD metabolite levels in the subjects may be associated with renal dysfunction.

Discussion

NGAL, also known as lipid carrier protein 2, is a glycoprotein found in a variety of cells and tissues, including the kidneys, liver, prostate, and heart. In healthy populations, NGAL predominantly exists in the form of a 46 kDa dimer, which can prevent infection and participate in the regulation of oxidative stress in the body. When the renal tubule is damaged, it will increase rapidly. Moreover, studies have shown that the stimulation of hypoxia and inflammatory cytokines in cancer patients can cause overexpression of NGAL. It has been shown to play a crucial role in the survival,

Table 3. Three different evaluation methods for evaluating subjects' VitD storage (ng/ml).

	Method 1 (ng/ml)	Method 2 (ng/ml)	Method 3 (ng/ml)
HC (n = 42)	31.12 (26.87–33.66)	29.60 (26.21–33.35)	28.42 (25.22–31.97)
RCC (n=44)	**26.33 (21.77–31.31)***	**24.90 (20.39–30.04)***	**24.40 (19.83–29.31)***
BC (n=39)	**20.71 (15.03–24.74)*^**	**19.66 (13.74–23.88)*^**	**19.05 (13.64–23.27)*^**
PC (n=45)	**20.15 (16.95–27.47)*^**	**18.70 (16.11–25.12)*^**	**18.07 (15.89–24.38)*^**
H	47.947	48.538	48.359
p-value	**<0.001**	**<0.001**	**<0.001**

Note: Method 1 = 25(OH)D_2 + 25(OH)D_3 + C_3-epi; Method 2 = 25(OH)D_2/3 + 25(OH)D_3 + C_3-epi; Method 3 = 25(OH)D_2/3 + 25(OH)D_3; Abbreviations: HC-healthy control; BC-bladder cancer; RCC-renal cell carcinoma; PC-prostate cancer; *p-value compared to HC; ^p-value compared to RCC

Table 4. Assessment of VitD nutritional status in the four groups [n (%)].

Subjects	Method 1			Method 2			Method 3		
	Deficiency	Insufficiency	Sufficiency	Deficiency	Insufficiency	Sufficiency	Deficiency	Insufficiency	Sufficiency
HC (n=42)	0 (0)	20 (47.6)	22 (52.4)	1 (2.4)	20 (47.6)	21 (50.0)	1 (2.4)	23 (54.8)	18 (42.9)
RCC (n=44)	**7 (15.9)**	**22 (50.0)**	15 (34.1)	**10 (22.7)**	**22 (50.0)**	12 (27.3)	**11 (25.0)**	**26 (59.1)**	7 (15.9)
BC (n=39)	**18 (46.2)**	**18 (46.2)**	3 (7.7)	**20 (51.3)**	**16 (41.0)**	3 (7.7)	**20 (51.3)**	**16 (41.0)**	3 (7.7)
PC (n=45)	**22 (48.9)**	**19 (42.2)**	4 (8.9)	**26 (57.8)**	**15 (33.3)**	4 (8.9)	**26 (57.8)**	**16 (35.6)**	3 (6.7)

Note: Method 1 = 25(OH)D_2 + 25(OH)D_3 + C_3-epi; Method 2 = 25(OH)D_2/3 + 25(OH)D_3 + C_3-epi; Method 3 = 25(OH)D_2/3 + 25(OH)D_3; Abbreviations: HC-healthy control; BC-bladder cancer; RCC-renal cell carcinoma; PC-prostate cancer

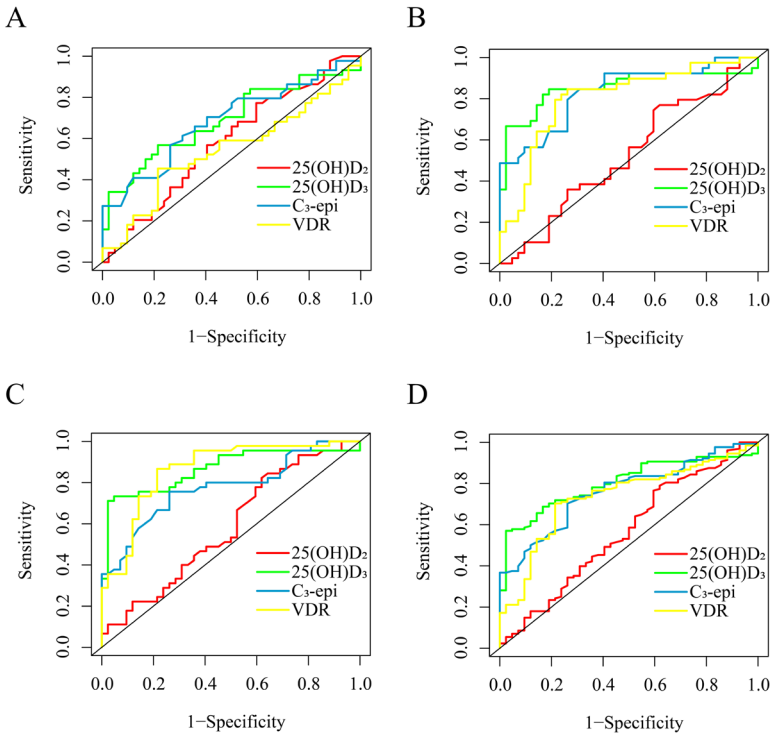

Figure 2. ROC analysis of VitD metabolites and VDR. A) shows the ROC curves of the RCC and HC groups; B) shows the ROC curves of the BC and HC groups; C) shows the ROC curves of the PC group and HC group; D) shows the ROC curves of RCC group, BC group, PC group, and HC group; Abbreviations: HC-healthy control; RCC-renal cell carcinoma; BC-bladder cancer; PC-prostate cancer; 25(OH)D_2 (ng/ml); 25(OH)D_3 (ng/ml); C_3-epi (ng/ml); VDR (ng/ml)

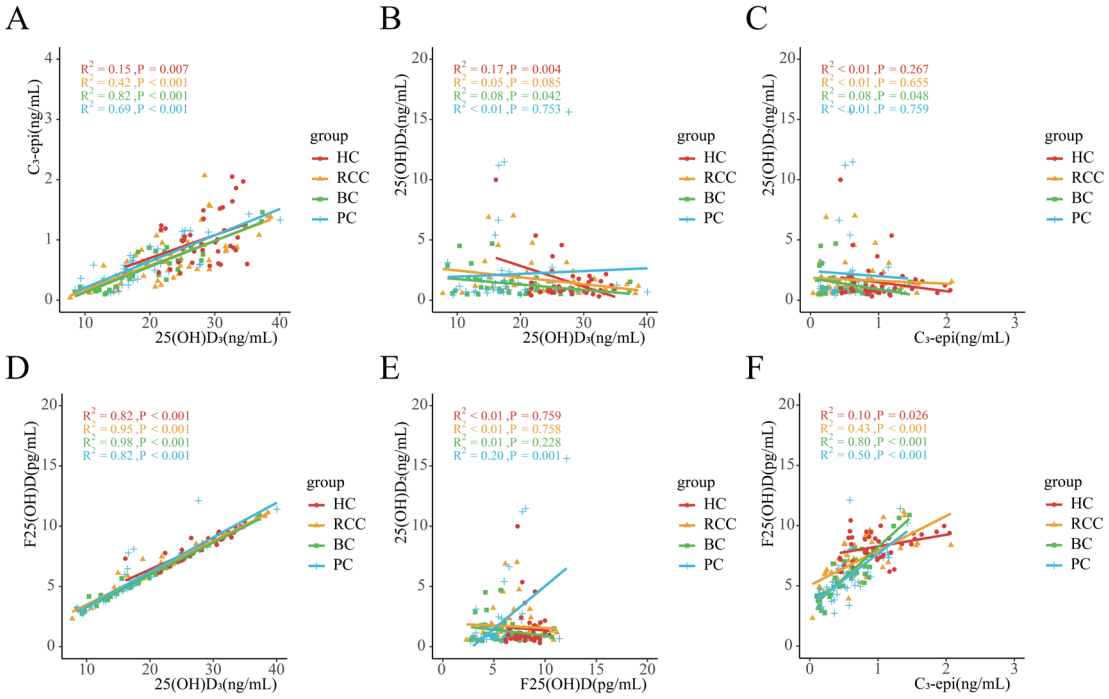

Figure 3. Correlation analysis between different VitD metabolites in four groups of subjects. A) Correlation between 25(OH)D_3 and C_3-epi; B) Correlation between 25(OH)D_2 and 25(OH)D_3; C) Correlation between 25(OH)D_2 and C_3-epi; D) Correlation between F25(OH)D and 25(OH)D_3; E) Correlation between 25(OH)D_2 and F25(OH)D; F) Correlation between F25(OH)D and C_3-epi; "R^2": Goodness of fit. Abbreviations: HC-healthy control; RCC-renal cell carcinoma; BC-bladder cancer; PC-prostate cancer

proliferation, and metastasis of tumor cells [11–13], and it may also be an important reason for the significant increase in serum NGAL levels in patients with urological cancer, including RCC, BC, and PC, as found in our study. Wang's research suggests that knocking down NGAL can lead to G1 cell cycle arrest, damaging cell proliferation, and clone growth. They believe that NGAL is a key regulatory factor for RCC cell proliferation [14]. Cancer cells require large amounts of iron to maintain an enhanced metabolic cycle and can increase cell proliferation, and studies by Rehwald et al. have shown that iron-containing NGAL has a tumor-promoting effect in RCC cell lines [15]. Similarly, Meier et al. also pointed out that iron-loaded NGAL enhances the survival of RCC cells by promoting the formation of reactive oxygen species and the activation of comprehensive stress response pathways, and subsequently increases the expression of SLC7A11, thereby reducing cancer cell death [16]. For PC patients, NGAL can induce stromal transformation of tumor cells through the ERK/SLUG axis, promoting the migration and invasion of prostate cancer cells [17]. This study found that NGAL showed an upward trend in three common types of urinary system cancer patients, and the increase was more significant. In this study, the levels of NGAL were significantly elevated in patients with common urological cancers compared to the healthy population. Concurrently, the levels of VitD metabolites, including 25(OH)D, 25(OH)D_3, F25(OH)D, and C_3-epi, were decreased, exhibiting a negative correlation. We posit that the significant increase in NGAL levels in patients with urological cancers suggests the presence of renal tubular damage. Since 25(OH)D_3 is primarily absorbed in the proximal renal tubules, this leads to a decline in the levels of VitD metabolites. Although NGAL is overexpressed in cancer and has been proposed as a biomarker for malignant tumors, studies have shown that most of the NGAL excreted by any cancer patient will be reabsorbed by the effective endocytosis mechanism in the proximal tubules after being freely filtered by the glomerulus in the kidneys. A study based on 20 RCC patients suggests that it cannot yet be used as an effective biomarker for predicting renal cancer [18]. Therefore, changes in kidney function are not good indicators of cancer.

The deficiency and insufficiency of VitD is a growing problem in our nutrition and health. At present, serum VitD detection methods in clinical laboratories are mainly divided into two categories. One is immunoassay including radioimmunoassay, enzyme-linked immunosorbent assay, chemiluminescence immunoassay, and so on [19]. However, the results obtained by immunoassay are susceptible to many factors, including temperature, time, accuracy, consistency of sample addition, etc. In addition, only the total amount of VitD can be detected, and 25(OH)D_2 and 25(OH)D_3 cannot be separated. As a result, the true value of VitD in patients is often lower than the result obtained by detection. In this study, the UPLC-MS/MS was used to simultaneously detect 25(OH)D_2 and 25(OH)D_3 and separate C_3-epi,

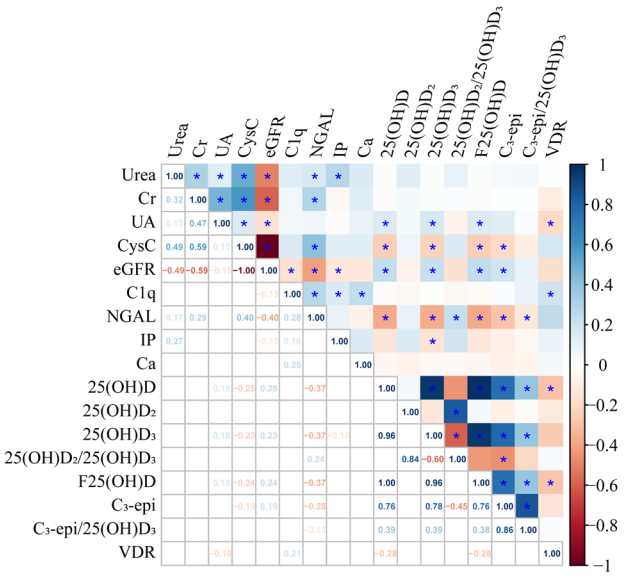

Figure 4. Correlation analysis of VitD metabolites and VDR with renal function indicators, Ca and IP. Notes: orange represented positive correlation and blue represented negative correlation; the Spearman correlation coefficient is shown in the figure; *p<0.05

which has high sensitivity, specificity, and accuracy, and is internationally recognized as the gold standard for the detection of 25(OH)D in serum. The detection of C_3-epi enables more accurate results and a more objective assessment of the patient's VitD status. The kidney is the main organ that affects VitD status and plays an important role in maintaining human health. Experiments and epidemiological studies at home and abroad have proved the importance of VitD for cancer prevention and the correlation between the lack of VitD and a variety of human cancers, so many anti-cancer properties of VitD have been proposed [6]. 25(OH)D_3 plays a crucial role in immune modulation and in shaping the commensal microbiota. It can inhibit the migration and invasion of cancer cells through various signaling pathways [20, 21]. In the tumor microenvironment, the secretion of cytokines and prostaglandins is crucial for the proliferation of cancer cells. VitD mitigates their secretion by downregulating nuclear factor kappa-B (NFκB) and cyclooxygenase-2 (COX-2), thereby inactivating their immune-modulatory functions. Additionally, VitD can also modulate the proportion of lymphocytes. Therefore, when using VitD for therapeutic purposes, its potential adverse effects should also be considered [4]. According to research reports, deficiency and insufficiency of VitD are associated with increased oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage, as well as the induction of systemic inflammatory responses. Such deficiencies and insufficiencies can promote the occurrence and progression of cancer [22–25]. In the bloodstream, 25(OH)D is present in both free and bound forms. The majority is tightly bound to VitD Binding Protein

(DBP), while a smaller fraction is loosely and non-specifically associated with albumin (ALB). F25(OH)D constitutes a very minor portion. According to the "free hormone hypothesis," only the free and non-specifically bound portions of the hormone can interact with target cells to exert their biological activity [26, 27]. In cancer, there may be differences in the mechanisms of action between 25(OH)D_3 and F25(OH)D, but there are currently few reports on this subject. Our research indicates a positive correlation between 25(OH)D_3 and F25(OH)D, which provides a certain reference for subsequent investigations into the potential mechanisms of F25(OH)D in cancer. Accumulated data indicates that the metabolism and function of VitD are dysregulated in many types of cancer. Therefore, understanding VitD metabolism and dysfunction in cancer is crucial for developing new options for early cancer diagnosis and treatment based on VitD metabolites [28]. The case-control study conducted by Li et al. on the Chinese Han population showed that higher circulating concentrations of 25(OH)D had a protective effect on RCC [29]. Similarly, results from a meta-analysis showed that higher levels of circulating VitD and higher intake of dietary VitD were protective in patients with RCC [30]. Our study found that serum VitD levels in common urogenital cancer patients (including RCC, BC, and PC) were significantly lower than those in the healthy group in both BC and PC patients, which is consistent with previous research results. We analyze that the possible reasons for the decrease in serum VitD in common patients with the urogenital system are: cancer patients may go out less and have insufficient exposure to sunlight; The adjustment of dietary structure leads to insufficient intake and so on.

As mentioned earlier, the activity of 25(OH)D_3 is 2–3 times that of 25(OH)D_2, and when our body experiences a certain factor that causes a significant decrease in 25(OH)D_3 and an increase in 25(OH)D_2. 25(OH)D can appear to have a total that remains unchanged but due to the difference in activity and the presence of isomers, the detection results may be different. In this experiment, we can see that when we consider these issues and convert them, the VitD content of both the disease group and the health group has decreased. If we only detect 25(OH)D, we cannot accurately reflect the body's VitD metabolite situation. We found that, compared with the healthy group, the disease group showed an upward trend in 25(OH)D_2 when both 25(OH)D and 25(OH)D_3 decreased, and the ratio of 25(OH)D_2 to 25(OH)D_3 was higher in all three groups than the healthy control group, indicating that the biological activity of VitD in patients with urogenital system cancer is decreased. In addition, our study found that the serum calcium levels of the three groups of patients were not significantly different from those of the HC group, but their VitD metabolite levels were significantly lower than those of the HC group, and there was no significant correlation between them. Therefore, although it is well known that VitD can regulate calcium and phosphorus metabolism, it does not mean that serum calcium level can reflect the normal VitD content of the body. Measuring serum calcium alone cannot reflect whether the body is deficient in calcium.

The formation of C_3-epi is due to the change of the spatial configuration of the -OH group at the 3rd carbon atom of 25(OH)D_3 from alpha to beta, and studies have shown that the biological activity of C_3-epi is much lower than that of its natural form, with no or only a small amount of biological activity. This means that if the same amount of 25(OH)D_3, the higher the proportion of C_3-epi, the less effective VitD will be [31]. Since the initial detection of C_3-epi in infants was reported [32], people's understanding of C_3-epi generally remains at the level of its impact on the assessment of the body's VitD storage levels, as described above [19, 31, 33]. Beyond this, the additional pathological significance of C_3-epi and its mechanism of action remain controversial and require further research for exploration. A cross-sectional study based on multiple diseases has suggested that C_3-epi could potentially serve as a biomarker for diseases [34]. However, further exploration is still required. In our study, 7.1%, 11.4%, and 2.2% of the subjects in the HC group, RCC group, and PC group, respectively, went from having sufficient VitD to having insufficient or deficient VitD simply because the C_3-epi was not included in the estimation method. This means that when the concentration of the isomers was excluded (i.e., no quantitative detection was performed), some subjects would cross the critical value for deficiency and appear to have sufficient VitD. At the same time, we also calculated the ratio of C_3-epi to 25(OH)D_3 in each group, and we found that the ratio of C_3-epi/25(OH)D_3 for each subject was lower in comparison with the HC group, and we hypothesize that this may be because the 25(OH)D_3 content in the cancer group was already significantly lower, so the conversion to C_3-epi resulted in a decrease in C_3-epi content, leading to a lower ratio.

VitD enters the body and works by binding to the VDR receptor. In a study by Song et al., it was found that the serum IL-6 level of renal cancer patients was higher than that of their control group, while the VDR level was related to the expression of the IL-6 gene. VDR activation can regulate cellular inflammation [35]. Xu et al. pointed out that NF-κB was activated in cancer tissue in RCC patients, and VDR may participate in regulating NF-kB activation, which has an important role in the expression of adhesion molecules and other pro-inflammatory genes [36]. Therefore, in this experiment, we found that the serum VDR level in patients with common urogenital system cancers, including (RCC, BC, and PC), was higher than that in the HC group, which may be related to inflammation in the disease group subjects. Currently, most studies on the serum VDR of subjects have used polymerase chain reaction technology for gene detection, and fewer have been detected by ELISA, so more experiments are needed for verification in the future.

Currently, research on body VitD is mostly conducted using immunological methods for detection without simul-

taneously separating 25(OH)D_2, 25(OH)D_3, and C_3-epi. Our study used UPLC-MS/MS technology to detect the serum metabolites of VitD in the HC, RCC, BC, and PC groups and found that compared to the healthy group, the VitD content in the three cancer patient groups was lower. However, we cannot accurately assess the true level of VitD in patients by only detecting the content of 25(OH)D. We should also consider the different active forms of VitD metabolites and the effects of isomers. However, there are still many factors that were not taken into account in this study, such as the fact that our study was conducted at a single center with a small sample size, we did not record the dietary VitD intake of the subjects, or the season, the duration of sunlight exposure in their living areas, or the use of sunscreen products, etc. Future studies are needed to investigate the changes in VitD levels in cancer patients and the mechanisms that cause these changes.

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