Total, Bioavailable And Free 25-Hydroxyvitamin D Are Associated With The Prognosis Of Patients With Non-Small Cell Lung Cancer

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Abstract

Purpose: To analyze the prognostic value of total, bioavailable and free 25-hydroxyvitamin D (25(OH)D) as well as vitamin D-binding protein (VDBP) in NSCLC patients.

Methods: We prospectively collected and analyzed data for 395 patients diagnosed with NSCLC between January 2016 and December 2018 in two university-affiliated hospitals. Total and free 25(OH)D and VDBP were measured directly, and bioavailable 25(OH)D was calculated using a validated formula. Their prognostic values were evaluated by Cox proportional hazards model, and hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated.

Results: Patients with NSCLC had significantly lower levels of total, bioavailable, and free 25(OH)D and higher VDBP levels in comparison to healthy controls (all $P < 0.001$). In multivariate analyses, higher levels of total, bioavailable, and free 25(OH)D were independently associated better overall survival (OS) and progression-free survival (PFS). For OS, the adjusted HRs were 0.58 (95%CI, 0.40-0.87; P for trend=0.008), 0.45 (95%CI, 0.30-0.67; P for trend<0.001) and 0.49 (95%CI, 0.33-0.73; P for trend<0.001) for the highest versus lowest tertile of total, bioavailable and free 25(OH)D, respectively. The corresponding adjusted HRs for PFS were 0.61 (95%CI, 0.43-0.86; P for trend=0.006), 0.56 (95%CI, 0.40-0.80; P for trend=0.001) and 0.60 (95%CI, 0.42-0.85; P for trend=0.004), respectively. However, VDBP was not associated with either OS or PFS.

Conclusion: The current study suggested that total, bioavailable and free 25(OH)D may be reliable prognosis indicators in NSCLC patients, though the optimal 25(OH)D form for NSCLC prognosis remains to be assessed in future studies.

Introduction

Lung cancer is the second most common cancer with the highest mortality rate worldwide [1]. Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer [2]. Although a wide range of treatments have been clinically available [3], the prognosis remains poor, with a 5-year survival rate of 21% in the United States [4]. It is of great clinical significance to explore valuable and adequate prognostic indicators to further guide individualized disease management in NSCLC.

Vitamin D, traditionally known as an essential nutrient, is a precursor of a potent steroid hormone that regulates a broad spectrum of physiological processes [5]. Increasing evidence indicates a significant anti-tumor effect of vitamin D through inhibiting cell proliferation, angiogenesis, and metastasis, while inducing apoptosis and differentiation [6]. However, vitamin D deficiency is evident globally and even more severe in cancer patients, including patients with lung cancer [7, 8]. Low levels of circulating vitamin D concentrations have been associated with poorer survival in NSCLC, but the findings are inconsistent [9-13].

As the major vitamin D circulating form in the human organism, total serum or plasma 25-hydroxyvitamin D (25(OH)D) is currently routinely used in clinical practice to assess vitamin D status [14]. About 85% to 90% of total 25(OH)D circulates bound to vitamin D-binding protein (VDBP), and this proportion is recognized as the biologically inactive form. The non-VDBP-bound fraction, consisting primarily of albumin-bound 25(OH)D (10 to 15%) and less than 0.03% of total 25(OH)D in the free form, is considered as bioavailable 25(OH)D. The free and albumin (with a 1000-fold weaker affinity for 25(OH)D than VDBP) forms are easier to be taken up by cell tissues, thereby exerting its biological actions [15]. Recent studies have reported that bioavailable or free 25(OH)D may be more representative indicators for vitamin D status as compared with total 25(OH)D [16, 17]. Current studies on vitamin D and lung cancer have been focused on total 25(OH)D and, to our knowledge, the relationships between circulating bioavailable and free 25(OH)D levels and prognosis of NSCLC are yet to be assessed.

In addition, VDBP may influence cancer progression in addition to being a carrier for vitamin D metabolites [18]. Therefore, we aimed to investigate the associations of total, bioavailable, and free 25(OH)D and VDBP with survival in NSCLC patients.

Methods

Study population

The Soochow Lung Cancer Cohort (SLCC) study is an ongoing patient cohort conducted in the first affiliated hospital and the second affiliated hospital of Soochow University. This study was designed to investigate the long-term prognosis factors among lung cancer patients in Southeast China. As described previously [19], a total of 525 primary lung cancer patients aged ≥ 18 years were enrolled in the cohort between January 2016 and December 2018. For the current study, we excluded patients with small-cell lung cancer ($n=62$). We further excluded patients lost to follow-up ($n=29$) and those with no sufficient data ($n=39$). Finally, the current study included 395 NSCLC patients. To compare the vitamin D indices with controls, 50 healthy volunteers (25 men and 25 women with a mean age of 60 years) were enrolled by Suzhou Industrial Park Centers for Disease Control and Prevention in October 2020.

Data Collection

An in-person interview was conducted by trained investigators using a structured questionnaire within one week after diagnosis to collect general information about age, gender, disease history [e.g., chronic obstructive pulmonary disease (COPD)], family history of cancer, body mass index (BMI), in addition to behavioral factors such as smoking status and alcohol consumption. Participants were asked to recall the frequency of alcohol consumption during the year prior to the baseline assessment. According to the obtained information, smoking status was categorized as never, former, or current smoker. Current smokers were defined as patients who had smoked continuously or accumulated for at least 6 months and continued to smoke during the survey period or those who gave up smoking for less than one year. Former smokers were defined as those who had quit smoking for more than one year at the study entry. Furthermore, medical records were also reviewed to extract their clinical data, including cancer

characteristics (TNM stage, histology, lesion and laterality), treatments (surgery, chemotherapy, radiotherapy and targeted therapy), and the levels of various blood analytes. Blood analytes included albumin, total cholesterol, neutrophils, lymphocytes, monocytes, carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE). Neutrophil-to-lymphocyte ratio (NLR) and lymphocyte-to-monocyte ratio (LMR) were calculated based on the collected data.

Determination of 25(OH)D Fractions and VDBP

A fasting plasma sample was collected from each patient at the time of recruitment. All samples were numbered and stored at -80°C until further analysis. The plasma circulating total 25(OH)D (D_2 and D_3) levels were measured with the use of liquid chromatography-mass spectroscopy (LC-MS/MS) at Suzhou Harmony Health Medical Laboratory Co.,Ltd. The mean intra-assay coefficient of variation (CV) was 1.7%. VDBP and free 25(OH)D were measured by commercial enzyme-linked immunosorbent (ELISA) assays (R&D Systems, Minneapolis, MN, USA and DAsource ImmunoAssays, Louvain-la-Neuve, Belgium, respectively). The mean CV values were 5.3% and 6.1% for VDBP and free 25(OH)D, respectively. Bioavailable 25(OH)D ([Bio D]) was calculated from measured free 25(OH)D, albumin, and affinity constant between 25(OH)D and albumin using the following formula [16]: $[\text{Bio D}] = [\text{D}_{\text{Free}}] + [\text{D}_{\text{Alb}}] = (\text{K}_{\text{alb}} \cdot [\text{Alb}] + 1) \cdot [\text{D}_{\text{Free}}]$, where $[\text{D}_{\text{Free}}]$ is the concentration of free 25(OH)D, $[\text{D}_{\text{Alb}}]$ is the concentration of albumin-bound 25(OH)D, $[\text{Alb}]$ is the concentration of serum albumin, and K_{alb} is the affinity constant between 25(OH)D and albumin ($6 \times 10^5 \text{ M}^{-1}$).

Follow-up

Follow up of patient outcomes began at the date of enrollment until the last follow-up date (December 2020) or until the date of death. The survival outcomes were evaluated semiannually. Information sources included the hospital inpatient or outpatient records, patient or family telephonic contact, in addition to local death registration system. Overall survival (OS) was defined as the time from the date of enrollment to the date of death, and progression-free survival (PFS) was defined as the time from the date of enrollment to the date of confirmed progressive disease (PD) or death.

Statistical Analysis

The 25(OH)D fractions (total, bioavailable, and free) and VDBP levels were categorized into three groups by the tertiles of the variables, and the Spearman correlation test was used to test their pairwise associations. One-way analysis of variance (ANOVA) with Tukey's Honestly Significant Difference (HSD) pairwise comparisons were used to evaluate the differences of the 25(OH)D fractions and VDBP levels among normal group, NSCLC survivors, and NSCLC non-survivors. The receiver operating characteristic (ROC) curve was used to determine the optimal cutoff value of the NLR and LMR. The chi-square test or Fisher's exact test was applied to explore differences in categorical variables. The Kaplan–Meier method with log-rank test was used to construct survival curves. Univariate and multivariate Cox proportional hazards regression models were used to identify variables associated with OS and PFS. Hazard ratios (HR) and 95% confidence intervals (CI) were then calculated for OS and PFS. Baseline variables that were considered clinically relevant or that showed a univariate relationship with outcome were entered into

multivariate analyses. P values for the linear trends were calculated by entering the median values of 25(OH)D or VDBP tertiles into the models. Further stratified multivariate analyses were performed according to age, gender, smoking, TNM stage, histology, lesion and surgery, while we also recognized that these analyses are subject to limited statistical power. Interactions were evaluated using the Wald test. Time-dependent ROC curves for the prognostic values of 25(OH)D fractions and VDBP were estimated using the R package survivalROC. All analyses were performed by IBM SPSS 25.0 and R software version 3.6.3. Two-sided P-value <0.05 was considered statistically significant.

Results

Baseline Clinical Characteristics

Of the 395 NSCLC patients included in the study, the mean age was 63.0 ± 10.5 years, and 63.8% of the participants were men, 49.4% were former/current smoker and 65.1% were classified as stage IIIB-IV (Table 1). The association between baseline clinical characteristics and total 25(OH)D were summarized in Table 1. Patients with higher total 25(OH)D levels had higher levels of albumin and LMR, and were more likely to collect blood in spring and autumn and to receive surgery or chemotherapy. Meanwhile, patients with higher bioavailable 25(OH)D levels had higher levels of BMI, albumin and LMR. Additionally, both bioavailable and free 25(OH)D were associated with season of blood-drawing (Supplementary Table 1).

Table 1

The relationship between total 25(OH)D and clinical characteristics in NSCLC patients [N (%)]

Variable	Category	Total 25(OH)D			P value
		Tertile 1 (N=132)	Tertile 2 (N=132)	Tertile 3 (N=131)	
Age(years)	<65	62(47.0)	72(54.5)	71(54.2)	0.380
	≥65	70(53.0)	60(45.5)	60(45.8)	
Gender	Male	84(63.6)	88(66.7)	80(61.1)	0.639
	Female	48(36.4)	44(33.3)	51(38.9)	
Smoking	Never	67(50.8)	65(49.2)	68(51.9)	0.910
	Former/current	65(49.2)	67(50.8)	63(48.1)	
Drinking	Yes	24(18.2)	31(23.5)	29(22.1)	0.550
	No	108(81.8)	101(76.5)	102(77.9)	
COPD	Yes	7(5.3)	4(3.0)	6(4.6)	0.649
	No	125(94.7)	128(97.0)	125(95.4)	
Family history of cancer	Yes	16(12.1)	23(17.4)	23(17.6)	0.384
	No	116(87.9)	109(82.6)	108(82.4)	
BMI(kg/m ²)	<18.5	12(9.1)	11(8.3)	11(8.4)	0.154
	18.5-24	94(71.2)	87(65.9)	76(58.0)	
	≥24	26(19.7)	34(25.8)	44(33.6)	
Season of blood-drawing	spring	74(56.1)	56(42.4)	51(38.9)	0.030
	Summer	16(12.1)	24(18.2)	20(15.3)	
	Autumn	23(17.4)	35(26.5)	45(34.4)	
	Winter	19(14.4)	17(12.9)	15(11.5)	
TNM stage	I-III A	41(31.1)	43(32.6)	54(41.2)	0.176
	IIIB-IV	91(68.9)	89(67.4)	77(58.8)	
Histology	AC	87(65.9)	94(71.2)	99(75.6)	0.309
	SCC	41(31.1)	31(23.5)	27(20.6)	
25(OH)D 25-hydroxyvitamin D, AC adenocarcinoma, BMI body Mass Index, CEA carcinoembryonic antigen, COPD chronic obstructive pulmonary emphysema, LMR lymphocyte-to-monocyte ratio, NLR neutrophil-to-lymphocyte ratio, NSE neuron-specific enolase, SCC squamous cell carcinoma					

Variable	Category	Total 25(OH)D			P value
		Tertile 1 (N=132)	Tertile 2 (N=132)	Tertile 3 (N=131)	
	Others	4(3.0)	7(5.3)	5(3.8)	
Lesion	Central	51(38.6)	46(34.8)	40(30.5)	0.385
	Peripheral	81(61.4)	86(65.2)	91(69.5)	
Laterality	Left	63(47.7)	62(47.0)	46(35.1)	0.069
	Right	69(52.3)	70(53.0)	85(64.9)	
Surgery	Yes	53(40.2)	71(53.8)	75(57.3)	0.012
	No	79(59.8)	61(46.2)	56(42.7)	
Chemotherapy	Yes	100(75.8)	119(90.2)	110(84.0)	0.007
	No	32(24.2)	13(9.8)	21(16.0)	
Radiotherapy	Yes	27(20.5)	20(15.2)	21(16.0)	0.473
	No	105(79.5)	112(84.8)	110(84.0)	
Targeted therapy	Yes	45(34.1)	34(25.8)	32(24.4)	0.167
	No	87(65.9)	98(74.2)	99(75.6)	
CEA (ng/mL)	<6.5	77(58.3)	86(65.2)	81(61.8)	0.522
	≥6.5	55(41.7)	46(34.8)	50(38.2)	
NSE (ng/mL)	<17	98(74.2)	104(78.8)	110(84.0)	0.153
	≥17	34(25.8)	28(21.2)	21(16.0)	
Albumin (g/L)	<40	58(43.9)	40(30.3)	38(29.0)	0.018
	≥40	74(56.1)	92(69.7)	93(71.0)	
Total cholesterol (mg/dL)	≤180	76(57.6)	63(47.7)	66(50.4)	0.253
	>180	56(42.4)	69(52.3)	65(49.6)	
NLR	≤2.63	55(41.7)	67(50.8)	66(50.4)	0.247
	>2.63	77(58.3)	65(49.2)	65(49.6)	
LMR	≤3.17	84(63.6)	60(45.5)	65(49.6)	0.008

25(OH)D 25-hydroxyvitamin D, AC adenocarcinoma, BMI body Mass Index, CEA carcinoembryonic antigen, COPD chronic obstructive pulmonary emphysema, LMR lymphocyte-to-monocyte ratio, NLR neutrophil-to-lymphocyte ratio, NSE neuron-specific enolase, SCC squamous cell carcinoma

Variable	Category	Total 25(OH)D			P value
		Tertile 1 (N=132)	Tertile 2 (N=132)	Tertile 3 (N=131)	
	>3.17	48(36.4)	72(54.5)	66(50.4)	
<i>25(OH)D</i> 25-hydroxyvitamin D, <i>AC</i> adenocarcinoma, <i>BMI</i> body Mass Index, <i>CEA</i> carcinoembryonic antigen, <i>COPD</i> chronic obstructive pulmonary emphysema, <i>LMR</i> lymphocyte-to-monocyte ratio, <i>NLR</i> neutrophil-to-lymphocyte ratio, <i>NSE</i> neuron-specific enolase, <i>SCC</i> squamous cell carcinoma					

Plasma levels of 25(OH)D Fractions and VDBP in NSCLC Patients

Plasma levels of total, bioavailable, and free 25(OH)D and VDBP in control group and NSCLC patient group were presented in Fig. 1. Mean total 25(OH)D concentration was 20.78 ± 7.81 ng/mL for all NSCLC patients. According to the reference ranges of clinical practice guideline [20], 37% of the patients were considered to be 25(OH)D insufficient (20 to 30 ng/mL) and 50.1% were deficient (<20 ng/mL). Compared with healthy controls, NSCLC patients had significantly lower levels of total, bioavailable and free 25(OH)D (25.56 ± 8.75 vs. 20.78 ± 7.81 ng/mL, 5.82 ± 1.06 vs. 2.87 ± 1.35 ng/mL, and 16.13 ± 1.82 vs. 7.69 ± 3.41 pg/mL, respectively, all $P < 0.001$), while had higher VDBP levels (175.69 ± 35.24 vs. 204.93 ± 50.47 µg/mL, $P < 0.001$). Meanwhile, NSCLC survivors had significantly higher total, bioavailable and free 25(OH)D levels than non-survivors (all $P < 0.001$). However, no significant differences in VDBP levels was observed between survivors and non-survivors. We then examined the relationships among vitamin D fractions. As shown in Table 2, total 25(OH)D had a moderate correlation with bioavailable 25(OH)D ($r_s = 0.60$, $P < 0.001$) and free 25(OH)D ($r_s = 0.58$, $P < 0.001$), while a weak correlation with VDBP ($r_s = 0.24$, $P < 0.001$). Notably, there was a strong correlation between bioavailable and free 25(OH)D ($r_s = 0.96$, $P < 0.001$). However, we did not find any association between VDBP and bioavailable or free 25(OH)D.

Table 2
Spearman correlation coefficients between plasma vitamin D-related markers in NSCLC patients

	Total 25(OH)D	Bioavailable 25(OH)D	Free 25(OH)D	VDBP
Total 25(OH)D	1	0.60 ^a	0.58 ^a	0.24 ^a
Bioavailable 25(OH)D		1	0.96 ^a	0.02
Free 25(OH)D			1	-0.01
VDBP				1
<i>25(OH)D</i> 25-hydroxyvitamin D, <i>VDBP</i> vitamin D-binding protein				
^a $P < 0.001$				

Impacts of 25(OH)D Fractions and VDBP on Survival

During a median 32 months (range: 1 to 60 months) of follow-up period, 179 (45.3%) patient deaths occurred. The Kaplan–Meier analysis showed that NSCLC patients with higher total 25(OH)D levels had better OS ($P < 0.001$, log-rank test) and PFS ($P < 0.001$, log-rank test) (Fig. 2A-B). Similarly, higher bioavailable and free 25(OH)D levels were associated with significantly increased OS ($P < 0.001$ for bioavailable and $P = 0.002$ for free 25(OH)D, log-rank test) and PFS ($P < 0.001$ for bioavailable and $P = 0.001$ for free 25(OH)D, log-rank test) (Fig. 2C-F). However, no significant association were found between VDBP and survival ($P = 0.610$ for OS and $P = 0.470$ for PFS, log-rank test; Fig. 2G-H)

Then, we further explored the association between 25(OH)D fractions or VDBP and survival through Cox regression analyses. Univariate analyses suggested that gender, smoking, drinking, COPD, BMI, season of blood-drawing, TNM stage, histology, surgery, CEA, NSE, albumin, total cholesterol, NLR and LMR were significant prognostic factors for OS (all $P < 0.2$; Supplementary Table 2). And in addition to the factors mentioned above, radiotherapy and targeted therapy were also significant prognostic factors for PFS (all $P < 0.2$; Supplementary Table 2). Furthermore, age was considered as an important factor due to its clinical relevance.

All three 25(OH)D factions were identified as independent prognostic indicators for survival after adjustment for factors mentioned above. The multivariable-adjusted HRs in the highest versus lowest tertile of total 25(OH)D levels were 0.58 (95%CI, 0.40-0.87; P for trend=0.008) for OS and 0.61 (95%CI, 0.43-0.86; P for trend=0.006) for PFS. Meanwhile, the multivariable-adjusted HRs in the highest versus lowest tertile of bioavailable 25(OH)D levels were 0.45 (95%CI, 0.30-0.67; P for trend<0.001) for OS and 0.56 (95%CI, 0.40-0.80; P for trend=0.001) for PFS. And the multivariable-adjusted HRs in the highest versus lowest tertile of free 25(OH)D levels were 0.49 (95%CI, 0.33-0.73; P for trend<0.001) for OS and 0.60 (95%CI, 0.42-0.85; P for trend=0.004) for PFS. However, VDBP were not significantly associated with either OS or PFS (all P for trend>0.05; Table 3).

Table 3

Hazard ratios for survival by tertile of plasma total, bioavailable, and free 25(OH)D and VDBP in NSCLC patients

	Tertile 1	Tertile 2	Tertile 3	<i>P</i> for trend
OS				
Total 25(OH)D				
Cut points, ng/mL	≤16.41	16.42-23.94	>23.95	
Deaths/total cases	77/132	58/132	44/131	
Unadjusted HR (95% CI)	1	0.63(0.45-0.89)	0.48(0.33-0.70)	<0.001
Multivariable-adjusted HR (95% CI) ^a	1	0.83(0.57-1.21)	0.58(0.40-0.87)	0.008
Bioavailable 25(OH)D				
Cut points, ng/mL	≤2.21	2.22-3.40	>3.41	
Deaths/total cases	76/132	60/132	43/131	
Unadjusted HR (95% CI)	1	0.73(0.52-1.02)	0.43(0.30-0.63)	<0.001
Multivariable-adjusted HR (95% CI) ^a	1	0.63(0.43-0.92)	0.45(0.30-0.67)	<0.001
Free 25(OH)D				
Cut points, pg/mL	≤6.04	6.05-9.12	>9.13	
Deaths/total cases	73/132	61/132	45/131	
Unadjusted HR (95% CI)	1	0.78(0.56-1.10)	0.53(0.36-0.77)	0.001
Multivariable-adjusted HR (95% CI) ^a	1	0.68(0.47-1.00)	0.49(0.33-0.73)	<0.001
VDBP				
Cut points, µg/mL	≤181.49	181.50-222.71	>222.72	
Deaths/total cases	64/132	55/132	60/131	
Unadjusted HR (95% CI)	1	0.86(0.60-1.23)	1.00(0.70-1.42)	0.966
Multivariable-adjusted HR (95% CI) ^a	1	0.67(0.46-0.99)	0.74(0.51-1.08)	0.114

25(OH)D 25-hydroxyvitamin D, CI confidence intervals, HR hazard ratio, OS overall survival, PFS progression-free survival, VDBP vitamin D-binding protein

^aAdjusted for age, gender, smoking, drinking, COPD, BMI, season of blood-drawing, TNM stage, histology, surgery, CEA, NSE, albumin, total cholesterol, NLR and LMR

^bAdjusted additionally for radiotherapy and targeted therapy

	Tertile 1	Tertile 2	Tertile 3	<i>P</i> for trend
PFS				
Total 25(OH)D				
Cut points, ng/mL	≤16.41	16.42-23.94	>23.95	
Deaths/total cases	94/132	71/132	62/131	
Unadjusted HR (95% CI)	1	0.56(0.41-0.77)	0.48(0.35-0.67)	<0.001
Multivariable-adjusted HR (95% CI) ^b	1	0.69(0.49-0.98)	0.61(0.43-0.86)	0.006
Bioavailable 25(OH)D				
Cut points, ng/mL	≤2.21	2.22-3.40	>3.41	
Deaths/total cases	89/132	77/132	61/131	
Unadjusted HR (95% CI)	1	0.80(0.59-1.08)	0.53(0.38-0.73)	<0.001
Multivariable-adjusted HR (95% CI) ^b	1	0.79(0.56-1.10)	0.56(0.40-0.80)	0.001
Free 25(OH)D				
Cut points, pg/mL	≤6.04	6.05-9.12	>9.13	
Deaths/total cases	85/132	78/132	64/131	
Unadjusted HR (95% CI)	1	0.85(0.62-1.15)	0.64(0.46-0.88)	0.007
Multivariable-adjusted HR (95% CI) ^b	1	0.74(0.52-1.05)	0.60(0.42-0.85)	0.004
VDBP				
Cut points, µg/mL	≤181.49	181.50-222.71	>222.72	
Deaths/total cases	78/132	73/132	76/131	
Unadjusted HR (95% CI)	1	0.87(0.64-1.20)	0.96(0.70-1.32)	0.780
Multivariable-adjusted HR (95% CI) ^b	1	0.73(0.52-1.02)	0.84(0.60-1.17)	0.290
<i>25(OH)D</i> 25-hydroxyvitamin D, <i>CI</i> confidence intervals, <i>HR</i> hazard ratio, <i>OS</i> overall survival, <i>PFS</i> progression-free survival, <i>VDBP</i> vitamin D-binding protein				
^a Adjusted for age, gender, smoking, drinking, COPD, BMI, season of blood-drawing, TNM stage, histology, surgery, CEA, NSE, albumin, total cholesterol, NLR and LMR				
^b Adjusted additionally for radiotherapy and targeted therapy				

We found that the inverse association of total, bioavailable, and free 25(OH)D with OS or PFS rate remained in most predefined subgroups, despite that statistical power was diminished. And no statistical

interaction was observed between three 25(OH)D fractions and above clinical characteristics (All P for interaction >0.05 ; Supplementary Table 3-5)

Prognostic performance of 25(OH)D fractions and VDBP

As shown in Fig. 3, we compared the prognostic performance of the total, bioavailable, free 25(OH)D and VDBP. The time-dependent ROC curves showed that bioavailable and total 25(OH)D obtained similar AUCs in dynamic trends within the follow-up time. In addition, the time-dependent ROC curves of bioavailable and total 25(OH)D were slightly higher than that of free 25(OH)D, but the difference was not significant. However, the AUCs of VDBP was significantly lower than that of 25(OH)D fractions. The AUC of the total, bioavailable, free 25(OH)D and VDBP for predicting three-year OS were 0.636, 0.620, 0.594 and 0.499, respectively, while the corresponding AUCs for predicting three-year PFS were 0.642, 0.620, 0.595 and 0.503, respectively.

Discussion

In this study, we evaluated the potential importance of total, bioavailable, and free 25(OH)D as well as VDBP as pre-treatment prognostic indicators for OS and PFS among patients with NSCLC. We found that all three 25(OH)D fractions had discrimination for long-term survival in NSCLC patients, as high levels of total, bioavailable or free 25(OH)D were associated with better OS and PFS. But we did not find a superior prognostic performance in bioavailable or free 25(OH)D as compared to total 25(OH)D. Moreover, no significant association was found between VDBP and survival in NSCLC.

Vitamin D deficiency is frequently observed in cancer patients, including lung cancer. Herein, we found that 50.1% of the patients had vitamin D deficiency (25(OH)D level <20 ng/mL) and 37.0% had insufficiency (20-30 ng/mL), suggesting a severe vitamin D deficiency status in the current NSCLC cohort, which is consistent with previous studies [11, 21]. In addition, compared with healthy controls, total 25(OH)D was lower in NSCLC patients, among whom survivors had a higher level of total 25(OH)D than non-survivors, indicating a latent impact of vitamin D on the disease prognosis of NSCLC.

Various mechanism may underlie the influence of vitamin D on the progression and prognosis of lung cancer. Vitamin D can inhibit lung cancer tumor growth, migration and proliferation, and promote apoptosis by downregulating Histidine-rich calcium-binding protein (HRC) [22]. As an active metabolite of vitamin D, 1,25-Dihydroxyvitamin D3 (1,25(OH)2D3) induces E-cadherin and decreases EMT-related molecules *SNAIL*, *ZEB1*, and *vimentin* in NSCLC cells, thus preventing NSCLC progression [23]. Besides, vitamin D deficiency may increase inflammation and proliferation of pre-malignant cells thereby enhancing progression of squamous lesions [24]. By contrast, vitamin D treatment in the EGFR mutant NSCLC cells may lead to inhibition of clonogenic growth in a dose-dependent manner [25]. And vitamin D or vitamin D derivatives play a powerful synergic role when used in combination with chemotherapy drugs, promoting the cytotoxic effects of drugs and reducing drug resistance on NSCLC cells compared to using the free single drug [26, 27].

Given the close affinity of vitamin D and NSCLC, the prognostic impact of vitamin D levels on NSCLC has been investigated previously, but the results are controversial. Several cohort studies addressed that higher circulating 25(OH)D showed an association with improved survival of patients with early- or advanced-stage NSCLC [9–11]. In contrast, another two studies demonstrated that there was no effect of 25(OH)D level on survival in advanced NSCLC [12, 13]. In addition, a nested case-control study suggested that patients with higher 25(OH)D was associated with significantly better survival among patients with lung adenocarcinoma but not among those with squamous cell carcinoma [28]. The inconsistent findings indicate that the role of vitamin D in NSCLC prognosis still deserves further exploration.

Recent studies have highlighted bioavailable or free 25(OH)D as a more meaningful marker of vitamin D function [16, 17]. In current study, besides total 25(OH)D, we measured bioavailable and free 25(OH)D levels and investigated their relationships with NSCLC prognosis for the first time. We found that plasma concentrations of bioavailable and free 25(OH)D both were significantly decreased as compared to healthy controls. Additionally, total 25(OH)D was positively with both bioavailable and free 25(OH)D, which may partly explain the decrement of bioavailable and free 25(OH)D. We also found that total, bioavailable, and free 25(OH)D levels were inversely related to both OS and PFS, indicating all three 25(OH)D forms as independent predictors for survival among NSCLC patients. Besides, the AUCs in bioavailable and free 25(OH)D were similar with total 25(OH)D in dynamic trend, thus we could not distinguish if bioavailable and free 25(OH)D were superior to total 25(OH)D in relation to NSCLC prognosis. Our results did not completely correspond with other cancers in previous studies. Chen et al reported that plasma bioavailable 25(OH)D was independently associated with PFS and OS, while total 25(OH)D was independently associated with PFS but not OS in diffuse large B-cell lymphoma [29]. Yang et al showed that higher bioavailable and free 25(OH)D, rather than total 25(OH)D, were associated with better OS in I-III stage colorectal cancer, though only free 25(OH)D was an independent prognostic factor for OS [30]. Similarly, Fang et al suggested that bioavailable 25(OH)D was a preferable biomarker over total 25(OH)D for the prognosis of hepatocellular carcinoma [31]. By contrast, Yuan et al demonstrated that bioavailable 25(OH)D levels were not more strongly associated with risk of advanced and lethal prostate cancer than total 25(OH)D levels [32]. There are several potential reasons for the inconsistent findings. First, the measurements for 25(OH)D fractions varied in these studies. More importantly, there is no uniform standard either measurements or cutoff values for bioavailable and free 25(OH)D at present, which may bias the association between 25(OH)D fractions and cancer prognosis. Second, circulating vitamin D concentration may be influenced by various factors such as liver function, kidney diseases, estrogens and genetic background [15], thus 25(OH)D fractions only at a time point could not comprehensively represent the status of vitamin D, thereby in part leading to the disagreement of current studies. And finally, total 25(OH)D levels may indeed differ in distinct populations. The mean level of total 25(OH)D in Fang et al study was much higher than Chen et al and our present studies (35.8 ng/mL vs. 16.0-20.8 ng/mL) [29, 31]. It is possible that total 25(OH)D with a small increment provides more benefit for patients with vitamin D deficiency (<20 ng/mL), but no meaningful impact on patients with sufficient vitamin D (>30 ng/mL). Taken together, high level of vitamin D may have a positive association with improved prognosis in various cancers. However, it still should be more prudent about the conclusion that

bioavailable or free 25(OH)D could be a better biomarker for the estimation of vitamin D status or the prediction of cancer prognosis.

In addition to different forms of vitamin D, another important biomarker for vitamin D status is VDBP. We found that VDBP levels were significantly higher in NSCLC patients compared with healthy controls. As VDBP is bound to most of vitamin D, the increase of its levels may reduce the free or bioavailable fractions of vitamin D. In other words, the bioavailability of vitamin D may be limited even though circulating vitamin D is sufficient. Previous studies showed an inverse association between VDBP and free or bioavailable 25(OH)D [32, 33], though the association is yet to be found in current study. Furthermore, VDBP per se has multiple biological activities beyond vitamin D transporter, including extracellular actin scavenger and enhancement of neutrophil chemotactic activity [18]. Particularly, the VDBP-derived macrophage activating factor (MAF) demonstrated potent inhibition of both proliferation and migration of tumor cells [34], and induced tumor cells apoptosis through stimulating macrophages [35]. To date, two study examined VDBP level and lung cancer prognosis. One study including 148 England operated NSCLC patients showed that patients with the highest quartile of VDBP ($>430.2 \mu\text{g/mL}$) tended to have better prognosis in comparison to those with the lowest quartile of VDBP ($<199.4 \mu\text{g/mL}$) [36]. The other study conducted in 500 Finland male lung cancer cases demonstrated that circulating concentration of VDBP was not associated with lung cancer specific survival [28]. In this study, no significant association was found between VDBP level and NSCLC survival. Of note, VDBP in the European studies are much higher than that in our study ($291\text{--}337 \mu\text{g/mL}$ vs. $204.9 \mu\text{g/mL}$), which could be partially attributed to the difference in race and genetic polymorphisms [16]. Whether a sufficiently high level of VDBP (such as $>430.2 \mu\text{g/mL}$) may have a positive effect on the progression of lung cancer to some extent remains to be further investigated.

Several limitations should be acknowledged in current study. First, the sample size was relatively small, thus the findings of the present study may need to be generalized with caution, and further studies with larger sample sizes are needed to precisely validate these results. Second, the bioavailable 25(OH)D level was calculated based on the total 25(OH)D level, albumin level, and VDBP level in the plasma. Recently, the direct measurement of the bioavailable 25(OH)D level was established [37], which may more accurately reflect the bioavailable 25(OH)D level in the participants. Finally, other factors affecting vitamin D levels, such as sunlight exposure and vitamin D supplementation, were not evaluated in our study. But season of blood drawing was included as a potential confounder factor, which may reflect sunlight exposure partially. Nevertheless, this study also has several strengths, including the prospective design to minimize the potential for reverse causality. Furthermore, to the best of our knowledge, this is the first study to explore of the relationship between bioavailable and free 25(OH)D and NSCLC survival.

Conclusion

In summary, total, bioavailable, and free 25(OH)D may serve as reliable biomarkers in predicting the OS and PFS of NSCLC patients. And further studies are needed to validate our findings in larger and different populations.

Declarations

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Data availability: All datasets generated for this manuscript can be obtained from the corresponding author on reasonable request.

Ethical approval: This study was approved by the Research Ethics Committee of Soochow University (Approval No. ESCU-2015–0002). All procedures performed in studies involving human

participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent to participate Written informed consent was obtained from all participants.

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Figures

Figure 1

Plasma levels of total, bioavailable, and free 25(OH)D and vitamin D-binding protein in controls, NSCLC survivors and NSCLC non-survivors. ^a $P < 0.05$; ^b $P < 0.001$

Figure 2

Kaplan-Meier survival curves for overall survival (OS) (A) and progression-free survival (PFS) (B) by tertiles of total 25(OH)D levels, for OS (C) and PFS (D) by tertiles of bioavailable 25(OH)D levels, for OS (E) and PFS (F) by tertiles of free 25(OH)D levels, and for OS (G) and PFS (H) by tertiles of vitamin D-binding protein levels

Figure 3

Time-dependent receiver operating characteristic curves of bioavailable 25(OH)D, total 25(OH)D, free 25(OH)D and VDBP for prediction of survival. The time dependence of each area under the curve (AUC)

for overall survival or progression-free survival is shown at 1, 2 and 3 years. (A) Overall survival; (B) Progression-free survival. *25(OH)D* 25-hydroxyvitamin D, *VDBP* vitamin D-binding protein

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